THE PROTECTIVE EFFECTS OF RESVERATROL ON HUMAN CORONARY ARTERY ENDOTHELIAL CELL DAMAGE INDUCED BY HYDROGEN PEROXIDE IN VITRO

Oya Sayin¹, Nur Arslan²,³ and Gul Guner¹,²

¹Research Laboratory, ²Department of Medical Biochemistry, School of Medicine, ³Institute of Health Sciences, Dokuz Eylul University, Izmir, Turkey

SUMMARY – Oxidative stress is defined as imbalance between the production and destruction of reactive oxygen species. The aim of this study was to investigate whether resveratrol could protect human endothelial cells against hydrogen peroxide damage in vitro. In this in vitro study on human coronary endothelial cells, the effects of resveratrol on the glutathione content in human coronary endothelial cells in vitro were evaluated with high performance liquid chromatography. The effects of resveratrol on protein expression of the glutamate cysteine ligase, glutathione peroxidase and glutathione reductase enzymes were evaluated with the Western blot method. Resveratrol increased the reduced glutathione contents significantly (p<0.05). Resveratrol increased protein expression of the glutamate cysteine ligase, glutathione peroxidase-1 and glutathione reductase enzymes (p<0.05). All data supported each other and suggested that resveratrol had a protective effect against human coronary artery endothelial cell damage. It is thought that these results could pave the way to the new therapeutic approaches to protect against oxidative stress that develops in cardiovascular diseases.

Key words: Resveratrol; Human coronary artery endothelial cell; Oxidative damage

Introduction

The endothelial cells localized between the blood and vascular smooth muscle cells play a role in the secretion of many vasoactive materials, in platelet aggregation, and in the control of vascular structure. Because of its position in the blood vessels, the endothelial layer works as a barrier between the blood and vascular smooth muscle cells, and functional integrity of the endothelium is important to prevent vascular leakage and atherosclerosis formation.

Under physiologic conditions, the formation and elimination of reactive oxygen species (ROS) are in balance. Increase in the oxidant enzyme activity and/or decrease in the antioxidant enzyme activity lead to oxidative stress. Oxidative stress plays an important role in cardiovascular diseases, cancer, neurologic diseases, diabetes, and aging. Oxidative stress is characterized by increase in endogenous reactive oxygen products such as hydrogen peroxide and superoxide. ROS include very bioactive, short living molecules originating from the reduction of molecular oxygen. Some of the common ROS are hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), and hydroxyl radical (OH). H₂O₂ does not possess non-coupled electrons and is less reactive than many ROS types. Besides activating many signal pathways in low pathophysiological concentrations (nano-micromolar), in high concentrations by reacting with heavy metals H₂O₂ produces the hydroxyl radical which oxidizes proteins...
and lipids, and causes fractures in DNA strands. High hydrogen peroxide level generally causes endothelial functional loss and/or cytotoxicity\(^8\).

Enzymatic antioxidant protection includes superoxide dismutase, glutathione peroxidase and catalase. Non-enzymatic antioxidants are ascorbic acid, \(\alpha\)-tocopherol, glutathione, carotenoids, flavonoids, stilbene and others. There is balance between the intracellular antioxidant level and efficiency under normal conditions. This balance is necessary for viability and health of the body\(^9\)-\(^12\). Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a phytoalexin and polyphenolic compound that can be found in the skin and seeds of grapes and red wine. It has antioxidant, anti-inflammatory, and anticarcinogenic effects\(^13\),\(^14\). Resveratrol has been shown to exert its antioxidant effects \(\text{via}\) two different cytoprotective mechanisms; it directly scavenges free radicals (i.e. hydroxyl radicals and superoxide anion) and increases the activity of certain antioxidant and cytoprotective enzymes\(^15\).

