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Resveratrol mediates anti-atherogenic effects on cholesterol flux in human macrophages and endothelium via PPARγ and adenosine

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ABSTRACT

Resveratrol is a bioactive molecule used in dietary supplements and herbal medicines and consumed worldwide. Known cardioprotective and anti-inflammatory properties of resveratrol have spurred investigation of the mechanisms involved. The present study explored potential atheroprotective actions of resveratrol on cholesterol metabolism in cells of the arterial wall, including human macrophages and arterial endothelium. Using QRT-PCR and Western blotting techniques, we measured expression of the proteins involved in reverse cholesterol transport (ABCA1, ABCG1 and SR-B1) and the scavenger receptors responsible for uptake of modified cholesterol (CD36, SR-A1 and LOX-1). We analyzed the effect of resveratrol on apoA-1-and HDL-mediated cholesterol efflux in human THP-1 macrophages. The effect of resveratrol on oxLDL internalization and foam cell formation were evaluated using confocal and light microscopy. Our data indicate that resveratrol regulates expression of major proteins involved in cholesterol transport, promotes apoA-1 and HDL-mediated efflux, downregulates oxLDL uptake and diminishes foam cell formation. Mechanistically, resveratrol effects were dependent upon PPAR-γ and adenosine 2A receptor pathways. For the first time we demonstrate that resveratrol regulates expression of the cholesterol metabolizing enzyme cytochrome P450 27-hydroxylase, providing efficient cholesterol elimination via formation of oxysterols. This study establishes that resveratrol attenuates lipid accumulation in cultured human macrophages via effects on cholesterol transport. Further in vivo studies are needed to determine whether resveratrol may be an additional resource available to reduce lipid deposition and atherosclerosis in humans.

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1. Introduction

Over the last decade, the search for natural compounds with the ability to prevent cholesterol accumulation in macrophages has been a main focus for many investigators. Several lines of evidence suggest that resveratrol (3,5,4′-trihydroxystilbene), a plant-derived polyphenol and phytoalexin, exhibits cardioprotective and anti-inflammatory properties (Fan et al., 2008; Palmieri et al., 2011; Prasad, 2010; Zhu et al., 2011). Current study explores its potential atheroprotective impact on cholesterol efflux and influx in cells of the arterial wall, including macrophages and arterial endothelium.

The balanced flow of cholesterol into and out of the macrophage is necessary to avoid lipid overload, and ultimately, atheroma development (Tabas, 2002). Cholesterol efflux from cells of the arterial wall to extracellular acceptors involves the ATP binding cassette transporters (ABC) A1 and G1 (Voloshyna and Reiss, 2011). ABCA1 promotes the transfer of cholesterol and phospholipids to lipid-poor apolipoprotein (apo) A-1 (Oram et al., 2001; Rust et al., 1999) contributing to the formation of high-density lipoproteins (HDL) in the liver. ABCG1 is critically involved in regulation of lipid-trafficking mechanisms in macrophages and participates in net efflux of cellular free cholesterol (FC) and phospholipid to lipid-free HDL (Wang et al., 2004). Expressions of ABCA1, ABCG1 and apolipoprotein E (apoE) are mediated through the nuclear receptors, liver X receptor (LXR) α and peroxisome-proliferator-activated receptor (PPAR) γ (Chawla et al., 2001; Ricote et al., 2004). Scavenger receptor class B type I (SR-BI)-mediated cholesterol efflux occurs as a bidirectional flux to phospholipid-containing acceptors (HDL and lipidated apolipoproteins), which depends on the gradient of FC (Yancey et al., 2003).

Synthesis of 27-hydroxycholesterol by the mitochondrial cytochrome P450 27-hydroxylase (CYP27A1) in arterial endothelium and macrophages provides a pathway for elimination of intracellular cholesterol. 27-hydroxycholesterol has statin-like properties, potently inhibiting HMG CoA reductase, suppressing smooth muscle cell proliferation and diminishing macrophage foam cell formation (Babiker et al., 1997; Reiss et al., 2000). An increase in 27-hydroxylase expression promotes cholesterol elimination both directly (by catabolism of cholesterol) and indirectly (by modulation of ABCA1 and apoE expression).

