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ORIGINAL ARTICLE

The Effect of Resveratrol on mRNA Levels of DNA polymerase Beta and Oxidative DNA Damage In H2O2-Induced Human Colon Cancer HT-29 Cells

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ABSTRACT

Introduction: Resveratrol (3,4,5-trihydroxystilbene), a polyphenol found in high levels in grape skin, has recently attracted huge attention because of its anti-carcinogenic properties. Protective effects of resveratrol against oxidative damage in DNA may be due to its ability to stimulate DNA repair pathways such as the base excision repair (BER).

Methods: This study aimed to investigate the effect of resveratrol on gene expression of DNA polymerase beta (DNA pol β), the primary polymerase involved in BER, in H2O2-inducedoxidative human colon cancer HT-29 cells. The 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) level and mRNA expression level of DNA polymerase beta (pol β) was measured after human colon cancer HT-29 cells were treated with 100 µM H2O2for 30 min followed by exposure with 75 µM of resveratrol for 48 h.

Results: The level of 8-OHdG was significantly increased by H2O2 treatment, but resveratrol pretreatment of cells prior to H2O2 treatment led to a significant reduction of 8-OHdG to the levels similar to those observed in controls. Analysis of qRT -PCR data by one way ANOVA revealed that resveratrol pretreatment also caused a measurable increase in the mRNA expression of DNA polymerase beta (DNA pol β) comparing to that of H2O2-treated and control cells.

Conclusion: The cancer-preventive effects of resveratrol may be due in part to stimulation of base excision repair processes. Our data confirm that resveratrol exerts its anti-oxidant and scavenging properties through a reduction in 8-OHdG level.

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INTRODUCTION

Increased production of reactive oxygen species (ROS) is one of the main forces driving the onset and progression of cancer [1]. ROS are chemically active molecules generated continuously in cell as natural by products of the normal metabolism of oxygen including the respiratory chain, xanthine oxidase, and cytochrome P450 protein [2, 3].

Additionally, environmental sources such as chemicals and radiation can induce ROS production [4]. Increased levels of ROS production can lead to lipid peroxidation of cellular membranes, oxidative modification of proteins and DNA damage [5]. Single strand breaks (SSB) and double strand DNA breaks (DSB) were considered as the most significant DNA damage. Eight-oxoguanine G (8-OHdG), an oxidized form of guanine, is the most common biomarker for the evaluation of oxidative DNA damage by ROS [6].

Mammalian cells possess several mechanisms to repair DNA damage including direct repair, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination repair (HR) and non-homologous end joining repair (NHEJ) [7]. Defects in DNA

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repair mechanisms have been associated with several human diseases such as cancer [8]. Among these DNA repair mechanisms, BER is a highly conserved biological pathway in human and is responsible for repairing most of the oxidative DNA base lesions such as 8-oxoG [9]. In order to repair DNA base lesions, BER is initiated by8-oxoguanine DNA glycosylase (OGG1), a bifunctional glycosylase which recognizes and cleaves 8-oxoguanine and also catalyzes 3' of a basic site (AP site). Following this initiation step, basic site is incised by apurinic endonuclease 1 (APE1), producing SSB intermediate, which is a substrate for DNA repair synthesis, and 5'-end clean-up by DNA polymerase beta (DNA pol β) and/or Pol Lambda (Pol λ), followed by nick ligation by a DNA ligase [10]. Plant polyphenols as dietary antioxidants protect intracellular components from oxidative damage induced by ROS and possesses anti-cancer property [11, 12].

Among polyphenols, resveratrol (trans-3,40,5-tri-hydroxystilbene), is one of the best known studied dietary antioxidants, found in various plant species such as mulberries, peanuts and red grapes [13]. Several investigations have cited the positive effects of resveratrol as a powerful anti-inflammatory and anti-diabetic [14, 15]. Resveratrol is an inhibitor of ribonucleotide reductase resulted in suppression of DNA synthesis and cell proliferation, tumor initiation, promotion, and progression [16]. Resveratrol have had antioxidant affects against chemical-induced oxidative stress including ethanol and hydrogen peroxide (H2O2) [17]. Resveratrol is able to prevent the increase in ROS following exposure to tobacco-smoke condensate and H2O2 [18]. H2O2 causes SSBs and DSBs in variety of cells [19].

