Food and Chemical Toxicology 47 (2009) 1362–1367

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Resveratrol toxicity: Effects on risk factors for atherosclerosis and hepatic oxidative stress in standard and high-fat diets

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ARTICLE INFO

Article history:
Received 4 June 2008
Accepted 10 March 2009

Keywords:
Resveratrol
Liver
Oxidative stress
Oxidized-LDL
High-fat diet

ABSTRACT

The beneficial action of moderate wine consumption is increasingly being attributed to resveratrol (*trans*-3,4′,5-trihydroxystilbene). To test the safety of resveratrol use as a dietary supplement, 24 male Wistar rats were initially divided into three groups: (C, $n = 6$) was given standard chow and water; (R, $n = 6$) received standard chow and 6 mg/l resveratrol in its drinking water (1 mg/kg/day), and (HFD, $n = 12$) received high-fat diet and water. In order to more appropriately study the effects of resveratrol on high-fat diet, after 30 days of treatments, HFD-rats were divided into two subgroups ($n = 6$/group):(HFD) remained receiving high-fat diet and water; (HFD-R) given high-fat diet and 6 mg/l resveratrol in its drinking water (1 mg/kg/day). The total experimental period was 45 days. The resveratrol dose took into account its average concentration in wine, the time variability of wine ingestion, and so of resveratrol consumption in humans. HFD-rats had hyperglycaemia, dyslipidemia, increased serum oxidized-LDL (ox-LDL) and hepatic oxidative stress. Comparing HFD-R and HFD-rats, resveratrol improved lipid profile and glucose level, enhanced superoxide dismutase, thus reducing ox-LDL and hepatic oxidative stress. Resveratrol, in standard-fed-rats reduced glutathione-antioxidant defense system and enhanced hepatic lipid hydroperoxide. In conclusion, based on the results of this single dose preliminary study with resveratrol in the drinking water of male Wistar rats for 30 days, it may be concluded that resveratrol may have beneficial effects in high-fat diets (e.g. ox-LDL, decreased serum and hepatic oxidative stress), but not in standard-fed diets (effects produced include enhanced hepatic oxidative stress). Further studies are indicated.

1. Introduction

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) a polyphenol from grape, *Morus* species, has been shown to be responsible for the cardiovascular benefits associated with moderate wine consumption, and so, with the "French paradox", a very low mortality rate due to cardiovascular disease despite high-fat diet intake (Stojanovic et al., 2001; Baur et al., 2006). However, the mechanisms that are triggered are not yet well understood, and it is questionable whether resveratrol powerful and beneficial *in vitro* activities are reproduced *in vivo*.

Oxidative modification of low-density lipoprotein (LDL) producing oxidized-LDL (ox-LDL) is the key step in the sequence of events leading to atherosclerosis and cardiovascular disease. Data on resveratrol effects and ox-LDL have been reported, but in most of these studies, only the lag time for LDL oxidation *in vitro* was examined (Benguendouz et al., 1998; Frémont et al., 1999; Stein et al., 1999; Zou et al., 2000; Frémont 2000; Ou et al., 2006; Milde et al., 2007). Since the majority of metabolic changes due to high-fat diet intake remain clinically silent, and only become manifest when health damage is effectively installed, studies to discover the relative potency of resveratrol on LDL oxidation *in vivo* may have particular importance.

Furthermore, experimental studies have pointed beneficial effects of resveratrol such as, improvement of lipid profile and lipoprotein metabolism (Wang et al., 2005; Milde et al., 2007), enhanced insulin sensitivity (Baur and Sinclair, 2006; Park et al., 2007), antioxidant activity (Ray et al., 1999; Stojanovic et al., 2001; Oak et al., 2005) and prolonged survival (Baur et al., 2006).

Abbreviations: BHT, butylated hydroxytoluene; C, standard-fed rats; CAT, catalase; EDTA, ethylenediamine tetraacetic acid; EI, energy intake; GSH, reduced glutathione; GSH-peroxidase, glutathione peroxidase; GSH-reductase, glutathione reductase; GSSG, oxidized glutathione; HFD, high-fat diet fed rats; HFD-R, high-fat diet fed rats receiving 6 mg/l resveratrol in its drinking water; HDL, high-density lipoprotein; H_2O_2, hydrogen peroxide; LDL, low-density lipoprotein; LH, lipid hydroperoxide; NADP, oxidized nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; ox-LDL, oxidized low-density lipoprotein; R, standard-fed rats receiving 6 mg/l resveratrol in its drinking water; ROS, reactive oxygen species; SOD, superoxide dismutase; TAS, total antioxidant substance; TG, triacylglycerol; VLDL, very low-density lipoprotein.

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doi:10.1016/j.fct.2009.03.010

However, some reports point adverse effects of resveratrol administration (Wilson et al., 1996; Fukuhara and Miyata, 1998; Crowell et al., 2004; Seve et al., 2005; Szkudelski, 2006; Bandele et al., 2008), so that, the use of resveratrol as an adjunct therapy for high-fat diet-induced adverse effects is still a controversial issue.