Glutathione is a non-protein thiol compound involved in cell protection from ROS and free radicals, and maintains the cellular redox state. Glutathione is present in mammalian cell types at millimolar concentrations and plays a role in detoxification of xenobiotics, transportation of amino acids, stabilization of cell membranes, and synthesis of proteins and DNA\(^16\)-\(^18\). Glutathione peroxidases (GPxs), which contain selenocysteine at the active site, protect the cells from oxidative stress by reducing \(\text{H}_2\text{O}_2\) and a wide range of organic hydroperoxide substrates. Glutathione peroxidase-1 (GPx-1, EC 1.11.1.9) is known as cellular or cytosolic form, and being the most abundant isoform within the eukaryotic cells, is a major intracellular antioxidant enzyme\(^19\),\(^20\). Glutamate cysteine ligase (GCL, EC 6.3.2.2), which is a rate limiting enzyme in \(\text{de novo}\) synthesis of glutathione, was previously known as \(\gamma\)-glutamyl cysteine synthase. GCL is a heterodimer which consists of heavy subunit, catalytic subunit (GCLC) and light subunit, regulatory subunit (GLCLR). The heavy subunit contains substrate binding sites and the light subunit regulates the affinity of the heavy subunit for substrates and inhibitors\(^21\)-\(^23\). Glutathione reductase (GR, EC 1.6.4.2) is a homodimer which is bound by disulfide bond and is found in the cytoplasm and mitochondria. Glutathione reductase is a flavin adenine dinucleotide (FAD) containing oxidoreductase belonging to the pyridine nucleotide disulfide family. The activated enzyme is composed of two subunits and each one has binding sites for FAD, NADPH and oxidized glutathione (GSSG). This enzyme converts oxidized glutathione to its reduced form\(^24\). In the literature, various injury models with rat cardiomyocytes\(^25\), rat aorta smooth muscle cells\(^26\) and bovine aorta endothelial cells\(^26\) were used to investigate the protective effect mechanisms of resveratrol \(\text{in vitro}\). However, there are no reports on the use of \(\text{in vitro}\) oxidative injury model of human coronary artery endothelial cells. Moreover, the effect of oxidative stress on protein expression of the glutamate cysteine ligase, glutathione peroxidase and glutathione reductase enzymes, which all work in the glutathione cycle, has not yet been evaluated.

In this study, we used a hydrogen peroxide injury model optimized for \(\text{in vitro}\) human coronary artery endothelial cells and investigated the possible effects of resveratrol by Western blotting of glutamate cysteine ligase, glutathione peroxidase-1 and glutathione reductase.

**Materials and Methods**

**Human coronary artery endothelial cell culture and materials**

Human coronary artery endothelial cells (HCAE cells) were obtained from Clonetics Lonza USA (CC-2585). HCAE cells were grown in the culture medium (EGM-2MV Bullet kit, Clonetics Lonza USA, CC-3202). EGM-2MV bullet kit is composed of EBM-2 (CC-3156) and EGM–2 MV growth factors (CC-4147). The reagent pack was purchased from Cambrex [(one reagent pack containing trypsin/EDTA, trypsin neutralizing solution, HEPES buffered saline solution (CC-5034)] and cell culture was performed according to the manufacturer’s instructions. Seeding density was 5000 cells/cm\(^2\) in HCAE cells. HCAE cells were maintained in the endothelial growth medium supplemented with 25% fetal bovine serum, hydrocortisone, hFGF-B, VEGF, R\(^3\)-IGF-1, ascorbic acid, hEGF and GA-1000 (Clonetics). The cells were incubated in a humidified atmosphere containing 5% CO\(_2\) at 37 °C. The medium was changed every other day and passaged every 5-9 days. In all experiments, HCAE cells were used in 5th-7th passages.
**In vitro experiments**

We used the oxidative injury model optimized in our previous studies as described below. The optimum time and concentration of hydrogen peroxide, which caused 30%-40% injury in *in vitro* human coronary artery endothelial cells, were determined as 750 µM; 1 hour. Using this model, we selected 50 µM for the resveratrol concentration to study its effects.

In this study, the cells were incubated with 50 µM resveratrol for 24 hours and then with 750 µM H$_2$O$_2$ for 1 hour. At the end of the incubation period, cell lysates were prepared. We evaluated reduced glutathione (GSH), oxidized glutathione (GSSG) and glutamate-cysteine ligase, glutathione peroxidase-1, glutathione reductase enzymes to investigate the protective effect of resveratrol during hydrogen peroxide injury. Glutathione level was determined with high performance liquid chromatography (HPLC) and enzyme expression with Western blotting. After concentrating the cells by 70%-80%, four groups were formed: control, H$_2$O$_2$ group, resveratrol group and resveratrol + H$_2$O$_2$ group. Triple group analysis was used for the glutathione, glutamate-cysteine ligase, glutathione peroxidase and glutathione reductase enzymes. Each experiment was repeated three times in order to standardize the process. The study protocol was approved by the local Research Ethics Committee of Dokuz Eylul University School of Medicine.

