International Journal of Health Sciences
March 2015, Vol. 3, No. 1, pp. 39-59
ISSN: 2372-5060 (Print), 2372-5079 (Online)

Published by American Research Institute for Policy Development
DOI: 10.15640/ijhs.v3n1a3
URL: http://dx.doi.org/10.15640/ijhs.v3n1a3

The Underlying Mechanisms for Resveratrol' Superinduction Effects

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Abstract

The superinduction effects of resveratrol was defined by its ability to activate estrogen response element (ERE) regulated luciferase reporter genes 2-3 fold higher than observed with 17β-estradiol (E_2). In HeLa cells, the superinduction effects of resveratrol with transfected estrogen receptor α (ERα) are cell density-dependent, but they are cell density-independent with ERβ. The superinduction effects of resveratrol are also shown to be independent of both ER and ERE. The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) pathway is critical for the superinduction effects of resveratrol in HeLa cells. The ERK1/2 inhibitor U0126 abolishes the superinduction effects of resveratrol, suggesting that this pathway is vital for resveratrol's superinduction effects. Western blotting results verified that resveratrol could activate ERK1/2 significantly 30 minutes after treatment. Resveratrol shows no effects on histone deacetylase (HDAC) activities both in vivo and in vitro by observing histone H_4 acetylation level changes. Our study suggests that the ERK1/2 pathway is involved in the superinduction effects of resveratrol in HeLa cells.

Keywords: resveratrol, ERK1/2, HDAC, superinduction, HeLa

Introduction

Resveratrol is a polyphenolic compound that is found naturally in grapes, peanuts, mulberries and a variety of medicinal plants (Romero-Perez et al., 1999). In plants, resveratrol functions as a phytoalexin that protects against fungal infections (Hain et al., 1990; Romero-Perez et al., 1999).

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Resveratrol has two geometric isomers: the (E) - or trans-isomer and the (Z) - or cis-isomer (Basly et al., 2000). Both of the two geometrical isomers are present in red wine (Goldberg et al., 1996).

Resveratrol's health benefits can be grouped into three main categories: it has cardioprotective activities (Das et al., 1999); it can act as an effective cancer chemopreventive agent (Jang et al., 1997); it can extend the lifespan in lower organisms and enhance the survival rates of some human cell lines (Finkel, 2003; Howitz et al., 2003; Wood et al., 2004).

Resveratrol binds to estrogen receptor preparations from MCF-7 cell extracts and rat uterine cytosol extracts (Basly et al., 2000; Bowers et al., 2000; Gehm et al., 1997). The relative binding affinities (RBA) of resveratrol for ERα and ERβ are shown to be not statistically different (Bowers et al., 2000). In 1997, Gehm et al. classified resveratrol as a phytoestrogen and reported that it is a superagonist in the MCF-7 cell line (Gehm et al., 1997). However, the superagonism of resveratrol is cell type dependent and the mechanisms for resveratrol's superagonist activity are still unknown. In our report, the superinduction effect of resveratrol also occurs in HeLa cells. The characteristics of this superagonism in HeLa cells are described and the underlying mechanisms are explored.

Resveratrol was reported to function as a superagonist in different cell lines (El-Mowafy and White, 1999; Gehm et al., 1997; Gehm et al., 2004). Since the definitions about resveratrol's superinduction effect are not consistent (Basly et al., 2000; Bowers et al., 2000; Gehm et al., 1997), three criteria have been proposed to determine if an agonist could work as a superagonist for estrogen receptors: 1) it could compete with certain receptor for its cognate ligand E_2; 2) it induces endogenous or luciferase reporter gene activation; 3) the difference between the gene activation activities must be statistically different.

Resveratrol has activation effects (Klinge et al., 2005; Miloso et al., 1999) or inhibition effects (El-Mowafy and White, 1999) on ERK1/2 in different experimental models. It appears that resveratrol activates ERK1/2 at low concentrations and inhibits it at high concentrations (Miloso et al., 1999).

Gene transcription is regulated by the change of chromatin structure. As the subunits of chromatin, nucleosomes are subjected to a variety of post-translational modifications, including phosphorylation, methylation, ubiquitination, ADP-ribosylation and acetylation.

Acetylation of the ε-amino groups of specific lysine residues is catalyzed by histone acetyltraferases (HATs) and hyperacetylation correlates with open, decondensed chromatin structure and gene activation (Vanhaecke et al., 2004). By contrast, histone deacetylase (HDAC) is responsible for removing these acetyl groups and causing gene repression. The possible mechanism for explaining resveratrol's superinduction effects was investigated to see if it has inhibition effects on HDAC activities.

Materials and Methods

Compounds and Hormones

17β-estradiol (E_2) and resveratrol were purchased from Sigma Chemical Company. All compounds were dissolved to make solutions of different concentrations by using absolute ethanol or dimethylsulfoxide (DMSO) vehicles. The typical concentrations are 1×10^{-8} M (E_2), 3×10^{-5} M (resveratrol), 5×10^{-3} M (methoxyacetic acid {MAA}), 3×10^{-7} M (trichostatin A {TSA}) and 5×10^{-6} M (1,4-diamino-2, 3-dicyano-1, 4-bis [2-aminophenylthio] butadiene {U0126}) respectively and as indicated in individual experiments.