It has been shown that resveratrol increases hepatic mitochondrial number and citrate synthase activity, a marker of aerobic metabolism (Baur et al., 2006), but mitochondria are the major production sites for reactive oxygen species (ROS), and so for oxidative stress, an imbalance between oxidants and antioxidants systems in favor of the former (Novelli, 2005).

Contradictorily resveratrol has also an antiangiogenic property, thus reducing the oxygen supply for aerobic metabolism in some cells (Oak et al., 2005). In this way, it was not demonstrated whether dietary resveratrol supplementation has hepatic adverse effect, mainly in high-fat diet condition, a situation characterized by enhanced oxidative stress. Because the possible link between resveratrol and hepatic oxidative stress, its dietary supplementation might enhance the adverse condition and further damage in high-fat diet intake. Interestingly, researches on resveratrol actions have been reported in a scattered fashion, but its effects on hepatic oxidative stress had drawn little attention until recently.

Thus, the major purpose of the present study was to investigate the effects of resveratrol supplementation to high-fat diet-induced adverse effects on lipid profile, serum glucose, *in vivo* serum ox-LDL, and markers of oxidative stress in serum and liver. The significance of this study has been mainly explained by potential application of its findings to preventive medicine. Thus in this experiment, resveratrol was given in standard-fed rats and in high-fat diet fed rats, as a model of experimental hyperglycemia, dyslipidemia and oxidative stress.

2. Materials and methods

2.1. Animals and diet

The Ethical Committee for Conduction of Animal Studies at the Institute of Biological Sciences, São Paulo State University (UNESP) approved the experimental protocol and all animals were cared for in accordance with the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care. Twenty four male Wistar rats, at 60-days-old were individually housed in polypropylene cages in an environmentally controlled clean-air room, with temperature of 22 ± 3°C, 12 h light/dark cycle, and a relative humidity of 60 ± 5%. The animals were initially divided into three groups. A control group (C, $n = 6$) was given free access to water and standard chow (Biobase, São Paulo, SP, Brazil) containing 19.80 g protein, 39.25 g carbohydrate, 4.41 g fat, 13.25 g fiber per 100 g of chow, and 2.75 kcal/g total metabolizable energy. To study resveratrol supplementation in standard-fed conditions, a (R, $n = 6$) group received standard chow and 6 mg/l resveratrol (*trans*-3,4′,5-trihydroxystilbene) in its drinking water, approximately 1 mg/kg body weight/day. In order to more appropriately study the effects of resveratrol on high-fat diet condition, a group (HFD, $n = 12$) received water and a high-fat diet containing 15.30 g protein, 43.34 g carbohydrate, 11.86 g fat, 10.20 g fiber per 100 g chow, and 3.41 kcal/g total metabolizable energy. After 30 days of the experimental period, the HFD group was randomly divided into two subgroups ($n = 6$/group): (HFD) group remained receiving high-fat diet and water, and (HFD-R) group given high-fat diet and 6 mg/l resveratrol in its drinking water. Therefore, the HFD had initially 12 rats, but was fatherly divided into two groups of six rats each.

The high-fat diet was formulated by mixing the supplemented ingredients with a previously triturated standard chow. The dietary ingredients were homogenized in 60 °C warm distilled water and the homogenate was used to prepare the pellets. Therefore, both control and experimental diet were given fresh each day as dry pellets, and there was no spillage. The high-fat diet was obtained by mixing 848 ml soy oil, 1310 g sucrose, 123 g cholesterol and 12 g cholic acid to 1000 g of the standard chow (Diniz et al., 2004). High-fat diet was hypercaloric according to the AIN-93 criteria (Reeves, 1997). Dietary crude protein ($N \times 6.25$) was determined using the micro-Kjeldahl/AOAC method. The carbohydrate proportion was carried out using 75 °C water as mobile phase to chromatographic determination (CG 17A chromatograph, Shimatzu Corporation Analytical Instruments Division, Kyoto, Kansai, Japan). Dietary fats were extracted in petroleum ether in a Soxhlet extractor (Diniz et al., 2006). The caloric value was calculated using the metabolic factor (4 × protein, 9 × fat and 4 × carbohydrate).

Rats in the C and R groups remained with the same treatment during all experimental period of 45 days. Food and drinking solutions consumption were measured daily at the same time (9:00–10:00 h). The body weights were determined once a week. The body weight was used to determine the surface area ($g^{0.7}$) = final body weight$^{0.7}$ (Novelli et al., 2007). The food intake was used to obtain the energy intake (EI, kcal/day) = (mean food consumption per day × dietary metabolizable energy).