**Measurement of cellular reduced and oxidized glutathione**

Intracellular contents of GSH and GSSG were determined using HPLC. The cells were first scraped with cold phosphate buffer solution (PBS) and centrifuged at 300 g for 7 min at 4 °C and then cell lysates were prepared, as shown in the Preparation of cell lysate section. The supernatants containing cytosolic proteins were used for protein content determination by the bicinchoninic acid (BCA) method (Thermo, Cat No: BCA, Protein assay kits, 23225, Rockford, USA). Then, the supernatants containing cytosolic proteins (100 µL) were deproteinized by adding 400 mL of 6% metaphosphoric acid (MPA) and centrifuged for 7 min at 10 000 g at 4 °C. The supernatants were stored at -80 °C until use. To determine reduced glutathione (GSH), 100 mL of MPA, 500 mM sodium phosphate, pH 7.00 were added to the supernatant to neutralize and dilute it. Neutralized samples were derived with 100 µL of *ortho*-phthalaldehyde (OPA) solution and then held for 5 min at room temperature; for neutralization 800 mL of 500 mM sodium phosphate was added to the product. For analysis of reduced glutathione, five microliters were injected into the HPLC system. For total glutathione (GSHt) determination, GSSG was reduced with dithiothreitol (DTT) and then protein precipitation was carried out. For this purpose, samples were treated with 100 µL of 25 mM DTT solution and 50 µL 0.1 M Tris buffer, pH 8.5 and then stored for 30 min at 4 °C. Samples (100 µL) were deproteinized by adding 400 mL of 6% MPA and centrifuged for 7 min at 10 000 g at 4 °C and the supernatants were removed. Derivationization procedure for the supernatants was performed as described above. Oxidized glutathione values were calculated by subtracting GSH values from total GSH and then dividing into two. The results were expressed as nmol/mg protein.

**Preparation of cell lysates and determination of protein expression of glutamate cysteine ligase, glutathione peroxidase-1 and glutathione reductase with Western blotting**

HCAE cells were treated with 50 µM resveratrol for 24 hours and then incubated for one hour at 750 µM H$_2$O$_2$. At the end of the incubation period, cells were scraped, centrifuged at 300 g for 7 min at 4 °C; then cell lysates were prepared: the supernatants were washed with ice-cold PBS, again centrifuged at 1500 rpm for 5 min, 200 µL lysis buffer was added ( NP-40 1%, 1 M Tris pH 7.4, 3 M NaCl, 20 mM EDTA, Protease inhibitors (Complete-mini protease inhibitors, Roche Diagnostics GmbH) and after incubating in an ice bath for 10 min, vortex-mixing was performed for 30 sec. To collect the supernatants containing cytosolic proteins samples were centrifuged at 10 000 g for 10 min. Protein concentrations in the supernatants were determined by BCA protein assay. Proteins in the cell lysate supernatants were analyzed in all samples using the BCA assay kit according to the manufacturer’s instructions. The supernatants were used to analyze expression of the glutathione and glutamate cysteine ligase, glutathione peroxidase-1 and glutathione reductase enzymes.
Cell lysates were prepared as described above. Total protein concentration was determined by the BCA assay. Total cell lysate (40 µg) was mixed with sample buffer (25 mM Tris HCl, 5% SDS, 2-mercaptoethanol 2%, glycerol 10%, bromphenol blue 0.002%) and then incubated for 5 min at 95 °C. Samples were loaded on 10% SDS-separating and 4% stacking polyacrylamide gel. Then, the proteins were transferred on polyvinylidene difluoride (PVDF) membrane for 2 h at 200 mA. After blocking for 30 min with nonfat dry milk in PBS containing NP-40 (for glutamate cysteine ligase, glutathione peroxidase-1, glutathione reductase and actin; 4%, 3%, 5%, 3%, respectively), the PVDF membrane was incubated overnight with polyclonal rabbit antibody [glutamate cysteine ligase (Santa Cruz, sc-22667), glutathione peroxidase-1 (Santa Cruz, sc-22145), glutathione reductase (Santa Cruz, sc-32408) and actin (Santa Cruz, sc-1615); 1:500, 1:200, 1:500, 1:500 in blocking buffer, respectively] at +4 ºC.

