Combined metformin and resveratrol confers protection against UVC-induced DNA damage in A549 lung cancer cells via modulation of cell cycle checkpoints and DNA repair

YONG-SYU LEE, BARBARA B. DOONAN, JOSEPH M. WU and TZE-CHEN HSIEH

Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA

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Abstract. Aging in humans is a multi-factorial cellular process that is associated with an increase in the risk of numerous diseases including diabetes, coronary heart disease and cancer. Aging is linked to DNA damage, and a persistent source of DNA damage is exposure to ultraviolet (UV) radiation. As such, identifying agents that confer protection against DNA damage is an approach that could reduce the public health burden of age-related disorders. Metformin and resveratrol have both shown effectiveness in preventing several age-related diseases; using human A549 cells, we investigated whether metformin or resveratrol, alone or combined, prevent UVC-induced DNA damage. We found that metformin inhibited UVC-induced upregulation of p53, as well as downregulated the expression of two DNA damage markers: γH2AX and p-chk2. Metformin also upregulated DNA repair as evidenced by the increase in expression of p53R2. Treatment with metformin also induced cell cycle arrest in UVC-induced cells, in correlation with a reduction in the levels of cyclin E/cdk2/Rb and cyclin B1/cdk1. Compared to metformin, resveratrol as a single agent showed less effectiveness in counteracting UVC-elicited cellular responses. However, resveratrol displayed synergism when combined with metformin as shown by the downregulation of p53/γH2AX/p-chk2. In conclusion, the results of the present study validate the effectiveness of metformin, alone or with the addition of resveratrol, in reducing the risk of aging by conferring protection against UV-induced DNA damage.

Introduction

The notion that aging and the quality of longevity of living organisms including humans may be improved can be found in century-old historical records (1). The topic is of interest to social and public health experts as well as basic and clinical scientists. On the one hand, life expectancy of humans has clearly benefitted from modern day medical advances that have eradicated several diseases that at one time plagued humankind (2,3); on the other hand, it is known that two thirds of people die daily from age-related causes pointing to aging as the single most significant risk factor for many human diseases (4).

Do interventions and dietary modalities exist that can delay the onset of aging, or counteract the deleterious effects of environmental insults impinging on the integrity of our genome, widely considered as a major risk factor for disease-associated aging? It is our hypothesis that disease-associated, subclinical aging (in relative terms) is a multistage biological process whose duration and manifestation can be dynamically regulated by environmental and dietary, as well as genetic factors. As a corollary, therefore, age-related diseases can be managed in humans using agents that attenuate cellular responses to external agents capable of damaging the integrity of the DNA in the genome.

Accumulation of DNA damage is regarded as a cause for aging, tumorigenesis and other inheritable diseases (5). Exposure of cells to ultraviolet (UV) radiation results in the generation of DNA damage and lesions, which, if left unrepaired, can directly or indirectly lead to dysfunctional cellular events and possibly disease-associated aging. Multiple changes occur to counteract UV-induced DNA damage, including the upregulation and activation of transcription factor p53. p53 is known to play an essential role in controlling various downstream target genes, frequently as different sets by a stimuli-specific [ionizing radiation, UV or reactive oxygen species (ROS)] mechanism (6,7). Thus, UV-induced p53 mediates cell cycle arrest and DNA repair and changes the expression of ataxia telangiectasia mutated (ATM) protein kinase and γH2AX (H2AX phosphorylated on Ser139) which then can be used as indicators to monitor the ongoing DNA damage induced externally by exposure to UV or by endogenously generated reactive oxygen species (ROS) (8-11).

In this study, we used human A549 cells to test and validate the ability of metformin and resveratrol, alone and in combination, to confer protection against exposure to UVC, known to contribute to aging by damaging genomic DNA. Metformin, with demonstrated efficacy to restore insulin sensitivity in type II diabetes (12,13), was selected for its activity

Correspondence to: Dr Tze-Chen Hsieh, Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA
E-mail: tze-chen_hsieh@nymc.edu

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in managing age-related diseases including cardiovascular disorders and cancer (14-19) by targeting the AMP-dependent protein kinase (AMPK) (20,21), and extension of lifespan (22). The choice of resveratrol (*trans*-3,4',5-trihydroxystilbene), found abundantly in grapes (23,24), was based on its plethora of biological activities (14-19), and documented antioxidant, anti-inflammatory (25-28) and anti-diabetic activities (29), as well as its ability to modulate and activate SIRT1, a key protein for the aging process (30-33), and prolongation of life span in mammals and other species (33-35). Results of our studies support the effectiveness of metformin, alone or combined with resveratrol, in reducing the risk of aging by conferring protection against UV-induced DNA damage.