Reporter Genes and Expression Vectors

The Vit^2-P36L luciferase reporter plasmid contains two copies of a 26-bp ERE from the xenopusvitellogenin A2 (Vit^2) gene linked to a minimal 36-bp promoter derived from the rat prolactin gene. By contrast, the plasmid P36 only has the 36-bp promoter and does not contain the ERE. Plasmid VitTK has a single ERE and a different promoter, the Hepes thymidine kinase promoter, rather than P36. The control for VitTK was the same plasmid lacking the ERE (TKSL). The full-length wild type human ERα cDNA, (Gly400), was obtained from P. Chambon (Tora et al, 1989). The full-length rat ERβ cDNA (Kuiper et al., 1996) was a gift from George G. J. M. Kuiper.

All estrogen receptors were also subcloned for expression into vectors containing the Rous Sarcoma Virus (RSV) promoter. RSV Neo was constructed in the same plasmid backbone, but expresses the neomycin phosphotransferase II gene instead of estrogen receptor.

Cell lines and Transfections

HeLa cells were obtained from American Type Culture Collection (Rockville, MD). All cells are routinely surveyed for mycoplasma using a polymerase chain reaction (PCR) method from Stratagene (La Jolla, CA). Cells are maintained and grown under estrogen-free conditions using media without phenol red and serum preparations that are treated with activated charcoal to remove endogenous steroid compounds.

Calcium-phosphate method was used for transfecting HeLa cells transiently after being modified for six well plates. Transient transfections were performed with a total 125 µl of the calcium phosphate solution for each well containing 2.5 µg DNA. Plates were then placed overnight in 5% CO_2. After 20 hours, cells were washed, fed with phenol red-free Dulbecco's Modified Eagle's Medium (DME) with 10% charcoal stripped newborn calf serum, treated with hormone or compounds as indicated, and returned to 10% CO_2. Each transfection was performed in duplicate and repeated at least three times.

After hormone treatment (E_2 or resveratrol) for 24 hours, cells were harvested in a triton lysis buffer, containing 50 mMTris, 50 mM 2 (N-morpholino) ethanesulfonic acids (MES) (pH 7.8), 1 mM 1,4-dithiothreitol (DTT), and 1% triton X-100. The lysate was assayed for luciferase activity, using a Monolight 2010 luminometer (Analytical Luminescence Laboratories, San Diego, CA). The luciferase activity of “none”, ethanol or DMSO treatment groups were taken as control and those of treatment groups were presented as relative fold changes.

Nuclear Extraction and HDAC Assay

HeLa cells were seeded in 100 mm dishes in the density of 1×10^6 cells/dish. On the third day, media was changed. 24 hours later, cells were washed and culture media was changed. Hormones or drugs were used to treat cells for 24 hours.

Nuclear extraction was prepared by following the protocol adapted from a mini-preparation technique (Deryckere and Gannon, 1994). HDAC activity was assayed by measuring the deacetylation of a fluorescent substrate (Jansen et al., 2004). HDAC activity was assayed according to the manufacturer's protocol for the HDAC fluorescent activity assay/drug discovery kit (Biomol, Plymouth Meeting, PA).

Western Blotting Assay

HeLa cells were seeded in 100 mm dishes in a density of 1.6×10^6 cells/dish. On the third day, media was changed. Cells were treated with hormones on the fourth day for desired time. Whole cell extracts were then prepared and cells were washed with ice-cold phosphate buffer solution (PBS). 0.5 ml 1X ice-cold cell lysis buffer plus 1mMphenylmethylsulfonyl fluoride (PMSF) was added to each plate (10 cm) after removing PBS. After 5 minutes incubation on ice, cells were scraped off the plate and transferred to microcentrifuge tubes. Cells were then sonicated on ice four times for 5 seconds each. Samples were centrifuged for 10 minutes at 4°C and the supernatants were transferred to new tubes. Protein concentrations were achieved by performing protein assays and equal amount of total protein was loaded on an SDS/4~12 % or 4~20% PAGE gel for separation at 105 volts for two hours. Then wet tank method was used to transfer proteins to a 0.2 μm nitrocellulose at 25 volts for 1 hour. After blocking in 5% milk (non-fat) for 1 hour, the membrane was incubated with different primary antibodies (as indicated in figures) overnight at 4°C. Primary antibodies are rabbit anti P42/44 antibody (Cell Signaling Technology, Inc.), anti-ERK1/2 antibody (Promega). Secondary antibody anti-rabbit IgG HRP (Cayman Chemical Company) and Enhanced Chemical Luminance Plus Western Blotting Detection System (Amersham Biosciences) were used for color development.

Histone Extraction and Immunoblotting

HeLa cells were seeded in 100 mm dishes in the densities of 0.8×10^6 cells/dish or 1.6×10^6 cells/dish. On the third day, media was changed. 24 hours later, cells were washed and culture media was changed. Hormones or drugs were used to treat cells for 30 minutes. Histone proteins from HeLa cells were isolated by acid extraction. Cells were washed with PBS and scraped off from the dishes. Then cells were pelleted by centrifugation and suspended in 300μl lysis buffer (10 mMHepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mMKCl, 0.5 mM DTT, and 1.5 mM PMSF.

Hydrochloric acid was added to a final concentration of 0.2M and the preparation was incubated on ice for 30 minutes. Samples were centrifuged at 11,000 g for 10 minutes at 4°C and supernatant was dialyzed against 200 ml 0.1M acetic acid twice (1~2 hours for each). Then the samples were dialyzed three times against 200 ml H_20 for 1hour, 3 hours and overnight, respectively. For immunoblotting, histones were separated on an SDS/4~12 % or 4~20% PAGE gel and were transferred to 0.2 µm nitrocellulose and probed with an anti-acetyl-histone H_4 antibody (UpState Cell Signaling Solutions).