The administered resveratrol dose was carefully evaluated. Assuming that the average concentration of resveratrol in wine is 5 mg/l, and that the moderate daily consumption of wine is 250 ml (Dong, 2003), the mean daily intake of resveratrol in these conditions is approximately 0.02 mg/kg in humans (Juan et al., 2002). However, more than two third of people living in occident countries drink more than just occasionally, and long-standing wine consumption is found in most populations (Ferreira and Willoughby, 2008). The kinetic parameters and bioavailability of resveratrol after oral administration was widely studied (Bertelli et al., 1998; Scalbert and Williamson, 2000; Juan et al., 2002). In accordance of these values, and to simulate a free living situation in which there is a wide time variability of resveratrol intake, we selected a single resveratrol dose that was ingested by rats during different times. This dose took into account the clinical effects of resveratrol dependence on their plasmatic levels, the plasmatic resveratrol levels after oral administration (Scalbert and Williamson, 2000), the time variability of wine ingestion, and so of resveratrol consumption in humans (Ferreira and Willoughby, 2008). The daily resveratrol intake was 1.07 ± 0.08 mg/kg body weight/day and 0.99 ± 0.01 mg/kg body weight/day for R and HFD-R rats, respectively (Table 1), and was calculated from the amount of aqueous solution ingestion.

2.2. Experimental procedure

After 15 days of the HFD group division (45 days of the experimental period), rats were fasted overnight (12–14 h). The animals were anaesthetized (0.1 ml ip of 1% sodium barbiturate) and sacrificed by decapitation. Blood was collected by a funnel into a centrifuge tube and allowed to clot to obtain the serum. The liver was rapidly removed. Hepatic samples of 200 mg were immediately used to estimate the reduced glutathione (GSH) and oxidized glutathione (GSSG). The remaining hepatic samples were frozen and stored at −86 °C for subsequent analysis. Hepatic samples of 200 mg were homogenized in 5 ml of a cold phosphate buffer (0.1 M, pH 7.4). Tissue homogenates were prepared in a motor-driven Potter Elvehjem, tissue homogenizer (1 min, 100 g) immersed in ice water. The homogenate was centrifuged at 10,000*g* for 10 min. The supernatant was analyzed for total protein, lipid hydroperoxide and antioxidant enzymes.

2.3. Biochemical determinations

The triacylglycerol, total cholesterol, high-density lipoprotein cholesterol (HDL) and glucose were determined in serum by enzymatic method (test Kit CELM diagnosis, Modern Laboratory Equipment Company, São Paulo, SP, Brazil). The very-low-density lipoprotein cholesterol (VLDL) was calculated by Friedewald et al. (1972), total protein was analyzed by Lowry et al. (1951) and antioxidant capacity, or total antioxidant substances (TAS) by test kit (Randox Laboratories Ltd., Crumlin, Co., Antrim, UK).

Low-density lipoprotein (LDL) was selectively precipitated from 60 µl of serum by adding 1 ml of phosphotungstic acid precipitating reagents (CELM diagnosis, Modern Laboratory Equipment Company, São Paulo, SP, Brazil) and separation by centrifugation at 1400*g* for 10 min. The LDL-pellet was dissolved in 600 µl of 0.015 mM NaOH, which does not contain interfering substances; therefore dialysis was not needed. The cholesterol concentrations in the dissolved LDL-pellet were measured (Scoccia et al., 2001), and 100 µl of the LDL-pellets were immediately used to measure the LH concentration (Jiang et al., 1991).

LH was measured in serum and in liver through hydroperoxide-mediated oxidation of Fe^{2+}, with 100 µl of sample and 900 µl of a reaction mixture containing 250 µM $FeSO_4$, 25 mM H_2SO_4, 100 µM xylenol orange and 4 mM BHT in 90% (v/v) methanol (Jiang et al., 1991).

GSSG was assayed in liver, using 2 mM DTNB, in 50 mM KH_2PO_4. GSH was measured by a kinetic assay in reaction medium containing 0.6 mM DTNB, 0.2 mM NADPH and 2 U of glutathione reductase in 50 mM KH_2PO_4 (Tietze, 1969). GSH-reductase (E.C.1.6.4.2.) activity was evaluated by monitoring NADPH oxidation at 340 nm. The assay mixture contained 1 mM Tris buffer pH 8.0, 5 mM EDTA, 33 mM GSSG and 2 mM NADPH (Miller and Blakely, 1992). GSH-peroxidase (E.C.1.11.1.9.) was assayed using 0.15 M phosphate buffer pH 7.0 containing 5 mM EDTA, 0.0084 M NADPH, 4 µg of GSH-reductase, 1.125 M sodium aside and 0.15 M GSH. GSH-peroxidase unit was defined as µmol of NADPH oxidized per minute per g protein (Nakamura et al., 1974). Catalase (E.C.1.11.1.6.) activity was determined in reaction mixture of 50 mM phosphate buffer pH 7.0 and 10 mM hydrogen peroxide at 240 nm. Catalase unit was defined as µmol of hydrogen peroxide decomposed per minute per g protein (Aebi, 1974). Superoxide dismutase (E.C.1.15.1.1.) activity was measured using superoxide radical (O_2^-)-mediated-nitro blue tetrazolium (NBT) reduction by an aerobic mixture of NADH and PMS. The complete reaction system consisted of 50 mM phosphate buffer pH 7.4, 0.1 mM EDTA, 50 µM NBT, 78 µM NADH and 3.3 µM PMS. One unit of SOD was defined as the amount of protein to decrease the reference rate to 50% of maximum inhibition (Ewing and Janero, 1995).