Materials and methods

Reagents. Fetal calf serum, Eagle's minimum essential medium, penicillin and streptomycin were purchased from Cellgro, Inc. (Herndon, VA, USA). Metformin (1,1-dimethylbiguanide chloride) and resveratrol were obtained from Calbiochem (La Jolla, CA, USA) and LKT Laboratories (St. Paul, MN, USA), respectively. All other chemicals and solvents used were of analytical grade. Primary antibodies: p53, cyclin B1, cyclin E, cdk1, cdk2, Rb, p53R2, cdc25C, actin and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Other antibodies for the present study were obtained from the following sources: serine-139-phosphorylated histone H2AX (Upstate Biotechnology, Inc., Lake Placid, NY, USA); p21 (Cell Signaling Technology, Inc., Beverly, MA, USA); plk1 (Invitrogen Corp., Carlsbad, CA, USA), and p-chk2 (Cell Signaling Technology, Inc.). All other chemicals and solvents used were of analytical grade.

Cell culture. The lung carcinoma cell line A549 was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in Eagle's minimum essential medium supplemented with 2 mM glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin and 10% fetal bovine serum. Cells were seeded at a density of $5x10^4$ cells/ml and passaged by washing the monolayers with phosphate-buffered saline (PBS) followed by a brief incubation with 0.25% trypsin/EDTA.

Preparation of chemicals and treatment. Metformin and resveratrol were dissolved in dimethyl sulfoxide (DMSO) and stored at -80˚C as 500 and 50 mM stock, respectively. Treatments included: 0, 2.5 or 25 μM of resveratrol or 5 mM metformin alone or in combination (5 mM metformin + 2.5 μM resveratrol or 5 mM metformin + 25 μM resveratrol). For UV irradiation experiments, the cells were first primed with metformin or resveratrol for 48 h and washed with PBS to remove the chemicals. The primed cells were exposed to 20 J/m^2 UVC for 10 sec, after which the UVC-exposed cells were maintained in culture for 4 h, and harvested for further analysis.

Preparation of cell extracts and western blot analysis. To determine the level of protein expression of various genes examined in the present study, control and treated cells were harvested and lysed in ice-cold RIPA buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM dithiothreitol and 10 μl/ml protease inhibitor cocktail from Sigma Chemicals (St. Louis, MO, USA)]. The protein concentration of the cell lysates was determined using the Coomassie protein assay kit (Pierce, Rockford, IL, USA) with BSA as the standard. The proteins in cell lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane as previously described (36). The blots were incubated overnight with various primary antibodies, followed by incubation for 1 h with secondary antibodies. The blots were detected with an ECL detection system (LumiGLO Peroxidase Chemiluminescent Substrate kit; KPL Biotechnology, Inc., Gaithersburg, MD, USA), quantified by densitometry and normalized against actin as the loading control as previously described (37).

Cell cycle analysis. Cell cycle phase distribution was assayed by flow cytometry as previously described (38-40); the histograms obtained were quantified for the percentage of cells in the respective phases (G_1, S and G_2/M) of the cell cycle.