All data was processed by usingPrism 3 (for graphing data) or ImageJ (for immunoblotting data). Statistical analyses are done with using One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests. Each experiment was repeated for at least three times. Statistical analyses were done and significance levels were shown as either "*" ($P< 0.05$) or "**" ($P< 0.01$).

Results

The agonistic effects of resveratrol with transfected ER(s)

As HeLa cells do not contain endogenous ER, they are transfected by exogenous ER(s) together with ERE bearing luciferase reporters. The ERα and ERβ subtypes have different expression profiles in the body and they are also functionally different (Kuiper et al., 1997; Sar and Welsch, 1999). The HeLa cells were transfected with either ER expression vector RSVhERα or RSVrERβ or both. The induction effects on luciferase activities are concentration dependent for both E_2 and resveratrol. For E_2, 10^{-8}M final concentration was chosen and it was shown to be the optimal concentration for being an estrogen receptor agonist (Gehm et al., 1997). However, for resveratrol, the final concentration of 3×10^{-5}M was chosen for all the experiments. Resveratrol is cytotoxic when it is used in higher concentrations in cell lines from different origins (Joe et al., 2002). Resveratrol acts as an agonist for both human ERα (hERα) (Figure 1A) and rat ERβ (rERβ) (Figure 1B). In addition, resveratrol showed superagonist activities in half of the experiments performed when HeLa cells were transfected with RSVhERα. By comparison, the superagonist activity was always observed when HeLa cells were transfected with RSVrERβ, but less potent than with RSVhERα (Figure 1A, B).

However, when the cells were transfected with both RSVhERα and RSVrERβ, the superinduction effects of resveratrol in HeLa cells were not observed anymore (Figure 1C). This suggests that the bioactivities of resveratrol are related closely with ER expression profiles in the body and could be tissue specific.

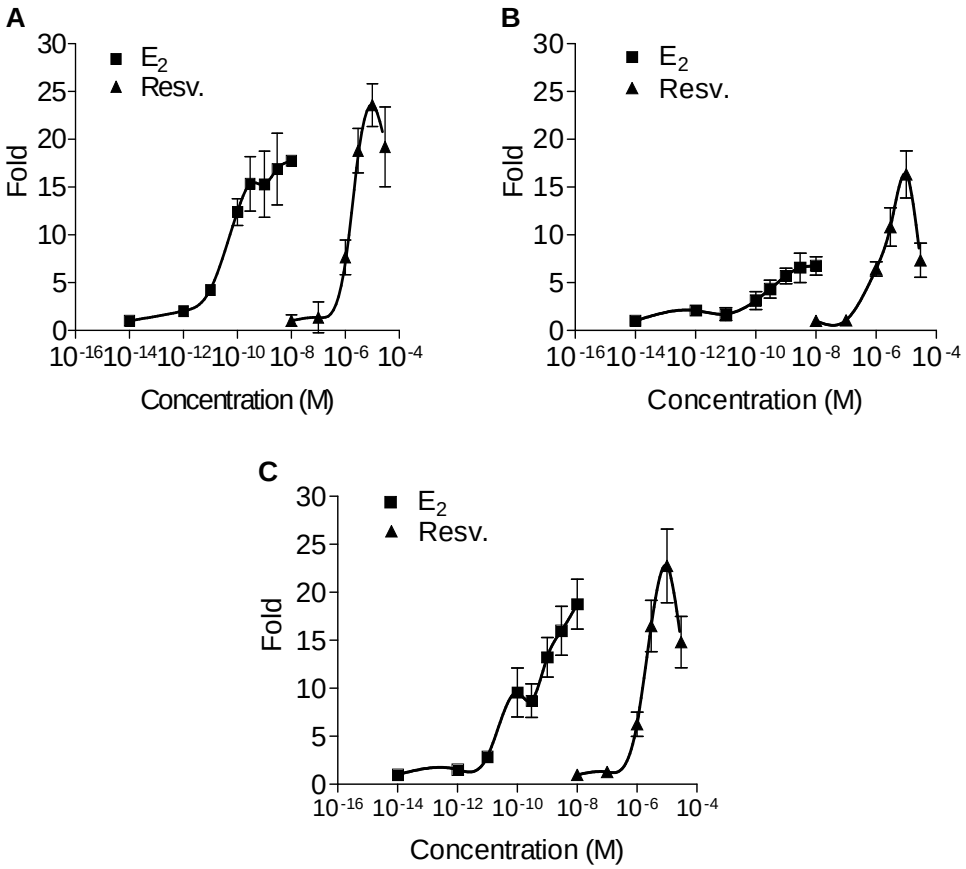

Figure 1: Both E_2 and Resveratrol Induce Luciferase Reporter Genes in a dose-dependent Fashion in HeLa Cells

HeLa cells were seeded in six well plates at the cell density of 0.15×10^6 cells/well. HeLa cells were transfected with three different plasmid combinations: Vit^{2}P36/RSVhERα (A), Vit^{2}P36/RSVrERβ (B), and Vit^{2}P36/RSVhERα/RSVrERβ (C). Different concentrations of E_2 and resveratrol were dissolved in 100% ethanol to treat cells as indicated and luciferase activity assays were performed.

