Anti-inflammatory compound resveratrol suppresses homocysteine formation in stimulated human peripheral blood mononuclear cells in vitro

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Abstract

Inflammation, immune activation and oxidative stress play a major role in the pathogenesis of cardiovascular disorders. In addition to markers of inflammation, moderate hyperhomocysteinemia is an independent risk factor for cardiovascular disease, and there is a link between the activation of immunocompetent cells and the enhanced formation of homocysteine in vitro. Likewise, anti-inflammatory drugs and nutrients rich in antioxidant vitamins are able to reduce cardiovascular risk and to slow down the atherogenic process. Resveratrol, a phenolic antioxidant synthesized in grapes and vegetables and present in wine, has also been supposed to be beneficial for the prevention of cardiovascular events. Apart from its strong antioxidant properties, resveratrol has also been demonstrated to act as an anti-inflammatory agent. In this study the influence of resveratrol on the production of homocysteine by stimulated human peripheral blood mononuclear cells (PBMCs) was investigated. Results were compared to earlier described effects of the anti-inflammatory compounds aspirin and salicylic acid and of the lipid-lowering drug atorvastatin. Stimulation of PBMCs with the mitogens concanavalin A and phytohemagglutinin induced significantly higher homocysteine accumulation in supernatants compared with unstimulated cells. Treatment with 10–100 μM resveratrol suppressed homocysteine formation in a dose-dependent manner. Resveratrol did not influence the release of homocysteine from resting PBMCs. The data suggest that resveratrol may prevent homocysteine accumulation in the blood by suppressing immune activation cascades and the proliferation of mitogen-driven T-cells. The effect of resveratrol to down-regulate the release of homocysteine was comparable to the decline of neopterin concentrations in the same experiments. The suppressive effect of resveratrol was very similar to results obtained earlier with aspirin, salicylic acid and atorvastatin; however, it appeared that doses of compounds needed to reduce homocysteine levels to 50% of stimulated cells were always slightly lower than those necessary to achieve the same effect on neopterin concentrations. The influence of resveratrol and all the other compounds on homocysteine production appears to be independent of any direct effect on homocysteine biochemistry.

Keywords: homocysteine; immune activation; peripheral blood mononuclear cells (PBMC); resveratrol.

Introduction

Cardiovascular disease is one of the major causes of morbidity and mortality in Western countries (1). Several epidemiological studies have demonstrated that mortality from coronary heart disease (CHD) can be reduced by moderate consumption of alcoholic beverages, especially red wine (2). The protective activity of wines has been attributed to polyphenolic and flavonoid substances, which have strong antioxidant properties (3, 4). Among these substances, resveratrol (2,3-dihydroxystilbene) is one of the most important compounds. It was found to interfere with platelet aggregation and coagulation; furthermore, the phytoalexin has been demonstrated to reduce oxidative stress, e.g., by decreasing the oxidation of lipoproteins (5, 6). In addition to its strong antioxidant activity, resveratrol also has anti-inflammatory properties, which may be partly due to the modulation of transcription factor nuclear factor (NF)-κB and the inhibition of cyclooxygenases (7). Inflammatory reactions and continuous activation of the cellular immune system are known to be involved deeply in atherogenesis (8, 9), and oxidative stress as well as moderate hyperhomocysteinemia are argued to represent major triggers of disease progression in cardiovascular disease (10, 11). Patients suffering from vascular diseases show elevated concentrations of pro-inflammatory cytokines such as interferon-γ and neopterin, which reflect well the extent of disease and on-going immune stimulation in these patients (12–14). An association between moderate hyperhomocysteinemia and increased neopterin concentrations is apparent in patients suffering from CHD (14), as well as in patients suffering
from several other diseases, including rheumatoid arthritis and neurodegenerative disorders (15). Recently human peripheral blood mononuclear cells (PBMCs) were observed to produce homocysteine and neopterin in parallel when stimulated with mitogens (16). Data suggest that elevated homocysteine concentrations may be related to immune activation (15), although moderate hyperhomocysteinemia is usually considered to be an independent risk factor for vascular diseases (11).

In this study, we investigated whether the antioxidant resveratrol can influence homocysteine production in human PBMCs. Results were compared to the previously established influence of the anti-inflammatory drugs aspirin and salicylic acid (17) and the HMG-Co reductase inhibitor atorvastatin (18), as well as to the changes in neopterin concentrations manifested in the same experiments (17–19).

**Materials and methods**

**Cell culture**

PBMCs were isolated from whole blood obtained from healthy voluntary blood donors by density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). Cells were maintained in RPMI-1640 (PAA Laboratories, Linz, Austria) supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 2 mM L-glutamine (Serva, Heidelberg, Germany) and 50 μg/mL gentamicin (Bio-Whitaker, Walkersville, MD, USA).