Results

Effects of resveratrol and metformin on DNA damage response under normal and UVC-induced conditions. DNA damage is an important factor contributing to carcinogenesis and the aging process. Resveratrol (18,19,23,41,42) and metformin (14,43) have each been reported to have beneficial effects against cancer cells, e.g., by suppressing proliferation and induction of apoptosis (44-47), and aging, e.g., prolonging life span in model systems (11,33,48-51). However, the effects of these two chemicals alone or in combination on p53 expression in the context of UV-induced DNA damage have not been investigated. Accordingly we monitored changes in the level of total p53. A pronounced increase (~2.3-fold) in p53 expression was observed in the UVC-induced control cells compared to the non-exposed control cells (Fig. 1A), suggesting that exposure to UVC resulted in the induction of total p53. Correspondingly, no significant change in p53 expression was observed in control cells treated with either resveratrol (2.5 and 25 μM) or metformin alone (5 mM) or in combination (2.5 or 25 μM resveratrol combined with 5 mM metformin) (Fig. 1A). In contrast, under the UVC exposed condition, treatments resulted in a decrease in p53 expression of 17-21% by resveratrol, ~60% by metformin and 59-74% by the combined treatment (Fig. 1A). These results are consistent with the interpretation that metformin alone or in combination with resveratrol can prevent UVC-induced p53 activation. Next, we tested whether resveratrol and metformin may induce DNA damage by affecting the integrity of genomic DNA by analyzing changes in the DNA damage marker γH2AX. In non-stressed cells, the combination of 5 mM metformin and 25 μM resveratrol resulted in ~15% decrease in γH2AX expression (Fig. 1B). Under the UVC-induced condition, a slight increase in γH2AX expression was observed in cells treated with 2.5 or 25 μM resveratrol (Fig. 1B). Surprisingly, metformin alone or in combination with resveratrol inhibited UVC-induced γH2AX expression (Fig. 1B). Thus, data on