Cell Density Affects Resveratrol's Superinduction Effects

Superinduction effects of resveratrol were always seen with transfected ERβ, but with ERα inconsistently (data not shown). Minor changes in cell growth from experiment to experiment might explain this inconsistency. In order to investigate if cell growth condition is an important factor for the agonistic activities of resveratrol in HeLa cells, different seeding densities were used and HeLa cells were tranfected with two different plasmid combinations: Vit^{2}P36/RSVhERα and Vit^{2}P36/RSVrERβ. E_2 and resveratrol were used to treat cells as indicated and luciferase activity assays were performed. Resveratrol showed superagonistic activities only at the cell density of 0.2 × 10^{6}cells/well with transfected Vit^{2}P36/ RSVhERα. But for Vit^{2}P36/RSVrERβ, the superagonistic activities of resveratrol are independent of cell growth conditions (Figure 2).

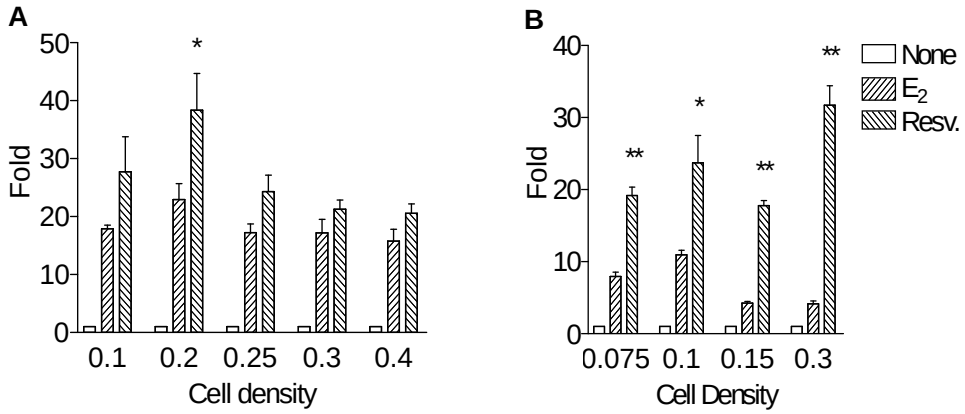

Figure 2: The Superinduction Effects of Resveratrol in HeLa Cells are Related to cell growth Conditions for Vit^{2}P36/RSVhERα, but not for Vit^{2}P36/RSVERβ

[A]: HeLa cells were seeded in six well plates with varying cell densities from 0.1 × 10^6 cells/well to 0.4 × 10^{6}cells/ well as shown. Cells were transfected with Vit^{2}P36/RSVhERα. [B]: HeLa cells were seeded in 6 well plates with varying cell densities from 0.075 × 10^6 cells/well to 0.3 × 10^{6}cells/ well as shown. Cells were transfected with Vit^{2}P36/RSVrERβ. All the cells were treated with either E_2 (10 nM) or resveratrol (30 μM) and luciferase activity assays were performed.

It is worth noting that our typical seeding cell density prior to these studies and presented in Figure 1 was 0.15 × 10^6cells/well for both ERα and ERβ. These results demonstrate that by changing cell density, more consistent superinduction effects can be achieved reproducibly.

Therefore, the experiments with RSVhERα were mostly performed at the cell density of 0.2 × 10^6cells/well. For the experiments with RSVERβ, the cell density of 0.1 × 10^6cells/ well was used.

The Superinduction effect of Resveratrol are Independent of ERs, EREs or Basal Promoters in HeLa cells

In order to determine whether ER was an obligatory component of superinduction, experiments were performed with ERα, ERβ or a control plasmid expressing the neomycin gene rather than ER. These studies were also performed using a control promoter P36, which is the same plasmid plus promoter as the estrogen responsive luciferase reporter Vit^2P36 but lacking the two tandem EREs (Figure 3A). The plasmid VitTK has a single ERE and a different promoter, the Hepes thymidine kinase promoter, rather than P36. The control for VitTK was the same plasmid lacking the ERE (TKSL) (Figure 3B and 3C). These experiments are used to determine the responses to 0, 1, or 2 EREs, responses on two different basal promoters and with either subtypes of ER, or no ER at all.