For stimulation, cells were seeded at a density of 1×10⁶/mL and stimulated with the mitogens concanavalin A (Con A; Sigma, Vienna, Austria; 5 and 10 μg/mL), and phytohemagglutinin A (PHA; Sigma 10 μg/mL) to induce interferon-γ formation. To examine the effects of resveratrol on PBMCs, cells were either pre-incubated with 10–100 μM resveratrol and stimulated after 30 min with mitogens, or resveratrol was added 2 h after stimulation. Then cells were incubated at 37°C in 5% CO₂ for 72 h, and supernatants were harvested by centrifugation (1500 rpm, 400×g, 4°C, 8 min) and then frozen at −20°C until measurement. Cells were counted manually by trypan blue exclusion or were used to monitor the cell cycle and apoptosis by fluorescence activated cell sorting (FACS) analysis. Cells were stained with the DNA-probe propidium iodide, which only crosses the membrane of necrotic cells, highlighting the DNA of these cells. Analysis was performed on a fluorescence-activated cell sorter (Coulter Epics XL-MCL, Beckman-Coulter, Krefeld, Germany).

Experiments were performed at least three times, with duplicates of controls and stimulated cells.

**Determination of homocysteine**

Homocysteine concentrations were determined by high-performance liquid chromatography (HPLC) as described previously (20). For measuring cell culture supernatants, 90-μL specimens were used. After reduction with tris-(2-carboxyethyl)-phosphine (TCEP) and derivatization with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F), separation was performed using a 55-mm cartridge (RP18 LiChroCART 55-4) and an RP18 precolumn (Merck, Darmstadt, Germany). Homocysteine and cysteine concentrations were monitored by fluorescence detection at wavelengths of 385 nm for excitation and 515 nm for emission.

**Statistical analysis**

For comparison of grouped data, the Mann-Whitney U-test was applied. Values of p<0.05 were considered to indicate significant differences.

**Results**

Unstimulated PBMCs produced small amounts of homocysteine (mean±SD, 0.7±0.2 μM). Pretreatment of unstimulated cells with 10–100 μM resveratrol only slightly decreased homocysteine production of resting cells (not significant; Figure 1). Stimulation of PBMCs with mitogens Con A and PHA induced an approximately eight-fold increase in homocysteine production compared to unstimulated cells (mean 5.7 μM, p<0.01; Figure 1). There was no difference between either mitogen and between dif-
different doses of Con A used: homocysteine concentrations were only slightly higher when 10 μg/mL Con A was used compared to 5 μg/mL Con A. Mitogen-induced homocysteine formation was influenced strongly by resveratrol in a dose-dependent manner: 10 μM resveratrol only tended to decrease homocysteine concentrations in comparison to stimulated PBMCs without resveratrol. In contrast, homocysteine formation was significantly inhibited when PBMCs were exposed to resveratrol concentrations ≥25 μM (p < 0.01; Figure 1). When cells were stimulated first and resveratrol was added 2 h after mitogen stimulation, the effects were nearly the same as in preincubation experiments, and there were no significant differences between the two sets of experiments.

When stimulated with mitogens, PBMCs also released approximately three-fold higher concentration of neopterin compared to unstimulated cells (8.2 ± 3.5 nM; p < 0.01 for all stimulations). In agreement with earlier data (18), resveratrol was effective in suppressing neopterin production rates dose dependently in parallel to homocysteine accumulation. Neopterin concentrations were also slightly lower in resting cells treated with resveratrol; at resveratrol concentrations above 50 μM they significantly differed from control cells without resveratrol (p < 0.05; data not shown in detail).

A resveratrol concentration of 23.9–32.1 μM was necessary to diminish homocysteine production to 50% of the maximum found in cells stimulated with the mitogens (Table 1), and there was no difference between the different mitogens and concentrations of Con A found. To reduce neopterin production to 50% of maximum levels, slightly higher resveratrol concentrations were needed in PBMCs stimulated with Con A (39.3–39.8 μM). For PHA-stimulated cells, the concentration of resveratrol needed to reduce neopterin production to 50% was comparable to that required to achieve the same effect on homocysteine accumulation (22.6 μM).

Whereas resveratrol concentrations up to 25 μM did not influence cell viability significantly, concentrations of 50 μM and above induced apoptosis. At resveratrol concentrations of 100 μM, up to 21% of cells were stained with annexin-FITC, an early marker of apoptosis, but only 4% were double-stained with annexin-FITC and propidium iodide, the late marker of apoptosis/necrosis.

When comparing the effects of the anti-inflammatory drugs aspirin and salicylic acid, millimolar concentrations were needed to diminish homocysteine and neopterin production to 50% of maximum in stimulated cells (Table 1). The concentrations required appeared to be slightly higher for neopterin (4.8–5.7 mM and 5.7–6.4 mM, respectively) than for homocysteine (2.9–3.3 mM and 3.0–4.4 mM, respectively). No such difference was apparent for atorvastatin, which was effective in reducing homocysteine (7.5 and 19.7 μM) and neopterin (9.8 and 13.8 μM) at similar concentrations.