Then the PVDF membrane was washed three times with 10% PBS containing 0.05% NP-40. The PVDF membrane was incubated with donkey anti-goat IgG-Horseradish Peroxidase Conjugated Affinity Purified Antibody (Santa Cruz, sc-2020) for one hour (glutamate cysteine ligase, glutathione peroxidase-1, glutathione reductase and actin, 1:3500, 1:3500, 1:5000, 1:3500 in blocking buffer, respectively). The PVDF membrane was washed three times with 10% PBS containing 0.05% NP-40. The transferred proteins were incubated with ECL substrate solution (Amer-}

### Statistical analysis

All experiments were repeated three times. All data were expressed as means ± SD. One way analysis of variance (ANOVA) was employed for comparison of significant differences among groups. The value of p≤0.05 was considered statistically significant.

### Results

**Resveratrol prevents hydrogen peroxide-induced glutathione decrease in HCAE cells**

H$_2$O$_2$ (at 750 µM) decreased the reduced glutathione content significantly after 1 hour in HCAE cells; on the contrary, resveratrol increased the reduced glutathione content. Also, resveratrol (at 50 µM, 24 hours) per se significantly increased the reduced glutathione content compared to control group (p<0.05) (Fig. 1A).

On the other hand, H$_2$O$_2$ (at 750 µM) increased the oxidized glutathione content significantly after 1 hour (p<0.05). There was no significant effect of resveratrol on the oxidized glutathione content (p>0.05) (Fig. 1B).

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**Fig. 1.** Effect of resveratrol on reduced and oxidized glutathione contents. Cells were treated with 750 µM resveratrol for 24 hours and then with H$_2$O$_2$ for 1 hour. Glutathione content was measured by HPLC; (A) resveratrol increased the reduced glutathione content (p<0.05); (B) no effect of resveratrol was observed on oxidized glutathione content. Data were presented as mean ± SD and these independent experiments were performed in triplicate (p>0.05).
Resveratrol induces glutamate–cysteine ligase, glutathione peroxidase-1 and glutathione reductase protein expression in HCAE cells

Western blot analysis was performed to determine the resveratrol-induced changes in glutathione-related enzymes of the human coronary artery endothelial cells. Representative blots and values of densitometric analysis of the blots are shown in Fig. 2A-2C. Resveratrol up-regulated glutamate cysteine ligase, glutathione reductase and glutathione peroxidase-1 protein expression in HCAE cells (p<0.05). β-Actin was used as the loading control.

Discussion

Oxidative stress is characterized by increased endogenous production of ROS such as superoxide and hydrogen peroxide, and changes the structure and function of vascular endothelial cells. Oxidative stress plays an important role in the pathogenesis of cardiovascular diseases, cancer, neurologic diseases, diabetes, aging, and many other diseases. Therefore, removal of excess ROS or suppression of their production with antioxidants is effective in preventing oxidative cell death. Resveratrol, which is present in high levels in the grapes, red wine, peanuts, cranberries, etc., has the potential to capture ROS4,30,31. Resveratrol has two different cell protective mechanisms for antioxidation: it captures free radicals directly (hydroxyl radical, superoxide anion radical) and its antioxidant capacity is due to the balance between the hydroxyphenolic groups and electron delocalization. It shows other antioxidant effects by increasing the cell protective and antioxidant enzyme levels15,25,32.