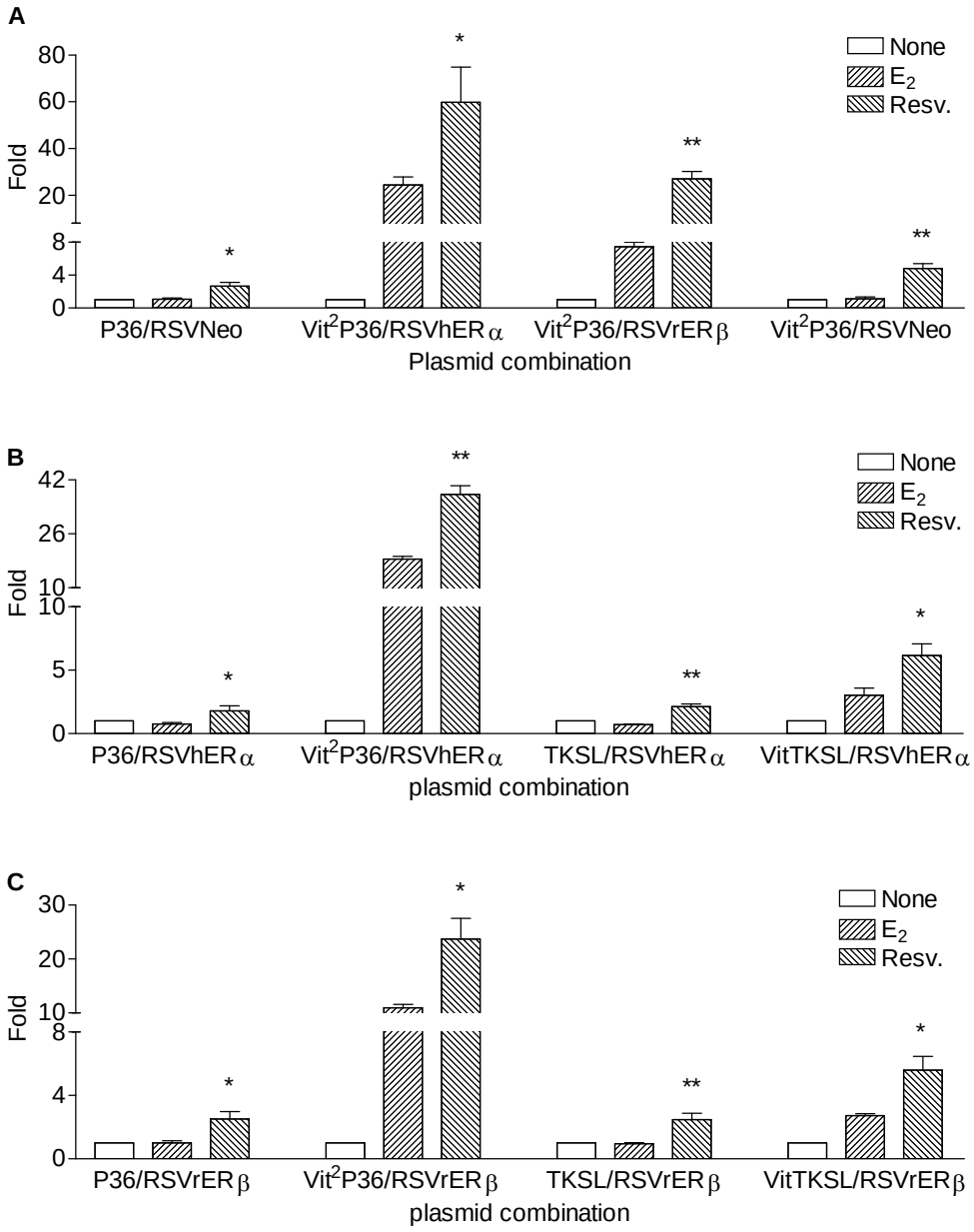

Figure 3: The Superinduction Effects of Resveratrol in HeLa cells are Independent of Transfected ERs, EREs or Basal Promoters in Luciferase Reporters

HeLa cells were seeded in six well plates at the cell density of 0.1×10^6 cells/well (for Vit^2P36/RSVERβ) or 0.2×10^6cells/ well (for others). Then cells were transfected with different plasmid combinations as shown. For each set (panel A, B or C) of experiments, at least three experiments were done. E_2 (10 nM) or resveratrol (30 μM) were used to treat cells as indicated and luciferase activity assays were performed. P36, Vit^2P36, TK and VitTK are plasmids with different promoters with or without ERE(s). The Vit^2-P36L luciferase reporter plasmid contains two copies of a 26-bp ERE from the xenopus vitellogenin A2 (Vit^2) gene linked to a minimal 36-bp promoter derived from the rat prolactin gene. P36 plasmid does not have ERE. The plasmid VitTK has a single ERE and a different promoter, the Hepes thymidine kinase promoter, rather than P36. The control for VitTK was the same plasmid lacking the ERE (TKSL). Statistical significance are shown as either "*" for $p<0.05$ or "**" for $p<0.01$.

The superinduction effects of resveratrol are independent of the basal promoters in luciferase reporters (P36, Vit^2P36, TK and VitTK) as all show superinduction with resveratrol compared to E_2 (Figure 3). It was not surprising that the more EREs (tandem) in the promoter region, the higher luciferase responses could be achieved by either E_2 or resveratrol. The superinduction effects of resveratrol in HeLa cells are independent of the existence of ERE in the promoter region of these luciferase reporter constructs (Figure 3), being seen with the basal promoters alone, without ERE. Furthermore, and even more unexpected, the superinduction was seen even in the absence of ER. This suggests that the responses observed with resveratrol are separable into two very different activities: one reflects resveratrol acting as an estrogen and the other shows resveratrol has a general multiplying effect over reporter gene activities.

Although the superinduction effect of reveratrol is independent of both ER expression and EREs in the promoter region of luciferase reporter genes, resveratrol induced higher luciferase activities with these reporter constructs which contain EREs than those do not (Figure 3).

The ERK1/2 Pathway Mediates the Superinduction Effects of Resveratrol

U0126, a specific inhibitor for ERK1/2 (Andrieux et al., 2004), was used to investigate the relationship between ERK1/2 pathway and the superinduction effects of resveratrol in HeLa cells. As seen before, resveratrol shows ER independent superinduction effects in HeLa cells (Figure 4). U0126, which inhibits ERK1/2 mediated cellular responses, was used together with resveratrol in different plasmid combinations (Figure 4). With the use of U0126 (5 μM), the superinduction effects of resveratrol were abolished by inhibiting the ERK1/2 pathway (Figure 4). These results suggest that ERK1/2 pathway is vital for resveratrol to show superinduction effects in HeLa cells.

Then the effects of resveratrol on the ERK1/2 pathway were studied by treating HeLa cells with resveratrol (30 μM) for different periods of time (0.5, 1, 2, or 3 hours) (Figure 5). Whole cell extractions were performed and antibodies against total or phosphorylated ERK1/2 were used to observe the effects of resveratrol on the ERK1/2 pathway. From the western results, it was seen that the phosphorylated form of ERK1/2 was increased about 4 folds over the control 30 minutes after the resveratrol treatment and the levels gradually lowered to the baseline level around 2 hours after resveratrol treatments (80% at 1 hour and 100% at 2 hours)(Figure 5).