### Discussion

Human PBMCs provide a reasonable model system for the interaction between different immunocompetent cells, namely T-lymphocytes and monocyte-derived macrophages, as pro-inflammatory cytokines such as interferon-γ or TNF-α are released and modulate the activity of each other. Resveratrol was found to suppress homocysteine production rates in mitogen-treated PBMCs, and the effects were quite comparable to those observed earlier for neopterin accumulation (19). Increased production of neopterin is indicative for the formation of the Th1-type cytokine interferon-γ, which is induced by both PHA and Con A in PBMCs, as is the pro-inflammatory NF-κB. Inflammatory cascades and chronic activation of the cellular immune system, with ongoing activation of immunocompetent T-cells and monocytes, are deeply involved in atherogenesis. Cellular immune activation and oxidative stress as a result of reactive oxygen production of monocyte-derived macrophages are important contributors to cardiovascular disease progression (8–10). The polyphenol resveratrol was demonstrated in several studies to have both antioxidant
and anti-inflammatory properties, modulating several pathways important in inflammation, such as cytokine expression and activation of cyclo-oxygenases (7, 10, 22). In PBMCs, homocysteine formation is induced by the mitogens Con A, PHA and pokeweed mitogen (PWM) (16), and therefore the possible influence of exogenously added immunomodulating compounds on biochemical pathways can easily be studied. This study shows that resveratrol concentrations >25 μM significantly suppressed mitogen-induced homocysteine formation. A trend towards lower concentrations was observed for 10 μM resveratrol, which may represent the concentration in humans.

When comparing our results with those obtained earlier for neopterin measurements (18), homocysteine production was not significantly altered by resveratrol concentrations >50 μM in the unstimulated controls, whereas neopterin formation was completely blocked. Thus, in resting cells, neopterin formation was more sensitive to exogenous influences than homocysteine production. In contrast, in activated cells, lower concentrations were able to suppress homocysteine concentrations than those needed to achieve a significant effect on neopterin production rates. In stimulated PBMCs, homocysteine accumulation appears to be mainly due to the proliferation of T-lymphocytes (18), enhancing their demand for folic acid, which represents an essential cofactor for the remethylation of homocysteine to methionine. Suppression of T-cell proliferation by resveratrol represents the most probable reason for the inhibition of homocysteine formation in our experiment, e.g., Gao and co-workers reported on reduced proliferation rates of PHA-stimulated spleen lymphocytes under resveratrol treatment (22). Well in line with this assumption, treatment of cells with aspirin and salicylic acid arrested cells in G0/G1 phase (18), thus suppressing homocysteine formation. In general, antioxidants such as flavonoids or polyphenols possess antiproliferative and apoptosis-inducing effects (23–25). In unstimulated human PBMCs, no significant toxicity of resveratrol concentrations below 50 μM was observed within 3 days of incubation; however, in earlier studies performed by Losa (26), resveratrol became slightly toxic over a period of 5 days. These data are also well in agreement with our results, in which concentrations ≥50 μM induced apoptosis.

An important question is whether effects of resveratrol observed in vitro may also be relevant in vivo. Two studies in animal models indicate that absorption of resveratrol after oral administration is sufficient to prevent liver injury in rats fed peroxidized oil (27) and to detect significant concentrations in plasma and several organs after oral administration (28). On the other hand, T-cell-mediated immune response was only marginally reduced, although the production of TNF-α was reduced in mice after intragastric administration of resveratrol for 4 weeks. Data indicate that the influence of resveratrol observed may be less expressed in vivo (29). As these studies were conducted with otherwise healthy mice, the question arises as to whether immunomodulating effects of resveratrol would be expressed more in mice with an activated immune system, where resveratrol might suppress overwhelming immune-system activation and reduce oxidative stress. In general, it is questionable to what extent we can extrapolate results from in vitro experiments. Compounds may not be efficiently resorbed, and a gradient exists between concentrations present in beverages and that established in the blood. However, at least in the gastrointestinal tract, the presence of all ingredients at reasonable concentrations can be assumed, which may mean that antioxidant reactions are more likely (30). In particular, the antioxidant capacity of ingested compounds could be of relevance in establishing or shifting the redox equilibrium in the gut, which could then be of benefit to the whole organism.

The effect of resveratrol on homocysteine production by stimulated PBMCs was very similar to that observed earlier with the anti-inflammatory drugs aspirin and salicylic acid, as well as atorvastatin. However, the concentrations needed to achieve similar effects were in the lower micromolar range for the statin and resveratrol, whereas approximately 100-fold higher concentrations were needed for aspirin and salicylic acid to achieve comparable effects. All these drugs/compounds are considered to possess anti-atherogenic potential. They may slow down inflammation and their antioxidant nature could be of crucial relevance (31); however, this is still questionable because of the high concentrations needed to achieve significant effects in vitro. The doses used are certainly far above the range of concentrations detectable in patients (32). However, the effect of resveratrol and the other compounds in slowing down homocysteine production in stimulated PBMCs could contribute to reduce the potential deleterious effects of hyperhomocysteinemia on the process of atheroma formation and vessel pathology. In studies examining the effect of wine consumption on homocysteine concentrations so far, diverse results have been obtained: some studies proposed an increase in homocysteine concentrations (33), while others found no effect (34) or a homocysteine-lowering effect (35). Further studies examining the issues addressed are needed before relevant conclusions regarding the efficacy of resveratrol in vivo can be drawn.

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References