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Proteins involved in lipid uptake into cells, particularly modified low-density lipoproteins (LDL), include the scavenger receptors CD36, scavenger receptor (SR)-AI and lectin-like oxidized low-density lipoprotein receptor (LOX)-1 (Reiss and Glass, 2006; Steinbrecher, 1999; Vohra et al., 2006). Their expression is not diminished upon exposure to excess cholesterol, leading to foam cell formation and early atherosclerotic lesions.

Here we report that resveratrol promotes expression of cholesterol efflux proteins – ABCA1 and G1 transporters, SR-B1 and 27-hydroxylase– in HAEC, human THP-1 and monocyte-derived macrophages (MDM). Mechanistically, the resveratrol effects were dependent upon PPAR-γ and adenosine 2A receptor pathways. Changes in cholesterol efflux were accompanied by suppression of foam cell transformation in cholesterol-loaded macrophages. Although resveratrol significantly decreases uptake of oxidized LDL (oxLDL) by THP-1 macrophages, its effect on scavenger receptors requires further exploration.

2. Materials and methods

2.1. Cell culture and reagents

HAEC and basal medium were purchased from Lifeline Cell Technology (Walkersville, MD). HAEC were grown at 37 °C in a 5% CO_2 atmosphere in Vasculife Basal Medium supplemented with Lifeline's growth factors. HAEC were between passages 3 and 6 when the experiments were performed. When cells reached 80% confluence they were subjected to incubation under conditions described in the next section.

THP-1 monocytes (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 50 μg per ml of penicillin–streptomycin at 37 °C in a 5% CO_2 atmosphere to a density of 10^6 cells per ml. Cell culture media and supplementary reagents were obtained from Invitrogen (Grand Island, NY). Differentiation of the monocytic THP-1 cells into adherent macrophages was stimulated by 48 h exposure to 100 nM phorbol 12-myristate 13-acetate (PMA), obtained from Sigma-Aldrich (St. Louis, MO). When differentiated phenotype was achieved, the PMA-containing medium was removed, and replaced with complete RPMI 1640 supplemented with 10% FCS. The macrophages were cultured for another 24 h before treatment.

Under a protocol approved by the Winthrop University Hospital IRB, human peripheral blood mononuclear cells (PBMC) were isolated from fresh blood obtained from healthy normolipemic donors by OptiPrep Density Gradient Media (Sigma-Aldrich, St. Louis, MO). For macrophage differentiation, isolated PBMC were cultured in RPMI with 10% FCS and cultured for 6–7 days to reach 80% confluence. The percent of the living cells was determined by trypan blue (Sigma-Aldrich, St. Louis, MO) exclusion assay. Cell cultures with viability more than 97% were used for experiments.

Resveratrol, 3,4′,5-trihydroxy-*trans*-stilbene, 5-[(1E)-2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol, and PPAR-γ antagonist, 2-chloro-5-nitro-N-phenylbenzamide (GW6992) were purchased from Sigma-Aldrich (St. Louis, MO).

The selective A_2AR antagonist 4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a] [1,3,5]triazin-5-ylamino]ethyl)phenol (ZM-241385) was purchased from Tocris Cookson (Bristol, UK).

2.2. Experimental conditions

Resveratrol stock solution was prepared in ethanol. GW6992 and ZM-241385 stock solutions were prepared in DMSO.