The ERK1/2 pathway is essential for resveratrol's superagonistic effect in HeLa cells. All these experiments implied that resveratrol showed its superagonistic effect in HeLa cells through non-classic estrogen gene activation pathway or pathways. It is clear that U0126 could abolish the superinduction effect of resveratrol in HeLa cells. On the other hand, when ER exists in the cells, using of U0126 could not knock the reporter activity all the way down to the level of DMSO control. In other words, although the ERK1/2 pathway is vital for resveratrol's superinduction effects in ER negative cell lines, there are still some other pathways or mechanisms to explain the gene induction effects of either E_2 or resveratrol in ER positive cell lines such as the classic ER mediated pathway (Figure 4C).

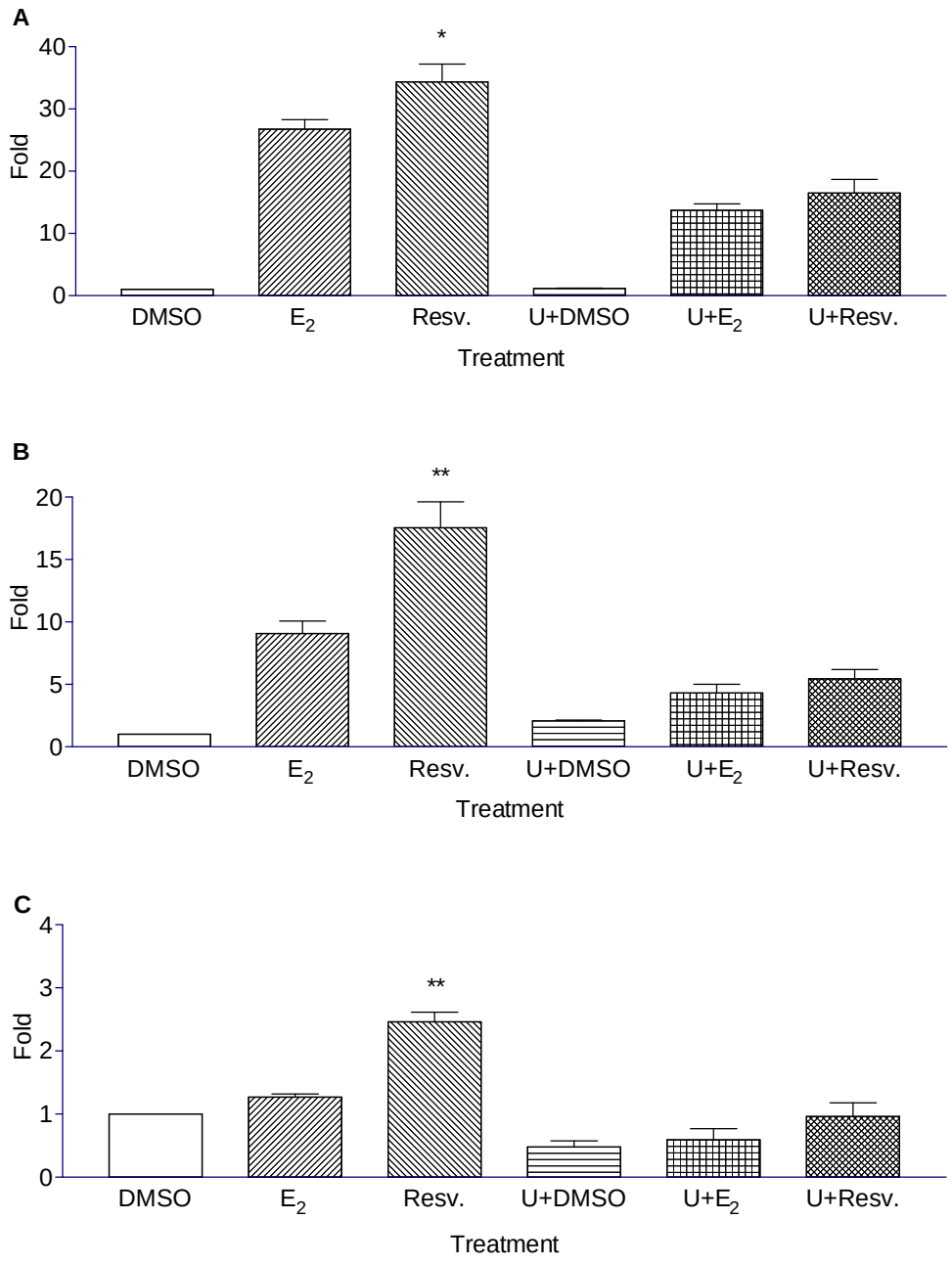

Figure 4: The ERK1/2 is involved in the Superinduction Effects of Resveratrol in HeLa cells

HeLa cells were seeded at the cell density of either 0.2 × 10^6 cells/well for Vit^{2}P36/RSVhERα [A] or 0.1 × 10^6 cells/well for Vit^{2}P36/RSVrERβ [B] or Vit^{2}P36/RSVNeo [C]. Hormones or drugs were used to treat cells solely or together as indicated and luciferase activity assays were performed. U stands for MEK1/2 inhibitor U0126. Statistical significance are shown as either "*" for $p<0.05$ or "**" for $p<0.01$.