The most prominent pathway for the auto-repair of DNA damages induced by H2O2 is BER [20]. Although the DNA auto-repair system exits in the cells, the frequent repairing of damaged DNA increases the ratio of mistakes made in the pairing of bases, which is the basis of the molecular pathology of many diseases [17]. DNA Pol β is a main enzyme in BER pathway and subsequently DNA protection [21]. Experimental evidence suggests a possible linkage between enzyme expression of BER pathway and dietary antioxidants [22]. Protective effects of resveratrol could be related to increased expression the mRNA level of repair enzymes including hOGG1 [17].

The effect of resveratrol on gene expression of DNA pol β is not fully established. Therefore, the current study aimed to examine the relationship between induction of cellular DNA damage by H2O2 treatment and mRNA expression level of DNA pol β following pretreatment with resveratrol in colon cancer cell linesHT-29. Additionally, we measured 8-OHdG level, as a biomarker for the evaluation of oxidative DNA damage by H2O2, in resveratrol treated HT29 cells.

MATERIALS AND METHODS

In **Cell Culture**

The human colon adenocarcinoma HT29 cell line was purchased from Pasteur Institute of Iran and cultured in Dulbecco's modified Eagle's medium (DMEM; Euroclone,) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 100 units/ml penicillin and 100 µl/ml streptomycin (both from Sigma, Germany) at 37 °C in humidified atmosphere containing 5% CO_2. The growing medium was changed every two days. After 80% confluence, cells were subcultured and plated into T-25 flasks and incubated until confluence.

Drug Treatment

Resveratrol (USA) was dissolved in ethanol and prepared immediately prior to use**.** When cells were 60% confluent, cells were pretreated for 48 h with resveratrol added to the culture medium in a final concentration of 75 µM [23]. Following incubation, to induce DNA oxidant damage, some cells were treated with fresh hydrogen peroxide (100 µM) for 30 min at 37 °C. After treatment with H_2O_2 for 30 min, the cells were washed with phosphate buffered saline (PBS). Harvested cells were kept at -80 ºC until use. Some cells were collected for 8-oxoG assay and the others were collected for RNA extraction. Each experimental treatment was performed in duplication at 3 different times.

Quantification of 8-OHdG

After various treatments, cell-conditioned medium was harvested and the level of 8-OHdG was determined using ELISA kit (Hangzhou, Eastbiopharm Co., Ltd., Hangzhou, China) according to the manufacturer's instruction.

Measurement of expression of DNA repair enzymes

RNA isolation and cDNA synthesis

Cells were grown to ~80% confluence. Total cellular RNA was prepared from cells using RNX- plus reagent (Sinaclon, Iran) according to the manufacturer's instruction. In brief, starting from RNX- plus lysates, total RNA was extracted using chloroform, precipitated with isopropanol and washed with 75% ethanol. The resulting pellet was dissolved in nuclease-free water. DNA impurity was eliminated by incubation with RNAase free DNAase I (5 U) (Fermentas, Lithuania) in the presence of RNAase inhibitor

(5U) for 1 h at 37°C. RNA concentrations of extracted RNA were calculated from the absorbance at 260 nm. RNA quality was evaluated by spectrometry to determine the A260/A280 ratio and by electrophoresis on a 1% agarose gel. A260/A280 ratios of ≥ 1.8 were considered acceptable and agarose gel analysis indicated the presence of two major bands corresponding to the 18S and 28S rRNA. RNA solutions were stored at −80 °C prior to cDNA synthesis. Complementary DNA (cDNA) was synthesized from 2µg RNA using the First Strand cDNA Synthesis Kit with random hexamer and oligo (dT) primers according to the manufacturer's instructions (Parsazmon, Iran).