THP-1 monocytes and macrophages (density 10^6 cells/ml), HAEC, PBMC (at 80% confluence) were subjected to incubation for 18 h under the following experimental conditions: (a) cell culture media alone; (b) ethanol solvent control; (c) DMSO solvent control; (d) resveratrol (10 μM); (e) resveratrol (25 μM); (f) GW9662 (1 μM); (g) resveratrol (10 μM) for 18 h+GW9662 (1 μM) for another 18 h; (h) GW9662 (1 μM) for 18 h+resveratrol (10 μM) for another 18 h; (i) ZM-241385 (1 μM) 1 h+resveratrol (10 μM) for 18 h. To obtain total protein lysates, incubation was done for 24 h under conditions (a–i) described above.

2.3. Gene-silencing via transfection of small interfering (si) RNA

Transfection of THP-1 macrophages (70% confluence) was carried out after their differentiation followed by 24 h incubation in complete RPMI. Gene-silencing experiments via transfection were performed in serum-free OPTI medium using siRNA transfection reagent (Santa Cruz, CA, sc-29528). Cells were transfected for 6 h with 100 nM of human PPARγ small interfering RNA (siRNA) (Santa Cruz, CA, sc-29455), or irrelevant non-targeting control siRNA-A (sc-37007) according to the manufacturer's protocol. Cells were then further incubated for 24–72 h under standard growth conditions. At 24–72 h post-transfection, depletion of PPAR-γ was confirmed by quantitative real-time (QRT)-PCR and immunostaining.

2.4. RNA isolation and QRT-PCR

Immediately after the incubation period, total RNA was isolated with the Trizol reagent and dissolved in nuclease-free water. The quantity of total RNA from each condition was measured by absorption at 260 and 280 nm wavelengths by ultraviolet spectrophotometry (Hitachi U2010 spectrophotometer).

QRT-PCR analysis was performed using the FastStart SYBR Green Reagents Kit according to the manufacturers' instructions on the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN). cDNA was copied from 1 μg of total RNA using Murine Leukemia Virus reverse transcriptase primed with oligo dT. Equal amounts of cDNA were taken from each reverse transcription reaction mixture for real-time PCR amplification using gene specific primers for 27-hydroxylase, ABCA1, ABCG1, LXR-α, SR-B1, SR-A1, CD36 and LOX-1 as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Table 1).

QRT-PCR was performed using techniques standardized in our laboratory (Anwar et al., 2011). Each reaction was done in triplicate. To correct for differences in cDNA load among samples,

Table 1
The list of specific primers used for QRT-PCR.

Gene	Primer
ABCA1	F 5′–GAAGTACATCAGAACATGGGC-3′
	R 5′–GATCAAAGCCATGGCTGTAG-3′
ABCG1	F 5′–CAGGAAGATTAGACACTGTGG-3′
	R 5′–GAAAGGGGAATGGAGAGAAG-3′
27-hydroxylase	F 5′–AAGCGATACCTGGATGGTTG-3′
	R 5′–TGTTGGATGTCGTGTCCACT-3′
LXRα	F 5′–GGGGCCAGCCCCCAAAATGCTG-3′
	R 5′–GCATCCGTGGGAACATCAGTCG-3′
ScR-B1	F 5′–GGTCCCTGTCATCTGCCAA-3′
	R 5′–CTCCTTATCCTTTGAGCCCTTT-3′
PPARγ	F 5′–GCAGTGGGGATGTCTCATAATGC-3′
	R 5′–CAGGGGGGTGATGTGTTTGAA-3′
CD36	F 5′–GAGAACTGTTATGGGGCTAT-3′
	R 5′–TTCAACTGGAGAG-GCAAAGG-3′
LOX-1	F 5′–TTACTCTCCATGGTGGTGCC-3′
	R 5′–AGCTTCTTCTGCTTGTTGCC-3′
ScR-A1	F 5′–CTCGTGTTTGCAGTTCTCA-3′
	R 5′–CCATGTTGCTCATGTGTTCC-3′
GAPDH	F 5′- ACCATCATCCCTGCCTCTAC-3′
	R 5′–CCTGTTGCTGTAGCCAAAT-3′