In this study, glutamate-cysteine ligase, glutathione peroxidase and glutathione reductase enzymes in HCAE cells were evaluated after treating for 24 hours with 50 µM resveratrol and then incubating for 1 hour with 750 µM, H₂O₂. When compared with control group, the reduced glutathione level decreased significantly in the H₂O₂ group (p<0.05) and increased significantly when compared with resveratrol + H₂O₂ group (p<0.05) (Fig. 1A). Resveratrol had no effect...
on the oxidized glutathione levels (p>0.05) (Fig. 1B). Normally, the reduced glutathione level in the cells is regulated by two mechanisms: the synthesis rate and the extracellular export. GSH level is affected by cellular thiol balance changes or various agents18. The vast majority of intracellular glutathione (>98%) includes cytosol and a small amount of the nucleus reduced form (GSH) of glutathione conjugates (GS-R), mercaptides [Cr, Cu (I), Cu (II), Se, Zn], thioether, thioester and oxidized disulfide dimer forms (GSSG). As a powerful reductive agent, glutathione supplies protons or acts as a nucleophilic conjugate cofactor for the antioxidant pathways. GSH is transformed to oxidized glutathione (GSSG), which is an electron donor and reduces free radicals. GSH captures reactive products such as reactive oxygen and nitrogen products. GSH gives electrons and directly deactivates superoxide anion, hydroxyl radical, peroxynitrite and singlet oxygen. Also, glutathione detoxifies hydroperoxide, peroxynitrite and lipid peroxides by catalyzing glutathione peroxidase and peroxiredoxin reactions33.

In our study, when the findings of reduced glutathione and oxidized glutathione, which are the major components of oxidative stress and antioxidant balance, were compared, an increase in oxidized glutathione to correspond to a decrease in the level of reduced glutathione was not observed during treatment with H2O2 (p>0.05). This may be explained by the possibility that reduced glutathione may have been transformed into glutathione conjugates rather than into oxidized glutathione. In our study, we determined a decrease in the intracellular glutathione level in the damage model created by hydrogen peroxide. This may be due to the fact that GSH moved out of the cell, formed glutathione–S-conjugates, or was oxidized to GSSG. Subsequently, GSSG is reduced to GSH by NADPH dependent glutathione reductase.

Resveratrol and resveratrol + hydrogen peroxide groups showed significant increase in intracellular GSH (p<0.05). Resveratrol stimulates the increase in the intracellular GSH pool. This is directly due to the intracellular effect of resveratrol because resveratrol is removed before treating the cells with hydrogen peroxide. Preincubation with resveratrol increased the GCLC protein expression. It has been suggested that resveratrol increases GSH level via GCLC induction. On the other hand, the increase in cellular GSH concentration increases the glutathione peroxidase protein expression. Furthermore, the increase of glutathione reductase protein expression by resveratrol leads to increased GSH formation from GSSG, produced during GPx-catalyzed reduction of H2O2 in HCAE cells.

In our study, resveratrol induced glutamate-cysteine ligase, glutathione peroxidase and glutathione reductase protein expression in HCAE cells (p<0.05). Resveratrol increased the level of the glutamate-cysteine ligase enzyme protein more than the level of the glutathione reductase protein. This may be due to the fact that the GSH/GSSG ratio is regulated by the glutamate-cysteine ligase mediated GSH biosynthesis.

The above results demonstrated that resveratrol prevented cell injury induced by hydrogen peroxide in HCAE cells.

In the literature, the protective effect of resveratrol in different cells is shown by mRNA expression and/or activities of the enzymes related to the glutathione cycle in vitro. Rat aorta smooth muscle cells were preincubated with 25, 50 and 100 µM resveratrol for 24, 48 and 72 hours and it was found to induce GSH levels and to increase the glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase and NAD(P)H:quinone oxireductase-1 activities. These data are thought to support the cardioprotective effects of resveratrol26. It was found that resveratrol induced antioxidant enzymes in rat cardiomyocytes (glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and NAD(P)H:quinone oxireductase-1) and increased resistance to oxidative stress25.

We believe that it is logical to evaluate the mechanism of the protective effects of resveratrol by analyzing the glutathione levels and glutamate-cysteine ligase, glutathione peroxidase and glutathione reductase levels via nuclear factor E2-related factor 2 (Nrf2) transcription in HCAE cells in vitro.