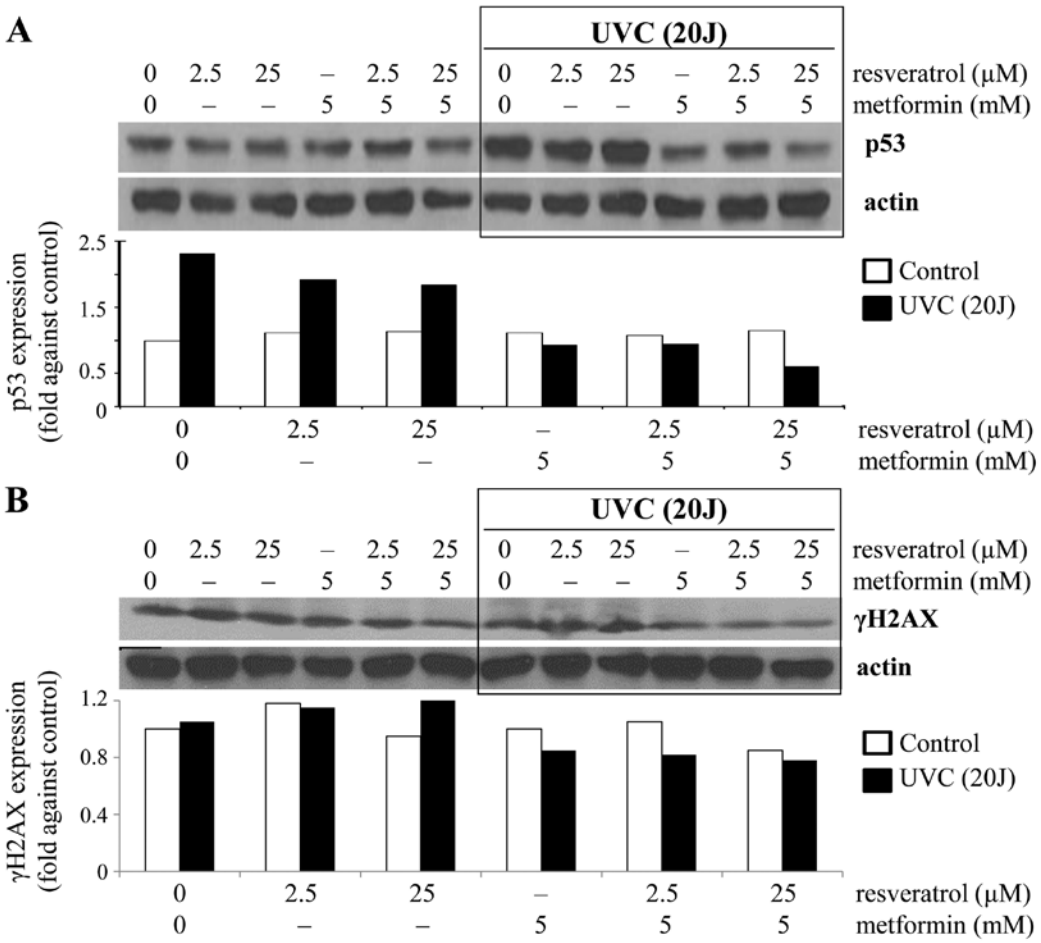

Figure 1. Effects of resveratrol and metformin on DNA damage response in A549 cells, with or without exposure to UVC. In non-UVC exposed conditions, cells were treated with resveratrol (2.5 and 25 μM), metformin (5 mM) or in combination (2.5 or 25 μM resveratrol combined with 5 mM metformin) for 48 h. For UVC-exposed experiments, cells were primed for 48 h as non-UVC exposed cells, which were then followed by exposure to 20 J/m^2 UVC for 10 sec. Both UVC exposed and non-exposed cells were maintained in culture for another 4 h and then harvested for further analysis. (A) Changes in p53 protein expression in cells treated with different agents with or without UVC exposure were analyzed by western blot analysis. (B) The total protein expression level of γH2AX was also determined. The intensity of the specific immunoreactive bands was quantified by densitometry and expressed as a fold difference against actin (loading control).

prevention of DNA damage by metformin and/or resveratrol resulting from the exposure to UVC as assayed by γH2AX expression generally agreed with measurements of p53 changes.

Changes in cell cycle phase transition and expression of specific signaling proteins impinging on cell cycle control by resveratrol and metformin under UVC-induced conditions. Alteration in p53 expression could induce an arrest in cell cycle progression. Since minimum effects on p53 resulted from treatment by resveratrol or metformin under non-UVC-induced conditions, we next focused only on cells exposed to UVC. We first determined the effects of resveratrol and metformin on cell cycle progression by flow cytometry. Metformin alone and in combination with a low dose of resveratrol caused a significant decrease in the S phase cell population (13.7% in control vs. 8.8 and 7.3% in cells treated with 5 mM metformin alone and combined with 2.5 μM resveratrol, respectively). This decrease was accompanied by a concomitant accumulation in the G_1 phase cell population (59.1% in control vs. 69.2 and 70.9% in 5 mM metformin without and with addition of 2.5 μM resveratrol) (Table I). To gain additional information on the underlying causes for the observed cell cycle phase transition change, we measured levels of cell cycle regulatory proteins cyclin E/cdk2 specifically required for G_1 and S phase transition by western blot analysis. Results in Fig. 2A showed that

Table I. Effect of resveratrol or metformin on cell cycle distribution.

Treatment	UV (20 J/m^2) G_1	S	G_2/M
Control	59.1	13.73	22.60
Resveratrol (2.5 μM)	60.90	14.00	21.01
Resveratrol (25 μM)	53.90	13.14	21.13
Metformin (5 mM)	69.24	8.82	13.68
Resveratrol (2.5 μM) + metformin (5 mM)	70.87	7.26	14.81
Resveratrol (25 μM) + metformin (5 mM)	65.48	11.57	15.54