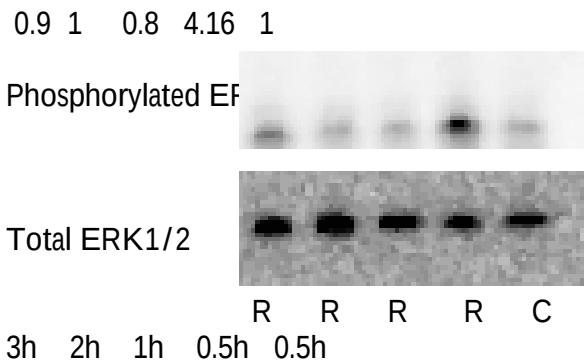

Figure 5: Resveratrol Increases Phosphorylated ERK1/2 level Significantly after a 30 Minute Treatment

HeLa cells were seeded in 100 mm dishes at the cell density (1.6 × 10^6cells/dish). Whole cell protein extractions were done after cells were treated with resveratrol (30 μM) or DMSO (control) for different time periods (30 minutes, 1 hour, 2 hours or 3 hours). Antibodies against total ERK1/2 (control) or phosphorylated ERK1/2 (P42/44) were used to show the effects of resveratrol on activating ERK1/2 along with time. Average fold changes of phosphorylated ERK1/2(P42/44) were shown on the top of the figure.

Comparisons among Resveratrol and two HDAC Inhibitors: MAA and TSA

MAA was shown to potentiate the transcriptional efficacy of several ligand activated nuclear receptors by both activating the ERK1/2 pathway and by inhibiting HDAC activity (Jansen et al., 2004). TSA could relax chromatin structure and increase gene expression by inhibiting HDAC (Minucci et al., 1997). To explore if the chromatin structure changes are related with the superinduction effects of resveratrol, experiments were performed to see if resveratrol works through the same mechanisms as MAA or TSA to increase histone acetylation level by inhibiting the HDAC activity and relaxing the chromatin structures.

HeLa cells were seeded in 6 well plates with cell densities of 0.15 × 10^6 cells/well and transfected with ERα or ERβ as indicated (Figure 6). Resveratrol, MAA and TSA were used solely or combined to treat cells. Resveratrol shows superinduction effects for both Vit^{2}P36/RSVrERβ and for Vit^{2}P36/RSVhERα in HeLa cells. Both MAA and TSA could enhance gene transcriptions in HeLa cells as compared to DMSO. It is noticed that TSA always has a more significant enhancing effect than MAA on increasing gene transcription (Figure 6).

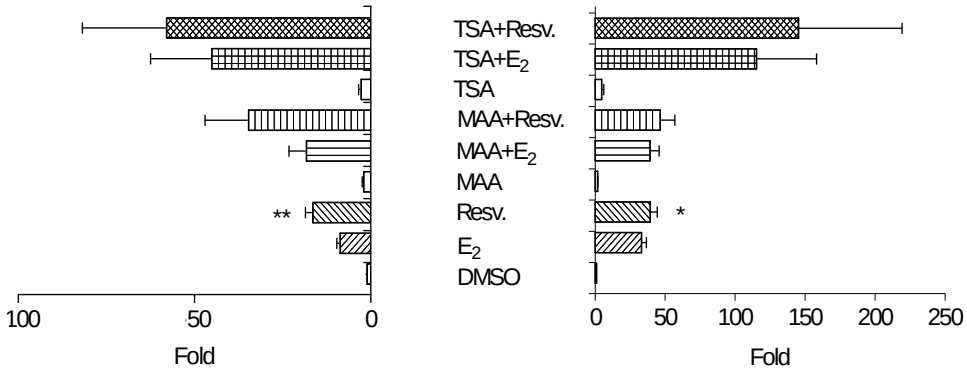

Figure 6: Both MAA and TSA have Synergistic effects with Resveratrol on Inducing Gene Expressions in HeLa cells

HeLa cells were seeded in 6 well plates with cell densities of 0.15 × 10^6 cells/well. For each well, HeLa cells were transfected with either RSVhERα (right panel) or RSVrERβ (left panel). DMSO, MAA (5 mM), TSA (0.3 μM), resveratrol (30 μM) or combinations of them were used to treat HeLa cells for 24 hours before the luciferase assay. Statistical significance are shown as either "*" for $p<0.05$ or "**" for $p<0.01$.

Resveratrol shows no Effect on HDAC Activities

From previous experiments (Figure 6), it was known that resveratrol increased gene expression through mechanisms different from those used by MAA or TSA (by inhibiting HDAC activity to loose nucleosome structures). Next the effects of resveratrol on general HDAC activity were investigated by using the HDAC fluorescent activity assay/ drug discovery kit with short chain fatty acids: MAA, TSA.

It was seen that HDAC activities from HeLa nuclear extracts are TSA sensitive (Figure 7). In addition, HDAC activities were also shown to be independent of the existence of NAD^+. MAA also inhibits HDAC activities, but the inhibition is not as complete as TSA does. MAA and TSA were shown to inhibit general HDAC activities from HeLa nuclear extracts and this is consistent with the observation that these two compounds could enhance gene transcription efficiency in HepG2 cell line (Kuiper et al., 1997). By contrast, resveratrol and U0126 do not have any effects on HDAC activities.

The difference between the inhibition effects of TSA and MAA explains the more profound gene activation effect of TSA compared to MAA. As expected, U0126 (a specific ERK1/2 inhibitor), has no direct effect on HDAC activity.

At last, the effects of resveratrol (30 μM) and E_2 (10^{-8} M) on general HDAC activity were compared (Figure 8). HeLa cells were treated with DMSO, E_2, resveratrol, MAA and TSA for 24 hours. MAA and TSA act as the positive controls to increase H_4 acetylation levels by inhibiting the HDAC activities. Histone preparation was done with acid extraction method. Anti-acetylated H_4 antibody was used to show the changes of H_4 acetylation level with various treatments. From the results, it was seen that E_2 treatment could increase H_4 acetylation and this may be related to its gene activation effects with transfected ERα or β. By contrast, resveratrol treatment has no effects on changing H_4 acetylation level and this is consistent with the results from previous studies (Figure 7).