Rubiolo et al.34 found that preincubation with resveratrol (25, 50 and 75 µM; 6, 24 and 48 hours) protected primary rat hepatocytes from oxidative stress by increasing the glutathione reductase, glutathione peroxidase, superoxide dismutase, catalase and NAD(P)H:quinone oxireductase-1 and glutathione-S-transferase activities. When compared with control cells, Nrf2 transcription factor was increased in
the cells pretreated with resveratrol (50 µM; 6, 24 and 48 hours). The Nrf2 protein expression and increased mRNA were correlated with high activities of phase II enzymes and antioxidants after preincubation with resveratrol. Brito et al.\textsuperscript{16} showed that the cardioprotective effect of 50 µM resveratrol in bovine aorta endothelial cells (14 h incubation) was due to its regulatory role in cellular redox state (increasing intracellular GSH level and reducing peroxynitrite) besides the classical antioxidant effect. Resveratrol (50 µM, 30 minutes) reverses the total glutathione decrease due to hydrogen peroxide (100 µM H\textsubscript{2}O\textsubscript{2}; 30 minutes) in primary cortical astrocyte cells.\textsuperscript{35} Kode et al.\textsuperscript{14} showed the effects of tobacco extracts such as a decrease in GSH levels and GCLC activities to be reversed by 10 µM resveratrol. These results correlate with our data. Signal pathways of resveratrol to increase the antioxidant enzymes and phase II enzymes in human coronary artery endothelial cells are not clear. Further studies are needed to highlight the mechanisms of resveratrol in inducing protein expression of antioxidant enzymes.

Recently, Nrf2 has been described. Nrf2 is a transcription factor and under normal conditions, it is located in the cytoplasm with the actin binding protein kelch-like ECH associating protein 1 (Keap 1). As the cell is exposed to oxidative stress or electrophilic compounds, Nrf2 dissociates from its repressor protein (Keap 1), and after migrating to the nucleus, it binds to the antioxidant responsive elements and then activates phase II detoxifying and antioxidant genes.\textsuperscript{14,36-38} Nrf2 regulates the expression of many antioxidant genes, including glutamate-cysteine ligase (GCL), hemooxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1).\textsuperscript{39-42}

In conclusion, our \textit{in vitro} study is the first one showing that resveratrol increases the glutathione level and glutamate-cysteine ligase, glutathione peroxidase-1 and glutathione reductase enzyme protein expressions in HCAE cells. This feature of resveratrol facilitates the understanding of some mechanisms of its cardioprotective effect and supports the development of new therapeutic strategies.

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Resveratrol protects coronary endothelial cells

Oya Sayin et al.


Sažetak

ZAŠTITNI UČINCI RESVERATROLA OD OŠTEĆENJA LJUDSKIH ENDOTELNIH STANICA KORONARNE ARTERIJE IZAZVANOG VODIKOVIM PEROKSIDOM IN VITRO

O. Sayin, N. Arslan i G. Guner

Oksidativni stres definira se kao neravnoteža između nastanka i razgradnje reaktivnih kisikovih vrsta. Cilj ove studije bio je istražiti može li resveratrol zaštititi ljudske endotelne stanice od oštećenja vodikovim peroksidom in vitro. U ovoj in vitro studiji na endotelnim stanicama koronarne arterije učinci resveratrola na sadržaj glutationa određeni su visokotlačnom tekućinskom kromatografijom. Učinci resveratrola na ekspresiju proteina enzima glutamat cistein ligaze, glutation peroksidaze-1 i glutation reduktaze određeni su metodom Western blot. Resveratrol je statistički značajno povećao sadržaj reduciranog glutationa (p<0,05). Resveratrol je povećao i ekspresiju proteina enzima glutamat cistein ligaze, glutation peroksidaze-1 i glutation reduktaze (p<0,05). Svi dobiveni rezultati potvrđuju da resveratrol ima zaštitni učinak od oštećenja endotelnih stanica koronarne arterije. Rezultati ove studije daju smjernice prema novim terapijskim pristupima koji bi se rabili za zaštitu od oksidativnog stresa koji se razvija u bolestima krvožilnog sustava.

Ključne riječi: Resveratrol; Ljudske endotelne stanice koronarne arterije; Oksidativno oštećenje