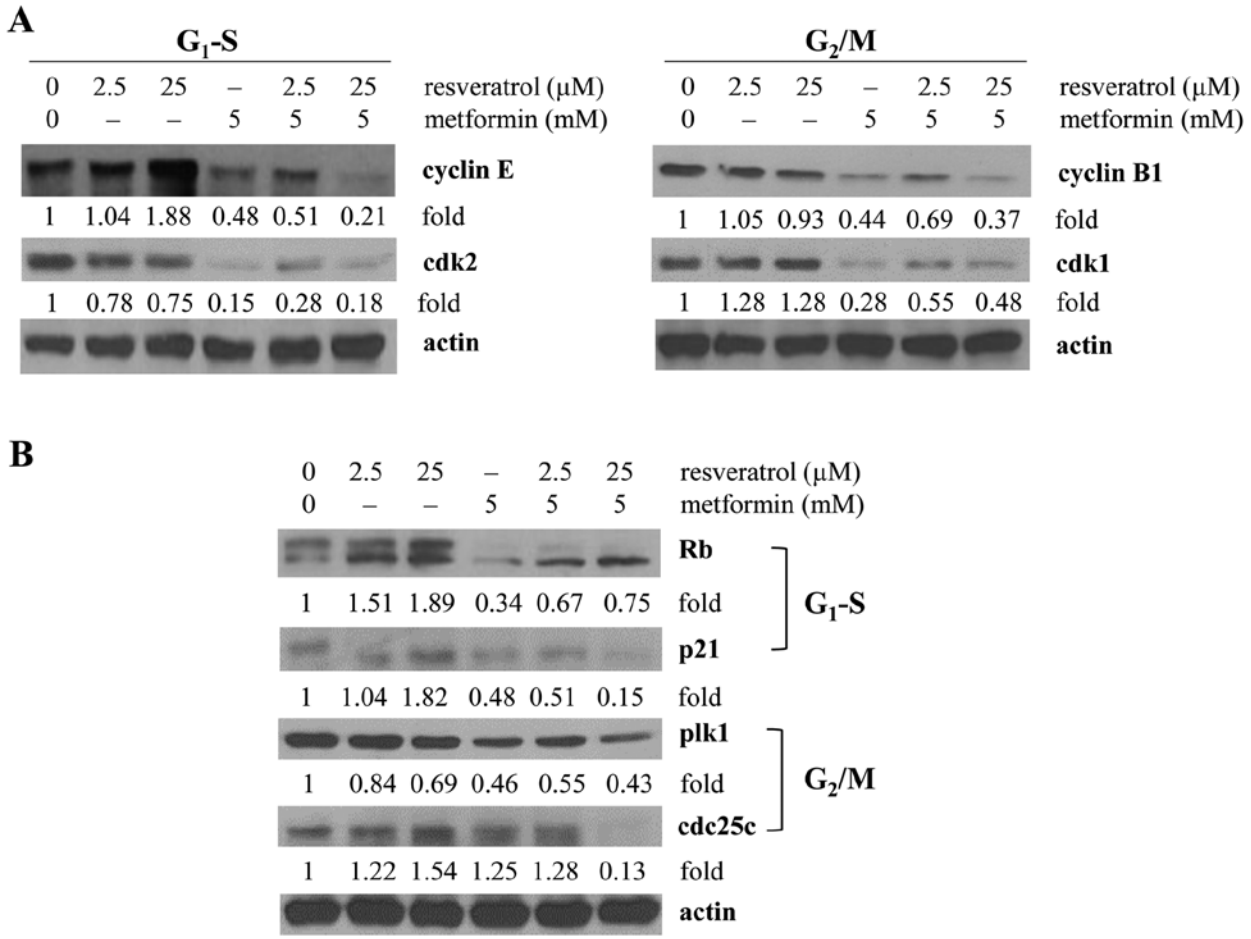

Figure 2. Changes in expression of various cell cycle regulatory proteins under UVC-exposed conditions following different treatments. A549 cells were primed with resveratrol (2.5 and 25 μM), metformin (5 mM) or in combination (2.5 or 25 μM resveratrol combined with 5 mM metformin) for 48 h and exposed to 20 J/m^2 UVC for 10 sec. Then cells were maintained in culture for another 4 h and harvested for further analysis. (A) Western blot analysis of the changes in cyclin E, cdk2, cyclin B1 and cdk1 protein expression. (B) The total protein expression levels of Rb, p21, plk1 and cdc25c were also determined by western blot analysis. The intensity of the specific immunoreactive bands was quantified by densitometry and expressed as a fold difference against actin (loading control).

metformin alone or in combination with resveratrol resulted in 52-79 and 72-85% suppression of cyclin E and cdk2, respectively. Since resveratrol alone did not significantly change cell cycle phase distribution under the conditions of exposure to UVC, the observed increase in cyclin E expression along with decreasing cdk2 expression following treatment by resveratrol may reflect may reflect a compensatory regulatory adjustment by cyclin E/cdk2 (Table I). As expected, however, a more pronounced decrease in the expression of cyclin E as well as cdk2 was detected in the cells treated with 5 mM metformin alone or with the addition of 25 μM resveratrol (Fig. 2A). Since metformin-treated cells also showed alterations in G_2/M progression, we assayed the changes in cyclin B/cdk1 expression. Following metformin treatment, downregulation of cyclin B1 (~56%) and cdk1 (~72%) was observed, but no further reduction on cdk1 expression occurred in cells treated with metformin combined with 2.5 or 25 μM resveratrol (Fig. 2A).

