The Role of GSH Depletion in Resveratrol Induced HeLa Cell Apoptosis

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***Abstract*—The dual role of Resveratrol (Rsv) in cell apoptosis was recently reported by its anti/pro-oxidant activities. The involvement of ROS and GSH was thus investigated in Rsv-induced HeLa cell apoptosis. Rsv, higher than 10μM, elevated the intracellular ROS but reduced $O_2^{\cdot-}$ and GSH levels. ROS scavengers (Tempol, catalase) could not inhibit the apoptosis. Treatment with GSH modulators DTT or BSO were resulted up-regulation or down-regualtion GSH levels, but both enhanced Rsv-induced HeLa cell apoptosis. However, BSO could not prevent the DTT+Rsv treated HeLa cells from apoptosis. Further, Rsv-induced HeLa cell apoptosis was accompanied by activation of caspase 3 but not caspase 9, neither did the loss of mitochondrial membrane potential. Conclusively, the changes of ROS by Rsv were not tightly correlated with apoptosis in HeLa cells. However, intracellular GSH levels are partially related to Rsv-induced HeLa cell apoptosis via a mitochondrial independent manner.**

Keywords- Resveratrol; ROS; Apoptosis; GSH; ROS scavenger; GSH modulator

I. INTRODUCTION

Converging data from Pharmaceutical and clinical studies have shown that Rsv, as an antioxidant, could reduce the risk for some types of human cancers [1,2]. Rsv could alter the expression and/or activities of signaling molecules, mitochondria-associated factors, transcriptional factors, tumor suppressor genes, and cellular glutathione, leading to apoptosis to prevent cancer development [2,3]. Nevertheless, it has been shown that Rsv could also exhibit pro-oxidant properties in term of ROS production [5,6,7]. The ROS depleted GSH and permitted overactivation of protein kinases, that in turn overactivate redox-sensitive transcription factors and responsive genes that promote cancer cell survival, growth, and proliferation [8,9,10]. The exact mechanisms involved in redox-mediated cell apoptosis were not fully understood and the protective effects of Rsv remained controversial. In this paper, we evaluated the role of GSH depletion in Rsv-induced HeLa cell apoptosis and investigated whether ROS scavengers or GSH modulator could rescue Rsv-induced HeLa cells apoptosis. We also studied the participation of caspases-3, caspases -9 and the changes of mitochondrial membrane potential.

II. MATERIALS AND METHODS

A. Reagents and Cell culture

The Resveratrol (Rsv), ROS scavengers catalase (CAT) and Tempol, dithiothreitol (DTT), and GSH modulator L-S,R-buthionine sulfoximine (BSO), 0.25% trypsin-EDTA solution, and other chemicals were purchased from Sigma (Sigma-Aldrich Chemical Co., St. Louis, MO). Rsv was dissolved in sterile dimethylsulfoxide (DMSO) as a stock solution of 200 mM. HeLa cells were obtained from Cell Bank (Shanghai IBCB, China) and cultured in DMEM supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin at 37 °C with 5% CO_2. Cells were routinely grown in tissue culture flasks and harvested with trypsin-EDTA in near-confluence.

B. Cell viability and apoptosis

Cell viability was determined by MTT assay [11]. Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by Hoechst-33342 staining. Briefly, HeLa cells treated with or without Rsv were detached with trypsin-EDTA in DMEM and washed in PBS, resuspended in 50 μl of 3% paraformaldehyde. Then the cells were stain by 8 μg/ml of Hoechst-33342 in PBS. Apoptotic cells were recorded by fluorescence microscope (Zeiss, Germany). A minimum of 400 cells were counted for each experiment for Apoptotic rates calculation. Fragmented apoptotic DNA was quantitatively analysed by bisbenzimide spectrofluorometry with fluorescence microplate reader (Ex/Em=365nm/460nm) (Varioskan Flash, Thermo, USA) [12]. Cell numbers were calculated by Hoechst/propidium-iodide stain [13,14]. Standard curve were made by a series of cell numbers in 96 well plates from 5×10^3 to 5×10^6.Cells were lysed, sonicated and mixed with 1.15 μg/ml Hoechst-33342 or 20 μg/ml Pi dye at a 1:9 ratio and kept in 37 °C for 30min. DNA-associated Hoechst/Pi fluorescence was measured at 460nm/620nm. Subtracting necrotic cells (Pi stained cells) from the total number of cells (Hoechst stained cells) gave the number of non-necrotic cells in each treatment.

