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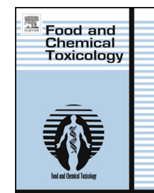

Resveratrol alters human endothelial cells redox state and causes mitochondrial-dependent cell death

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A R T I C L E I N F O

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A B S T R A C T

Studies analyzing the impact of natural antioxidants (NA) on Endothelial Cells (ECs) have dramatically increased during the last years, since a deregulated ECs redox state is at the base of the onset and progression of several cardiovascular diseases. However, whether NA can provide cardiovascular benefits is still a controversial area of debate. Resveratrol (RES), a natural polyphenol found in grapes, is believed to provide cardiovascular benefits by virtue of its antioxidant effect on the endothelium. Here, we report that tissue-attainable doses of resveratrol increased the intracellular oxidative state, thus affecting mitochondrial membrane depolarization and inducing EC death. Cyclosporine A, a mitochondrial permeability transition pore inhibitor, prevented oxidative-mediated cell death, thus implicating mitochondria in resveratrol-induced EC impairment. The specific cytochrome P450 (CYP) 2C9 inhibitor, sulfaphenazole, counteracted both oxidative stress and mitochondrial membrane depolarization, providing EC protection against resveratrol-elicited pro-oxidant effects. Our findings strongly suggest that CYP2C9 mediates resveratrol-induced oxidative stress leading to mitochondria impairment and EC death.

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1. Introduction

A substantial body of evidence indicates that oxidative stress-induced endothelial cell (EC) dysfunction is an important pathogenic factor of cardiovascular diseases (CVD) (Cai and Harrison, 2000). The term “vascular oxidative stress” refers to increased levels of reactive oxygen species (ROS) within the vascular wall, where production is due to disease-associated aberrant functioning of several ROS-generating enzymes, including NADPH oxidase, the mitochondrial electron transport system and cytochrome p450 (Cai and Harrison, 2000; Puntarulo and Cederbaum, 1998). In this context, the regular consumption of natural antioxidants (NA) from different sources, such as vegetable foods and some beverages like coffee, tea and red wine has been associated with a reduced incidence of risk factors for CVD (Cucciolla et al., 2007; Willcox et al., 2008). The most interesting study performed in this context is the so called “French Paradox” (Boin et al., 2014), whose cardiovascular benefits have been attributed to the polyphenol resveratrol, a naturally occurring antioxidant present in red wine (Cucciolla et al., 2007).

Multiple evidence, coming from observational, in vitro or ex vivo, and animal studies, indicate that resveratrol may exert cardiovascular protection by reducing oxidative stress (Ladurner et al., 2014; Li et al., 2012). However, the human clinical trials available so far have shown conflicting or controversial results concerning the protective effect of resveratrol against CVD and its sequelae (Carrizzo et al., 2013; Ponzo et al., 2014). The reason for these disappointing findings is unclear, but differences in the characteristics of the patients enrolled, doses and duration of resveratrol supplementation, have been proposed, at least in part, as responsible causes (Ponzo et al., 2014; Tome-Carneiro et al., 2013). In particular, the resveratrol dosage capable of maximizing its health effects without raising toxicity problems remains an area of controversial debate (Carrizzo et al., 2013; Rocha et al., 2009). In fact, while there is a consistent body of literature on the protective effects of Resveratrol against diseases or toxic drugs, there are relatively few reports on its possible toxicity.

We have previously reported that resveratrol can dose-dependently induce pro-oxidant damage of human ECs, and that

Abbreviations: MMP, mitochondrial membrane potential; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; CYPs, cytochrome P450.

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flavin-containing oxidases are the major source of the resveratrol-induced ROS increase (Pasciu et al., 2010). In this study, we further dissected the molecular mechanism of resveratrol induced EC-damage by demonstrating that resveratrol-induced intracellular oxidation elicits mitochondrial damage and EC death. In addition, the cytochrome P450 (CYP) 2C9 appears to be the main source of the resveratrol-induced oxidative stress. Our results also suggest that identification of an optimal dosage is absolutely required in order to have a high benefit–risk ratio in all the forms of resveratrol consumption.

2. Experimental procedures

2.1. Reagents

Resveratrol, sulfaphenazole (SPZ), and Cyclosporine A (CsA) were supplied by Sigma.

2.2. Cell culture and treatments

Human umbilical vein endothelial cells (ECs) were obtained and cultured as previously described (Pintus et al., 2003). ECs were subcultured at a split ratio of 1:2 and used within three passages. In order to mimic physiological vessel wall conditions, before experimentation, cells were grown until confluence to reach contact-dependent growth inhibition before experimentation. Unless not specified otherwise, cells were plated in 96-well plates (Corning) and processed for experiments in endothelial cell defined medium (ECACC). In selected experiment cells were pre-incubated for 30 minutes with either the mitochondrial permeability transition pore inhibitor CsA (Zhao et al., 2011) or the specific CYP2C9 inhibitor SPZ (Viswanathan et al., 2003).