Cyclin E/cdk2 also plays a pivotal role in controlling Rb and entry into the S phase. We also examined whether control of cyclin E/cdk2 by metformin may result in a change in Rb. We found that under the same treatment conditions a ~66% suppression of Rb was observed that could contribute to the partial G_1 and S arrest elicited by metformin (Fig. 2B). Additionally, we also investigated the effects of metformin and resveratrol on the p53-p21 axis of the G1 and S checkpoint control in response to the UVC stimuli. Resveratrol (25μM) increased p21 expression (1.8-fold); whereas metformin alone or in combination with resveratrol resulted in >50% downregulation of p21 (metformin alone or with 2.5 μM resveratrol) and ~85% decrease of p21 (metformin with 25 μM resveratrol) (Fig. 2B). Activation of cyclin B1 and the cyclin B1/cdk1 complex is tightly controlled by phosphorylation and de-phosphorylation via plk1 and cdc25c, respectively (52,53). Therefore, cyclin B/cdk1-mediated G_2/M progression by metformin and resveratrol was further analyzed by the changes in plk1/cdc25c. A more pronounced decrease in the expression of plk1/cdc25c was detected in the cells treated with 5 mM metformin when combined with 25 μM resveratrol (Fig. 2B); in agreement with the cyclin B1/cdk1 changes we observed (Fig. 2A).

Control of DNA damage checkpoint and repair by resveratrol and metformin under UVC-induced conditions. DNA repair plays an important role in DNA damage responses during anti-carcinogenesis and anti-aging. Two tumor suppressor proteins, checkpoint kinase 2 (Chk2) and p53R2, associated

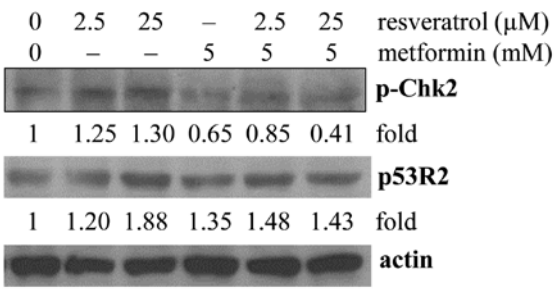

Figure 3. Control of DNA damage checkpoint and repair by resveratrol and metformin under UVC-exposed conditions in A549 cells. Conditions of priming, treatment and analysis were identical to those described in Fig. 2 legend. The total protein expression levels of p-chk2 and p53R2 were determined. The intensity of the specific immunoreactive bands was quantified by densitometry and expressed as a fold difference against actin (loading control).

with DNA damage checkpoint and DNA repair were further analyzed in response to UVC. In cells treated with resveratrol, p-chk2 and p53R2 were upregulated (Fig. 3), suggesting the activation of DNA repair by resveratrol under UVC treatment as a cellular protective mechanism from DNA damage. Metformin alone or in combination with resveratrol also resulted in the upregulation of p53R2 (Fig. 3) while downregulation of p-chk2 was found in cells treated with metformin alone or combined with resveratrol (Fig. 3). These results suggest that in cells exposed to UVC, metformin alone or with addition of resveratrol likely induces DNA repair via the upregulation of p53R2 without concomitantly invoking the activation of chk2.

Discussion

In previous studies, we examined multiple gero-preventive agents by focusing on their activity in controlling the mTOR/S6 signaling pathway (54). Both metformin and resveratrol were found to reduce constitutive DNA damage as indicated by the inhibition of the phosphorylation of H2AX (γH2AX) and ribosomal S6 protein expression (54). These results suggest that metformin and resveratrol, previously regarded as prime candidates for treating and preventing type II diabetes (12,13) and coronary heart disease (25-28), may offer the potential to be repositioned as candidate anti-aging drugs via the modulation of intrinsic aging factors. Indeed, the interest in resveratrol and metformin as gero-active chemicals may have started much earlier stemming from efforts to identify and develop caloric restriction mimetics (CRMs) based on the longstanding observation of McCay in the 1930s that a reduction in caloric intake retarded aging and extended median and maximal life span (55,56). Metformin, a biguanide currently considered the primary stay of management for diabetes, acts as a CRM in whole organisms by a multitude of mechanisms including at the metabolic level, the facilitation of fatty acid oxidation and glucose uptake in peripheral tissues as well as the suppression of hepatic gluconeogenesis. Molecularly, metformin not only serves as a sensor/modulator of cellular energy status, but also as an activator and inhibitor of AMPK and mTOR, respectively, in line with the vital role it plays in growth control. In tumorigenic settings, ample evidence has been obtained that exposure to metformin shows efficacy in patients diagnosed with malignant diseases including pancreatic (57) and breast cancer (58), and colorectal polyps (59). Since cancer is a disease associated with aging, it is not totally surprising that metformin also harnesses the capacity to improve life expectancy as befitting of a gero-protective agent. The same considerations may apply to resveratrol (14-19); its efficacy has been verified in all three stages of carcinogenesis (initiation, promotion and progression) in UVB and chemically induced skin tumor growth in mice (23,42) and in numerous animal models of human types of cancers (18,19). To date, the roles of resveratrol and metformin in preventing environmentally induced external damage that contribute to aging remain largely unknown.