C. Detection of intracellular ROS , $O_2^{\cdot-}$ and glutathione (GSH) concentration

Intracellular ROS was detected by 2',7'-Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Ex/Em =

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488nm/525 nm). $O_2^{\bullet-}$ was detected by Dihydroethidium (DHE) (Ex/Em=300nm/610 nm). GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) (Ex/Em =492nm/517nm). In brief, cells were incubated with the designated dose of Rsv with or without the ROS scavengers and GSH modulator for 48 h. They were then incubated with 20 μM H_2DCFDA or 5 μM DHE or 5μM CMFDA at 37°C for 30 min and washed with PBS, and detected using fluorescence microplate reader. ROS and $O_2^{\bullet-}$ levels were calculated as mean fluorescence intensity (MFI) per 1,000 non-necrotic cells.

D. Quantification of Caspase -3 and -9 activities and mitochondrial membrane potential (ΔΨm)

The activities of caspase-3 and caspase-9 were assessed using the Colorimetric Assay Kits by the labeled Ac-DEVD-pNA and Ac-LEHD-pNA (BYT, Jiangsu, China). Briefly, cells in each treatment were collected and suspended in 5 volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM EDTA, 0.5% NP40, 0.5 mM DTT). Results were shown as the absorbance at 405nm per 100μg protein. The changes in mitochondrial membrane potential were analyzed using a mitochondria-selective dye JC-1. Cells were treated as indicated drugs for 48h at 37°C then stained with JC-1 (10μg/ml) for 15 minutes, washed once and analyzed in green / red fluorescence (Ex/Em = 485/530nm / Ex/Em=550/595nm) detection buffer. The ratio of red to green fluorescence intensity was determined as a measure of ΔΨm.

III. RESULTS AND DISCUSSION

A. Effects of Rsv on ROS, $O_2^{\bullet-}$ and GSH levels in HeLa cells

In present work, Rsv treatment caused inhibition of HeLa cell growth in concentration- and time-dependent manner (Fig. 1A). The formation of apoptotic bodies was observed obviously at concentrations as low as 20 μM Rsv in HeLa cells (Fig. 1B).After 48h treatment with Rsv DNA fragmentation was found significantly at 40 μM, also an IC_{50} (Fig 1A), within the range found in Leukemia cells from 32-100μM [15,16]. Thus the concentration was considered to be suitable to differentiate the levels of apoptosis in the presence of Rsv versus apoptosis in the presence of Rsv and ROS scavengers or GSH modulators.

Our data showed that the intracellular ROS levels were significantly increased in Rsv-treated HeLa cells at 48h, as shown in Fig. 2A. $O_2^{\bullet-}$ levels were rising with a relative low concentration of Rsv in HeLa cells till 10μM (Fig. 2A). These results are consistent with other reports, which showed that increased intracellular ROS plays an important role in Rsv-induced apoptosis in T-lymphoblastic leukemia CEM-C7H2 cells [17], in prostate cancer PC-3 and DU-145 cells [18], and in human prostate cancer DU-145 cells [19]. Ahmad et al. observed that exposure of human leukaemia (HL-60) cells to low concentrations of resveratrol (4–8μM) inhibited caspase activation and DNA fragmentation induced by incubation with H_2O_2 [6]. Likewise, in rat hepatocytes exposed to ferrylmyoglobin-induced oxidative stress, physiological concentrations (100pM–100nM) of resveratrol exerted pro-oxidant activities [20]. At these concentrations, resveratrol elicited pro-oxidant properties as evidenced by an increase in intracellular $O_2^{\bullet-}$ concentration.

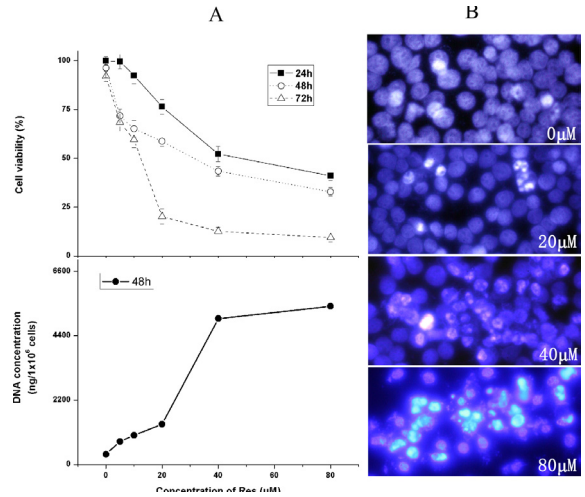

Figure 1. (A) Inhibition of proliferation of HeLa cells and quantitative analysis of DNA fragmentation by Rsv treatment as the indicated time. A total number of $5x10^6$ cells for each concentration of Rsv were centrifuged and lysed in lysis bufer. Lysate and medium preparation were diluted in Tris Sodium EDTA buffer containing 1mg/ml Hoechst-33342. The results shown are representative of 6 independent experiments. (B) The apoptosis in HeLa cell by Rsv treatment at 48h.

