Resveratrol May Protect Plasma Proteins from Oxidation under Conditions of Oxidative Stress In Vitro

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Introduction

Oxidative stress is considered as the main cause in development of several chronic human diseases including cancer, diabetes, hypertension, cardiovascular diseases and aging. Reactive oxygen species (ROS) are generated under normal metabolic processes and are involved to some extent as signaling molecules and defense mechanisms as seen in phagocytosis, neutrophil function, and shear-stress induced vasorelaxation, however their excess/uncontrolled generation is deleterious which leads to oxidative stress. ROS may damage all types of biological molecules. Oxidative damages to proteins, lipids or DNA may all be seriously deleterious and may be concomitant. However, proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators; hence, the effect of damage to one molecule is greater than stoichiometric.

It is well established that exposure of proteins to reactive oxygen species (ROS) can alter the physical and chemical structure of the proteins causing oxidation of side-chain groups, protein scission, backbone fragmentation, cross-linking and unfolding. These alterations are consequent to formation of new reactive groups, generation of protein carbonyls (PCO), oxidation of -SH groups, formation of dityrosine containing cross-linked protein products known
as advanced oxidation protein products (AOPPs) and many others.\textsuperscript{5-7}

There is overwhelming evidence to suggest that nutritional sources of antioxidants, such as fruits, vegetables, tea or wine would attenuate tissue damage caused by oxidative challenges.\textsuperscript{8} Polyphenolic compounds, abundant in these nutritional sources, could play a major role in enhancing the antioxidant system.\textsuperscript{9,10} Resveratrol, \textit{(trans-3,5,4′-trihydroxystilbene)} is a polyphenolic phytoalexin present naturally in grapes, peanuts and red-wine. It has been reported to possess diverse biochemical and physiological actions, including estrogenic, antiplatelet, and anti-inflammatory properties.\textsuperscript{11} Recently resveratrol is found to be a highly potent antioxidant which could inhibit free radical generation in brain, spinal cord, liver and red cell membrane.\textsuperscript{12-15} However, depending on the concentration of the phytoalexin and the cell type, it has also been shown that resveratrol can exhibit pro-oxidant properties, leading to oxidative breakage of cellular DNA in the presence of transition metal ions such as copper. It has also been proposed that such a pro-oxidant action could be a common mechanism for anticancer and chemopreventive properties of resveratrol.\textsuperscript{16,17}

In the present study, we have evaluated the \textit{in vitro} effect of resveratrol on markers of protein oxidation: PCO, AOPPs and -SH groups (total thiol), in human plasma subjected to \textit{in vitro} oxidative stress. The study was aimed to test the efficiency/efficacy of resveratrol to maintain redox status of plasma proteins.

\section*{Experimental}

\subsection*{Materials and methods}

\textit{Collection of blood, isolation of plasma}

The protocol of study was in conformity with the guidelines of the Allahabad University Ethical Committee. Human venous blood from seventeen different healthy volunteers was obtained by venipuncture in heparin. Plasma was obtained by centrifuging the blood at 1800 x g for 10 min at 4 °C.

\textit{Induction of oxidative stress and \textit{in vitro} experiments with resveratrol}

Oxidative stress was induced \textit{in vitro} by incubating the plasma with 10$^\text{-5}$ mol L$^{-1}$ tert-butylhydroperoxide (t-BHP) for 30 min at 37 °C. The procedure of induction of oxidative stress was similar to already published reports.\textsuperscript{15,18} The effect of resveratrol was evaluated by co-incubating the plasma with t-BHP and resveratrol at different doses for 30 min at 37 °C. Parallel control experiments were also performed in which resveratrol was replaced with an equal amount of solvent.

\textit{Determination of advanced oxidation protein products}

Determination of advanced oxidation protein products (AOPPs) in plasma was based on spectrophotometric detection according to Witko-Sarsat \textit{et al.}.\textsuperscript{5} Briefly, 200 µL of diluted plasma (\textit{i.e.} one volume of plasma + five volumes of phosphate-buffered saline (PBS)) as test; 200 µL of chloramine-T solution (0-100 µmol L$^{-1}$) for calibration and 200 µL of phosphate buffered saline (PBS) as blank were taken in three separate tubes. 10 µL of 1.16 mol L$^{-1}$ potassium iodide and 20 µL of acetic acid were added making the final volume of the assay medium to 2.3 mL, absorbance was measured immediately at 340 nm. The chloramine-T absorbance at 340 nm was linear within the range of 0 to 100 µmol L$^{-1}$. Readings were taken in triplicates of 17 independent experiments. Concentration of AOPPs was expressed as µmol L$^{-1}$ of chloramine-T equivalents.

\textit{Determination of plasma protein carbonyls}

Plasma protein carbonyls (PCO) content was measured according to procedure of Levine \textit{et al.}.\textsuperscript{5} Plasma samples were taken in two tubes as test and control. 4.0 mL of 10 mmol L$^{-1}$ 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 mol L$^{-1}$ HCl was added to the test sample and 4.0 mL of 2 mol L$^{-1}$ HCl, alone was added to the control sample. The contents were mixed thoroughly and incubated for 1 h in the dark at 37 °C. The tubes were shaken intermittently every 10 min to facilitate the reactions with proteins. After that, 20% TCA (m/v) was added to both tubes and the mixture left in ice for 10 min. The tubes were then centrifuged at 3,500 rpm for 20 min to obtain the protein pellets. The supernatant was carefully aspirated and discarded. The protein pellets were washed three times with ethanol:ethyl acetate (1:1, v/v) solution to remove unreacted DNPH and lipid remnants. Finally protein pellets were dissolved in 6 mol L$^{-1}$ guanidine hydrochloride and incubated for 10 min at 37 °C. The insoluble materials were removed by centrifugation. PCO content was determined by measuring the absorbance of the supernatant at 370 nm. Each sample was read against the control. Readings were taken in triplicates of 17 independent experiments. The PCO content was calculated by using an absorption coefficient ($\varepsilon$) of 22,000 M$^{-1}$ cm$^{-1}$ and data was expressed in nmol L$^{-1}$ of plasma.