In the present study, we focused on the roles resveratrol and metformin play in modulating the cellular and molecular changes in cancer cells elicited in response to UV challenge using as a model UVC-stressed and unstressed A549 cells. Specifically, DNA damage responses by resveratrol and metformin were assessed using changes in the level of expression of p53 and γH2AX. Our results showed that metformin at 5 mM significantly prevented the UVC-induced upregulation of p53; relatively, much less inhibition on UVC-induced p53 expression was observed in cells treated with resveratrol (Fig. 1A). In addition, inhibition of UVC-induced γH2AX expression was only observed in metformin and not resveratrol treatment conditions (Fig. 1B). These findings suggest that metformin has better preventive potential against UVC-induced DNA damage compared to resveratrol.

Flow cytometric analysis showed differential effects on cell cycle control by metformin and resveratrol in UVC-exposed cells (Table I). Metformin resulted in G_1 arrest and prevention of S and G_2/M entry accompanied by the inhibition in cell cycle-associated regulatory proteins, vis-à-vis, cyclin E/cdk2, Rb, p21 cyclin B1/cdk1 and plk1 (Fig. 2A and B). As well, metformin downregulated p-chk2, known to be involved in a p53-dependent cell cycle checkpoint for DNA damage (Fig. 3). In contrast, no significant change in cell cycle transition occurred in UVC-induced cells following resveratrol treatment (Table I), and only moderate changes to the above mentioned G_1 and S and G_2/M cell cycle regulatory proteins as well as p-chk2 were observed (Figs. 2A and B, and 3). It is also notable that the metformin induced cell cycle arrest at the G_1 and S checkpoint under UVC conditions was not mediated via the p53-p21 axis, but did show a correlation with the reduction in cyclin E/cdk2 and Rb (Fig. 2). Since metformin-mediated cell cycle control is decoupled from p53/p21, we also tested control of p53-mediated DNA repair by the changes in p53R2, a recently discovered DNA repair regulatory protein (60-62). Upregulation of p53R2 expression by metformin after UVC exposure was also observed (Fig. 3). The effects of metformin on UVC-induced cells may therefore be summarized as to include: i) the prevention of UVC-induced DNA damage as supported by downregulation of p-chk2, p53 and γH2AX (Figs. 1 and 3); ii) induction of cell cycle arrest (Table I) decoupled from p53/p21; and iii) fortification of DNA repair through p53-independent control of p53R2 (Fig. 3).

Compared to metformin, resveratrol as a single agent is marginally effective in UVC-exposed cells, suggesting that it operates by a different mechanism. This possibility is supported by our results showing that, as related to the prevention of

DNA damage in UVC-exposed A549 cells, synergism occurs between these two agents since cells are more susceptible to the co-treatment regimen than to each individual agent. This conclusion is made evident by the following results: i) suppression of DNA damage based on the downregulation of γH2AX/p53/p-chk2 (Figs. 1 and 3); ii) inhibition of cell cycle progression via modulation of cyclin E/cdk2, Rb, p21 cyclin B1/cdk1 and plk1/cdc25c (Fig. 2A and B); and iii) enhancement of DNA repair indicated by the upregulation of p53R2 (Fig. 3).

In conclusion, our results revealed the mechanistic aspects that underlie or contribute to the beneficial effects of metformin and resveratrol, two readily available and widely used agents, regarding their potential as single or combined candidates for conferring protection against UV-induced DNA damage and hence reducing the risk of aging.

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