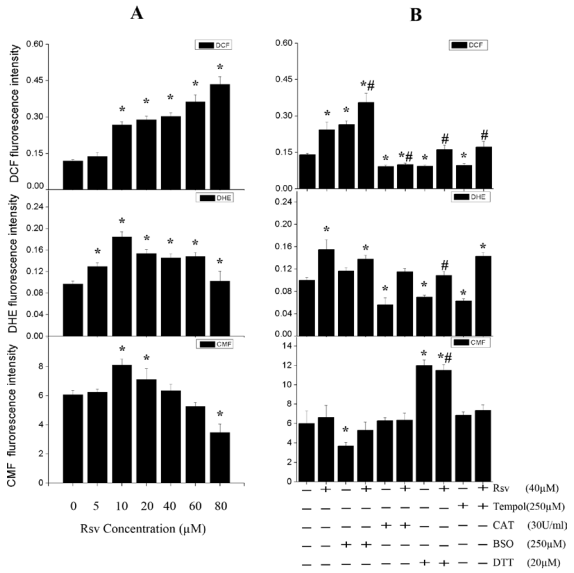

Figure 2. (A) Effects of Rsv on intracellular ROS, $O_2^{\bullet-}$ and GSH in HeLa cells at 48h. (B) Effects of ROS scavengers, GSH modulators on ROS, $O_2^{\bullet-}$ and GSH levels in Rsv-treated HeLa cells at 48h. *, $P < 0.05$ compared with the control group; #, $P<0.05$ compared with Rsv group. The results shown are representative of 3 independent experiments. (Student's t-test).

Being different with ROS, GSH levels were rising with lower concentration of Rsv in HeLa cells till 10μM (Fig 2A). Then, it dropped down steadily towards the highest concentration of Rsv. In a study by Losa [21], stilbene appeared to maintain the glutathione content in peripheral

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blood mononuclear cells with oxidative damage caused by 2-deoxy-D-ribose. In human blood platelets, resveratrol markedly decreased oxidation of thiol groups of proteins in these cells [22]. In a model of PMA application to mouse skin, resveratrol induced the restoration of ROS and glutathione levels, and also myeloperoxidase, glutathione reductase and SOD activities [23]. Those evidences and present findings have provided interesting insight into the systematic effect of Rsv on intracellular redox state. These data seem to support both anti- and pro-oxidant activity of Rsv, depending on the concentration of Rsv and the cell type.

B. The mechanism involved in Rsv- treated HeLa cell apoptosis.

We attempted to determine whether ROS scavengers prevent Rsv-induced cell apoptosis by reducing intracellular ROS levels. In contrast to our expectation, Tempol did not significantly reduce the level of $O_2^{\bullet-}$ in HeLa cells treated with 40μM Rsv at 48h (Fig. 2B). Further, Catalase inhibited ROS but did not inhibited insignificant $O_2^{\bullet-}$ production in Rsv-treated HeLa cells (Fig. 2B). None of these ROS scavengers could reduce apoptosis in Rsv-treated HeLa cells at 48h (Fig. 3A). It is possible that they did not prevent cell apoptosis because the higher dose effect of Rsv at 40μM. However, neither did we observe the anti-apoptotic results of elevated concentration of Tempol (500μM) and CAT (60 unit/ml) on 40μM Rsv-treated HeLa cells nor the anti-apoptotic results of both ROS scavengers on 20μM Rsv-treated HeLa cells (data not shown). Additionally, our other results indicated that the changes of ROS by Rsv were not entirely correlated with apoptosis in HeLa cells, since 80μM Rsv showed distinct drop down of $O_2^{\bullet-}$ content. Further, up to higher concentration of 100-160μM, Rsv induced strong apoptosis (data not shown) but did not show an increased level of ROS compared to that in 40μM Rsv treatment. With regard to the source of ROS, we had found that the Rsv did not change the mitochondrial membrance potential (ΔΨm), neither did the activities of caspase 9 (Fig. 3C) (Fig. 3D). However, the activation of the caspase 3 was confirmed in Rsv-treated HeLa cells (Fig. 3C), consistently with Rsv induced apoptosis in other cells [24,25,26].These results support that ROS separately could not play a critical role in Rsv-induce HeLa cell apoptosis.