Real-time quantitative RT-PCR (qRT-PCR)

Real-Time qRT-PCR was run in the light cycler real time PCR system (Roche Diagnostics, Mannheim, Germany). Each qRT-PCR reaction contained, 2µl of cDNA (10-fold diluted), 0.5 µL of 5 mmol/L solutions of each of the forward and reverse primers, and 10µL of 2**x** SYBR green DNA PCR Master Mix (YektaTajhisAzma Company, Iran) in a total volume of 20 µL. The samples were loaded in duplicates. All incubations included an initial denaturation at 95°C for 10 minutes and 40 cycles (15 seconds at 95 °C, 30 se at 56 °C and 30 s at 60 °C). Primer pairs were designed using Allel ID software (Applied Biosystems). The primer sequences used for amplifications related to DNA pol β were:5′-ACTGTGGTGTTCTCTATTTCA-3′ (forward) and 5′- CAAGGGACGGATGGTGTA -3′ (reverse) and those related to the housekeeping gene, RPLO (internal control), were as follows: 5′-GAAGGCTGTGGTGCTGATGG- 3′ (forward) and 5′-CCGGATATGAGGCAGCAGTT- 3′(reverse). Product specificity was confirmed by ethidium bromide- agarose gel (1.5% w/v) electrophoresis of the amplified products and by melting curve analysis (Tm). Target transcript levels were calculated by $2^{-\Delta\Delta CT}$ method [24].

Statistics

All experiments were performed three times independently and each experiment included two replicates. Results are the means ± SEM. Statistical evaluations were performed with SPSS16 analytic software (SPSS, Inc., Chicago). One-way ANOVA with a LSD post hoc test was used to determine the difference in the experimental variables between the studied groups. *P*-values less than 0.05 were considered significant.

RESULTS

Resveratrol Suppresses Formation of 8-OxoG by H_2O_2.

To evaluate the effect of resveratrol on oxidative damage in HT 29 cell line, we used H_2O_2 to induce oxidative DNA damage. 8-Hydroxydeoxyguanosine (8-OHdG), an accepted biomarker of ROS-induced oxidative base lesions in DNA, was determined in all groups. As shown in Figure 1, resveratrol pretreatment prior to H_2O_2 treatment significantly suppressed 8-OH-dG formation compare to that of H2O2 treatment cells ($P < 0.05$).

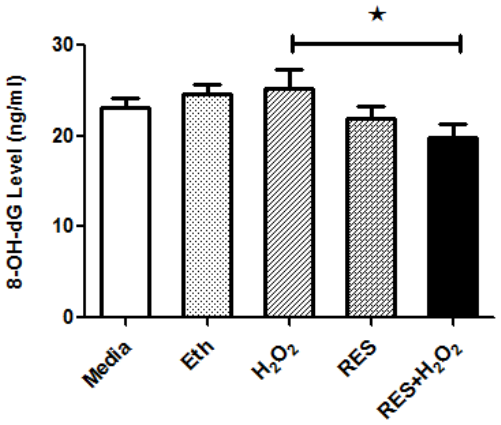

Figure 1. Resveratrol pretreatment reduces H2O2-induced 8-OH-dG formation ($P< 0.05$). Cells were pretreated with 75μM resveratrol for 48h and then some cells were incubated with 100 μM H2O2 for 30 min. Bars represent mean ± SEM (n=3). EtOH= Ethanol, RES= Resveratrol.

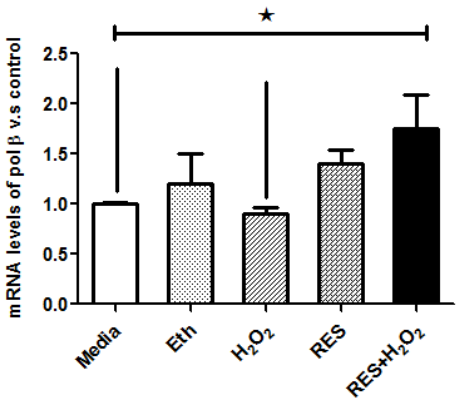

Figure. 2. Effects of resveratrol pretreatment on relative mRNA levels of DNA pol β under H_2O_2 challenge in HT-29 cell line. The ratio of the levels of mRNA for DNA pol β gene in H_2O_2, resveratrol and resveratrol plus H_2O_2 treated cells to those of saline treated cells (media) were assessed following the normalization of the expression levels of mRNA for DNA pol β to those for RPLO. * indicates that the different is significant (P<0.05). The histograms in each of the panels are the mean ± SEM., n=3.EtOH= Ethanol, RES=Resveratrol

DISCUSSION

Antioxidants play an important preventive role in the development of diseases such as cancer and diabetes [25, 26]. Resveratrol is an antioxidant and the free-radical scavenging properties of resveratrol are responsible for its protective effect against chemical-induced oxidative DNA damage and anti-mutagenic activity [27]. During the past decades, investigators have tried to elucidate details of the mechanisms underlying the antitumor activity of resveratrol [16].