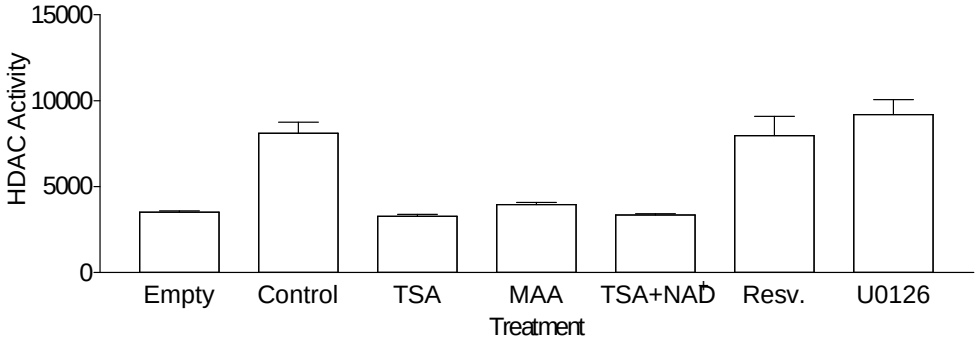

Figure 7: Resveratrol shows no Inhibition Effects on HDAC Activities from HeLa Nuclear Extracts

The HDAC fluorescent activity assay/drug discovery kit was used to examine the effects of TSA, MAA, NAD^+, TSA+ NAD^+, resveratrol, and U0126 on HDAC activities from HeLa nuclear extracts. The empty control has no Hela nuclear extracts. The control is the baseline HDAC activity level from the Hela nuclear extracts. TSA and MAA act as the positive control for inhibiting general HDAC activities. The concentrations for TSA, MAA, NAD^+, and resveratrol are 0.3 μM, 5 mM, 200 μM, and 30 μM respectively.

It was concluded that resveratrol could increase gene transcription to a higher level than E_2 at the concentration of 3×10^{-5} M. However, unlike E_2, it increases gene expression but not through enhancing the H_4 acetylation.

Figure 8: The Endogenous H_4 Acetylation Level is not Changed by Resveratrol Treatment

HeLa cells were seeded in 100 mm dish at the cell density of 1×10^6 cells /dish. Hormones or drugs were used to treat cells for 24 hours. Histone preparation was performed with acid extraction method. Anti-acetylated H_4 antibody was used to show the changes of H_4 acetylation level with various treatments. MAA and TSA act as the positive controls by increasing H_4 acetylation. The average H_4 acetylation level changes over the control are shown by ImageJ readings.

Discussion

Resveratrol is a compound that has received a great deal of attention in several decades for its health beneficial effects. Group research showed that beverage intake of resveratrol could result in sufficient absorption/ plasma levels for its beneficial effects (Bertelli et al., 1998).

For resveratrol, the superinduction effect with ERα is cell density-dependent, but with ERβ, it is cell density-independent. The superinduction effects of resveratrol, although initially identified using estrogen responsive genes, is both ER- and ERE-independent. In addition, the superinduction effects of resveratrol in HeLa cells are also independent of basal promoters in luciferase reporter constructs. Cell density was also shown to be a significant factor for inducing estrogen responsive genes by resveratrol. This may explain the inconsistency among reports on the ability of resveratrol to activate reporter or endogenous genes.

In our report, resveratrol has been shown to have superinduction effects in an ER-independent manner. This is consistent with other reports that resveratrol could work through ER-independent pathways (Levenson et al., 2003; Mgbonyebi et al., 1998; Opipari et al., 2004). It remains to be elucidated about the relationship between ER-independent pathways and the potential health benefits attributed to phytoestrogens but not to other estrogens including estradiol.

The ERK1/2 pathway is shown to be essential for the superinduction effect of resveratrol in HeLa cells. This is consistent with a report that stated both E_2 and resveratrol could activate ERK1/2 pathway through estrogen receptors in three different endothelial cells (Klinge et al., 2005). Yet, these results continue to raise a cautionary concern regarding the possibility of dramatic superinduction of estrogenic responses in vivo, or estrogen-like responses occurring at much lower concentrations than might be predicted based on data obtained under conditions that do not promote superagonist effects.

Human HDACs can be divided into two groups: trichostatin A-sensitive (Class I and II families) and trichostatin A-insensitive, NAD^+ dependent (sir2 family). The later group of HDACs consumes NAD^+ and couple lysine deacetylation to formation of nicotinamide and O-acetyl-ADP-ribose (Tanner et al., 2000; Tanny and Moazed, 2001). Resveratrol was shown by other groups that it has no activation or repression effects on Sirt1, one nuclear member of Sir2 family (Araki et al., 2004; Borra et al., 2005; Kaeberlein et al., 2005). So resveratrol seems to produce superinduction effects by mechanisms other than inhibiting HDAC activities.

Future work will be directed to further elucidate the molecular mechanisms involved in the superinduction phenomenon of resveratrol such as the roles of activators, coactivators activated by the ERK1/2 pathway or the changes of transcription machinery components. In addition, the biological relevance of these effects on endogenous genes is worth to be explored as well.

Acknowledgement

This study was funded by the promoting external applications for research grant and was reviewed by Dr. Stuart Warden.

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