\textit{Estimation of plasma -SH groups}

Plasma -SH groups (total thiols) were estimated according to Kitajima’s method,\textsuperscript{19} based on the ability of
the -SH group to reduce 5, 5'-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow colored anionic product whose OD is measured at 412 nm. Readings were taken in triplicates of 17 independent experiments. The concentration of the -SH group was expressed as µmol L\(^{-1}\) of plasma.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA. The results are reported as means ± SD. Statistical differences were analyzed using two-way ANOVA; and the differences were considered to be significant when \(p < 0.05\).

**Results and Discussion**

Reactive oxygen and nitrogen species such as superoxide anion, hydroxyl radicals, hydrogen peroxide, lipid peroxyl radicals, nitric oxide, and peroxynitrite, are generated in different cellular systems through enzymatic and non-enzymatic reactions. Many pathological conditions are associated with excessive production of these reactive species which can attack key proteins, lipids and DNA, alter signal transduction pathways, destroy membranes and subcellular organelles, and subsequently result in apoptosis and cell death.\(^{13}\)

Proteins are likely to be major targets, as a result of their abundance in cells, plasma, and most tissues, and their rapid rates of reaction both with many radicals and with other oxidants (e.g., peroxides, excited states, peroxynitrite, chloramines, and ozone). It is of importance to consider that the extent of damage to any particular component does not necessarily correlate with the importance of such damage, the output of damage depends more upon the type of molecule getting damaged in comparison to quantity of damage. Thus a low level of damage to a critical species may be of much greater significance than massive damage to a nonessential target.\(^{20}\)

Induction of oxidative stress in plasma in vitro by incubating with \(t\)-BHP (10\(^{-5}\) mol L\(^{-1}\)) caused a significant \((p < 0.001)\) increase in the PCO and AOPPs level and decrease in -SH group content compared with basal values. Incubation of plasma with resveratrol resulted in significant protection against the \(t\)-BHP-induced oxidative stress as evidenced by the decrease in AOPPs (Figure 1) and PCO levels (Figure 2) and increase in -SH groups content (Figure 3). It was observed that the effect of resveratrol was dose/concentration dependent, a higher effect was observed with increase in concentration. Resveratrol protected generation of PCO and depletion of -SH groups at lower concentration (0.1 µmol L\(^{-1}\)), however effect of resveratrol in protection of generation of AOPPs was not significant at this concentration.

The presence of carbonyl groups in proteins has been used as a marker of ROS-mediated protein oxidation. Generation of protein carbonyl derivatives occurs by oxidative modifications of proteins either by the \(\alpha\)-amidation pathway or by oxidation of glutamyl side chains, which leads to formation of a peptide in which the N-terminal amino acid is blocked by an \(\alpha\)-ketoacyl...
The levels and mutual relations between different redox forms of thiols in plasma are decisive for the plasma redox capacity, which determines its proper function. For all these reasons, plasma thiols should be considered not only as metabolites transported between organs and tissues, but also as functionally important plasma components. Reduced concentration of -SH groups has been reported in several chronic diseases including aging and diabetes. Protection of -SH group oxidation by resveratrol at lower concentration not only proves its strong antioxidant potential but may also help in preserving the correct structure of proteins.

Several in vivo studies also prove the health protective biological activities of resveratrol. Resveratrol has been shown to increase plasma antioxidant capacity and inhibitory effect on the colon carcinogenesis in wistar rats. Systemic administration of resveratrol blocked the increase in platelet aggregation that was induced in the rabbits by a hypercholesterolaemic diet. In stroke-prone, spontaneously hypertensive rats, resveratrol significantly reduces markers of oxidative stress such as glycated albumin in serum, and 8-hydroxyguanosine in urine. Furthermore, in guinea pigs, resveratrol was found to induce the activities of quinone reductase 1(QR1) and catalase in cardiac tissue, and decrease the concentration of ROS generated by menadione. Trans-resveratrol can be absorbed from grape juice in biologically active quantities and in amounts that are likely to cause reduction in the risk of atherosclerosis. In 1999, Blaridi et al. reported the stimulation of endogenous adenosine release by oral administration of quercetin and resveratrol in man. Resveratrol also causes an increase in plasma adenosine levels and blood nucleosides in human subjects. These results indicate that resveratrol can suppress pathological increases in the oxidation/peroxidation of macromolecules in vivo.

In recent years, the antioxidative activity of food constituents has been investigated extensively in an effort to characterize their efficacy as natural dietary antioxidants. Our observation on the protective effect of resveratrol at the concentration 10 to 0.1 µmol L$^{-1}$, on oxidation of plasma proteins under in vitro oxidative stress assumes significance because it has been reported that after consumption of resveratrol rich diet, the plasma value of resveratrol could be achieved in micromolar range. Also, the effects exerted by resveratrol get importance because the maintenance of protein redox status is of fundamental importance for cell function, since structural changes in proteins are considered to be among the molecular mechanisms leading to endothelial dysfunction and development of many chronic diseases including aging. On the basis of its antioxidant effects and its potential to suppress pathological increases in the oxidation/peroxidation of macromolecules in vivo, resveratrol may protect plasma proteins from oxidation.
of our experiments and effects shown by resveratrol we substantiate existing evidence for the strong antioxidant property of resveratrol. Our findings provide an additional evidence to the possible mechanism(s) by which resveratrol exerts health beneficial effects.

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References