The effect of resveratrol pretreatment for 48 h on 8-OHdG level has been studied, a biomarker of H_2O_2-induced oxidative DNA damage in HT 29 cell line. Pretreatment of cells with resveratrol significantly decreased 8-OH-dG production. Indeed, pretreatment of cells with resveratrol is able to prevent the accumulation of intracellular ROS and DNA breaks induced by H_2O_2. Similarly, resveratrol and its analogues have strong ability in decreasing 8-OHdG level and hydroxyl radical scavenging activity [28]. Resveratrol reduce the 8-OHdG level following induction of oxidative DNA damage [29, 30]. H_2O_2inducesoxidation of bases involved in formation of 8-OHdG in cell [31, 32]. Indeed, H_2O_2 is dissociated into two OH• groups, eventually, the reaction of OH•radical with guanine leads to the formation of 8-OHdG and facilitates mutagenic process and tumorigenesis [32]. The antioxidant mechanism of resveratrol may be related to its ability to scavenge hydroxyl radicals [33]. These findings are collectively in line with the suggestion that resveratrol has strong antioxidant activities by decreasing the 8-OHdG level. Finally, other flavonoids such as melatonin, quercetin with antioxidant activity, protect cells against H_2O_2-induced oxidative DNA damage [34, 35].

Some foods rich in antioxidants such as vitamin C, vitamin E, and polyphenols might have effects on regulation of DNA repair processes and thus might prevent oxidative damage of DNA [36]. The second approach was whether resveratrol may protect DNA from damage by enhancing DNA repair activity. Cellular DNA repair ability can be estimated through determination of mRNA or protein level, protein levels and by activity assay of DNA repair enzymes [37]. Base excision repair (BER] is an important cellular defense mechanism in eukaryotic cells, responsible for repair of oxidative DNA damage, and used to counter the genotoxic effects of ROS [38]. Therefore, an increase in expression of the involved genes in BER probably alters the cellular states because of oxidative stress conditions.

In our study, we focused on expression of DNA Polβ, the primary polymerase involved in BER. We found resveratrol pretreatment prior H_2O_2 exposure induces DNA Polβ expression. Therefore, induction of DNA Polβ gene expression by resveratrol is associated with reduced formation of nucleotide strand breaks and increased DNA repair process. To date, the role of resveratrol in DNA Pol β gene regulation in chemical -induced oxidative in vivo and in vitro is not fully established. Resveratrol and other antioxidant caused a measurable increase in the mRNA expression of human 8-oxoguanine DNA glycosylase (hOGG1], the most important

enzyme in base excision repair system, in cells with or without oxidant exposure [28, 30, 35].
Notably, in our experiment, when resveratrol was used alone, did not produce significant changes in the expression level of DNA Pol β and also 8-OHdG level compare to control group. Our results are in consistence with Ellen L.et al that found resveratrol treatment alone did not increase in activity of the DNA Pol β. DNA Polβ is a 39-kDa protein made up of a single polypeptide, which contains two domains, 8 and 31 kDa. DNA Polβ through its bifunctional deoxyribose phosphate lyase and polymerase activities maintains genomic integrity and stability maintains genomic integrity and stability [39].

One of the important purposes of the present study was to determine whether resveratrol pretreatment prior induction of oxidative stress in cells stimulated DNA BER activities. We found evidence of a resveratrol induced increase in expression of BER enzyme. In fact, DNA Pol β, which catalyzes the rate limiting step in BER, was significantly increase by resveratrol pretreatment. Accordingly, it is suggested that the antioxidant mechanism of resveratrol may be related to its ability induction of BER.
The limitation of our study was assessment the effect of resveratrol on DNA Polβ in expression level, not protein level or enzyme activity. Furthermore, we did not study dosing-time dependency of resveratrol onDNA pol β expression.

Conclusion

The present study shows the capability of resveratrol to prevent oxidative damage caused by H_2O_2 exposure. Our report is one of the earliest studies showing resveratrol affection of the BER capacity by regulating DNA pol β expression at the level of transcription. It might lead to a hypothesis that explains the molecular basis of anticancer properties of resveratrol.

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