Thiols are able to act as antioxidants by reacting with ROS at relatively high drug concentrations. Also it can be pro-oxidants producing ROS in vitro [27,28]. Interestingly, in our data, DTT (20μM) showing the significant augmentation of apoptosis in Rsv-treated HeLa cells did not significantly amplify the loss of mitochondrial membrane potential (ΔΨm) and ROS levels (Fig. 2B) (Fig. 3A) (Fig. 3D), consistently with work by Tartier et al. in DTT-induced HL-60 cell apoptosis [29]. Under physiological conditions, thiol-mediated antioxidant reaction had also been shown to result in intracellular generation of $O_2^{\bullet-}$ [30]. DTT had been shown to efficiently protect cells from apoptosis or death in other reports [30, 31]. Controversially, DTT intensified apoptosis accompanied with decreased $O_2^{\bullet-}$ levels in Rsv-treated HeLa cells (Fig. 2B). The antioxidant activity of DTT involved in those DTT-mediated inhibitions of cell death did not satisfy present finding.

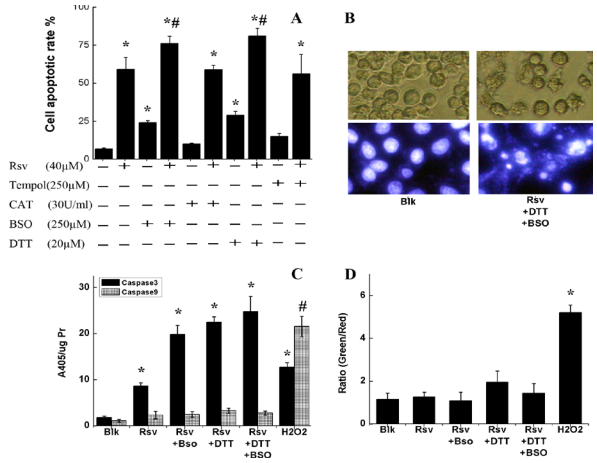

Figure 3. (A) Effects of ROS scavengers, GSH modulators on Rsv-induced apoptosis in HeLa cells at 48h. A minimum of 400 cells were counted for each experiment for Apoptotic rates. (B) The morphological changes in HeLa cells by BSO (500μM)+DTT+Rsv treatement at 48h. (C) Effects of GSH modulators on the activations of caspase 3 and 9 in Rsv-treated HeLa cells at 48h. (D) Effect of GSH modulators on mitochondrial membrane potential. The ratio of red to green fluorescence intensity was determined as a measure of ΔΨm. H_2O_2 (20μM) was used to induce HeLa cell apoptosis as a positive control at 48h. *, $P < 0.01$ compared with the control group; #, $P<0.01$ compared with Rsv group. The results shown are representative of 3 independent experiments. (Student's t-test).

Present study indicated the depletion of intracellular GSH content by 10-80μM Rsv in HeLa cells. In this context, we employed BSO to study whether the intracellular GSH depletion was involved into the increased ROS accumulation in Rsv-treated HeLa cells. BSO (250μM) enhanced Rsv-induced ROS accumulation and Rsv-treated HeLa cell apoptosis at 48h (Fig. 2B)(Fig. 3A). Obviously, the depletion of intracellular GSH was tightly correlated with ROS production. Then we focus on the effects of ROS scavengers and DTT on intracellular GSH levels in Rsv-induced HeLa cells apoptosis. Interestingly, DTT, not ROS scavengers, could alter the levels of intracellular GSH in Rsv-treated HeLa cell (Fig. 2B). However, the pro-apoptotic effects by the usage of DTT or BSO, seemed contradictory to each other, were both observed in Rsv-treated HeLa cells (Fig. 2B)(Fig. 3A). It challenged the idea that the GSH depletion alone is necessary for the Rsv-induced HeLa cell apoptosis. In order to understand further, we used BSO again in the treatment by Rsv plus DTT in HeLa cells at 48h (Fig. 3B) (Fig. 3C) (Fig. 3D). Interestingly, additional BSO (500μM) could not prevent the DTT plus Rsv treated HeLa cells from apoptosis. Those findings proved that the intracellular GSH rather than ROS were partially correlated with apoptosis in Rsv-treated HeLa cells via a mitochondrial independent manner (Fig. 4).There must be another paralleling way involved in Rsv-induced HeLa cell apoptosis without sensitivity to GSH depletion.

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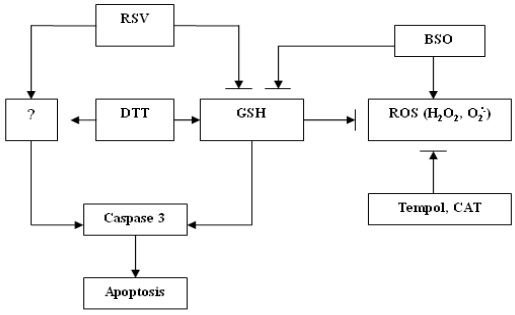

Figure 4. The diagram for Rsv-induced HeLa cell apoptosis via certain a pathway independent of mitochondria, show that the intracellular GSH rather than ROS are partially related to Rsv-induce cell apoptosis.

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