2.3. Determination of the intracellular redox state

Intracellular redox state was investigated by using the a lentiviral vector encoding for the redox-sensing green fluorescent protein (roGFP), which reports the redox state of the GSH/GSSG pool *in vivo* in both plant and mammalian cells (Cossu et al., 2012; Dooley et al., 2004; Meyer et al., 2007). The DNA coding for the cytoplasmatic form of roGFP was obtained starting with our previous plasmid pCDN3-roGFP (Posadino et al., 2011), which has been cloned into a pBluescript II KS using Kpn and Xho restriction enzymes to obtain pBluescript II KS-roGFP. From the latter, the roGFP cDNA was cloned into the lentiviral vector (LV) p156-RRL-sinPPT-GFP-pre (kindly provided by prof. Francesco Galimi, Department of Biomedical Sciences, University of Sassari) using BamH1 and Sal I restriction enzymes to obtain the LV p156-RRL-sinPPT-roGFP. 1.5 μl of lentiviral particles, obtained from p156-RRL-sinPPT-roGFP, were used to infect a T25 cell culture flask of primary HUVECs. Three days after virus infection, cultured cells showed a transduction efficiency of about 70% as depicted by Fig. 1A–C, which is representative of six photos taken with a fluorescence microscope (Olympus XI70). RoGFP has two fluorescence excitation maxima at 400 (oxidized form) and 485 nm (reduced form) and display rapid and reversible ratiometric changes in fluorescence in response to changes in ambient redox potential. The ratios of fluorescence from excitation at 400 and 485 nm indicate the extent of oxidation and thus the redox potential while canceling out the amount of indicator and the absolute optical sensitivity (Dooley et al., 2004). In place of confocal imaging analysis, we used a recently developed fluorometer-based method for monitoring roGFP oxidation (Cossu et al., 2012; Posadino et al., 2011; Rosenwasser et al., 2010). Fluorescence measurements were performed in clear 24-well plates (Corning, Lowell, MA) on a fluorescence plate reader GENios plus (Tecan, Männedorf, CH) from the upper side using multiple reads per well (the read pattern was

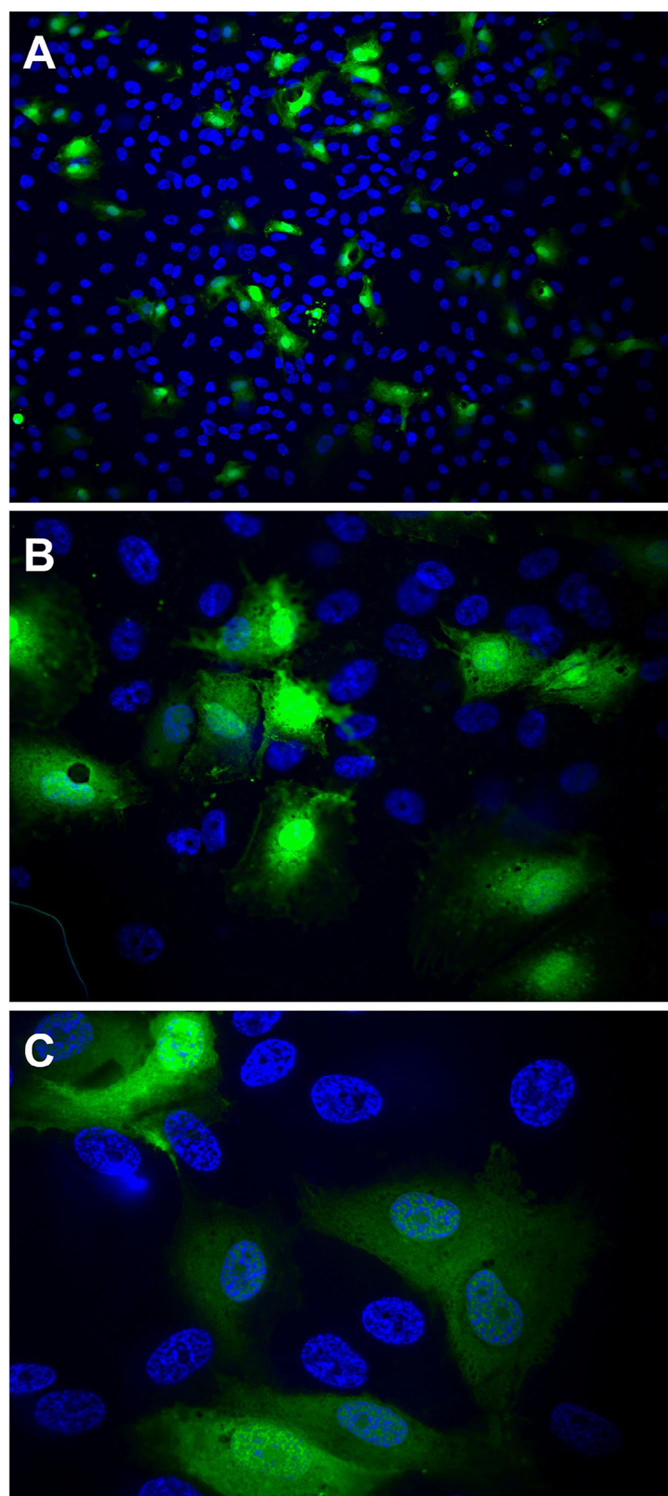

Fig. 1. HUVECs expressing the cytoplasmatic form of the redox-sensing green fluorescent protein (roGFP). Three days after virus infection, cells were plated in glass chamber slides at concentrations to allow 70–80% confluence in 24 hrs. On the day of experiments, cells were washed with PBS three times, fixed with 4% paraformaldehyde, counterstained with *Hoechst*, mounted and visualized by fluorescence microscopy. Images (A) depict cytoplasmic merged images of *roGFP* (green) and nuclear Hoechst staining (blue) of HUVECs at 20× magnification. Images (B) depict cytoplasmic merged images of *roGFP* (green) and nuclear Hoechst staining (blue) of HUVECs at 60× magnification. Images (C) depict cytoplasmic merged images of *roGFP* (green) and nuclear Hoechst staining (blue) of HUVECs at 100× magnification.

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