Resveratrol is pro-apoptotic and thyroid hormone is anti-apoptotic in glioma cells: both actions are integrin and ERK mediated

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The stibene resveratrol (RV) initiates p53-dependent apoptosis via plasma membrane integrin αVβ3 in human cancer cells. A thyroid hormone (t, thyroxine, T4) membrane receptor also exists on αVβ3. Stibene and T4 signals are both transduced by extracellular-regulated kinases 1 and 2 (ERK1/2); however, T4 promotes cell proliferation in cancer cells, whereas RV is pro-apoptotic. Thyroid hormone has been shown to interfere with RV-induced apoptosis. However, the mechanisms involved are not fully understood. In this study, we examined the mechanism whereby T4 inhibits RV-induced apoptosis in glioma cells. RV activated conventional protein kinase C and ERK1/2 and caused nuclear localization of cyclooxygenase-2 (COX-2), consequent p53 phosphorylation and apoptosis. RV-induced ERK1/2 activation is involved in not only COX-2 expression but also nuclear COX-2 accumulation. NS-398, a COX-2 inhibitor, did not affect ERK1/2 activation, but reduced the nuclear abundance of COX-2 protein and the formation of complexes of nuclear COX-2 and activated ERK1/2 that are required for p53-dependent apoptosis in RV-treated cells. T4 inhibited RV-induced nuclear COX-2 and cytosolic pro-apoptotic protein, Bcl-Xs, accumulation. Furthermore, T4 inhibited RV-induced apoptosis by interfering with the interaction of nuclear COX-2 and ERK1/2. This effect of T4 was prevented by tetradaloxyraacetic acid (tetrac), an inhibitor of the binding of thyroid hormone to its integrin receptor. Tetrac did not, in the absence of T4, affect induction of apoptosis by RV. Thus, the receptor sites on αVβ3 for RV and thyroid hormone are discrete and activate ERK1/2-dependent downstream effects on apoptosis that are distinctive.

Introduction

Thyroid hormone (t, thyroxine, T4; 3,5,3′-triiodo-l-thyronine) is a proliferation factor in vitro for a rat glioblastoma cell line (C6) via a cell-surface receptor for the hormone on integrin αVβ3 (1). This receptor is at or near the arginine–glycine–aspartate (RGD) recognition site on the integrin that is involved in the interactions of the integrin with extracellular matrix proteins. The intracellular domain of the integrin may activate extracellular-regulated kinases 1 and 2 (ERK1/2) (2–4) and we have shown that T4 rapidly increases cellular ERK1/2 activity via the integrin (5). Short integrin antagonist peptides that contain the RGD sequence have been designed as tools to demonstrate the role of integrins in transducing the signals of a number of extracellular matrix proteins. RGD peptides block thyroid hormone actions mediated by the integrin (1).

Abbreviations: COX-2, cyclooxygenase-2; cPKC, conventional protein kinase C; ELISA, enzyme-linked immunoadsorbent assay; ERK1/2, extracellular-regulated kinases 1 and 2; FBS, fetal bovine serum; pERK12, phospho-ERK1/2; PMA, phorbol 12-myristate 13-acetate; RGD, arginine–glycine–aspartate; RGE, arginine–glycine–glutamate; RV, resveratrol; TCA, trichloroacetic acid; tetrac, tetradaloxyraacetic acid; T4, t, thyroxine.
Cell culture
The rat glioma C6 cell line and human glioblastoma U87MG cell line were purchased from American Type Culture Collection (Manassas, VA). The rat glioma GL.261 cells were obtained from the Roswell Park Cancer Institute, Buffalo, NY. C6 cells were maintained in F12K medium supplemented with 18% fetal bovine serum (FBS). U87MG cells were maintained in Minimal Essential Medium supplemented with 10% FBS and GL.261 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS. All cell cultures were maintained in a 5% CO₂/95% air incubator at 37°C. Prior to treatment, cells were plated in 0.25% hormone-stripped FBS-containing medium for 2 days. In other sets of experiments, U87MG cells were either treated with 20 μM CGP or the MEK inhibitor, PD for 24 h. Exponentially growing C6 glioma cells were seeded in slide chambers. After treatment, cells were collected and stored for at least 18 h at −20°C. From each appropriately diluted sample, 100 μl were added to a 96-well plate coated with a DNA-binding protein and incubated at room temperature for 3 h. After three washes with wash buffer, antibody detector was added for 1 h. Streptavidin conjugate was added and incubated for 0.5 h before adding substrate. Plates were read at 450 nm.

Apoptosis/nucleosomes
An early event in apoptosis is DNA fragmentation followed by release of nucleosomes into the cytoplasm. The nucleosome is the basic unit of chromatin and results from the ordered association of histones and DNA (21). The double-antibody sandwich ELISA is based upon the specific recognition of nucleosomes by a pair of monoclonal antibodies and detects cytoplasmic nucleosomes onto the ELISA plate. Cells were treated with different reagents for 48 h. The medium was harvested, spun down and pellets were washed twice with phosphate-buffered saline. Pelleted cells were lysed. Supernatants were collected and stored for at least 18 h at −20°C. From each appropriately diluted sample, 100 μl were added to a 96-well plate coated with a DNA-binding protein and incubated at room temperature for 3 h. After three washes with wash buffer, antibody detector was added for 1 h. Streptavidin conjugate was added and incubated for 0.5 h before adding substrate. Plates were read at 450 nm.

Immunoblotting and immunoprecipitation
Nuclear and cytosol protein extracts were prepared, quantitated and separated on discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and then transferred by electroblotting to nitrocellulose membranes (Millipore). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween, and then incubated with selected antibodies overnight. Secondary antibodies were either goat anti-rabbit IgG or rabbit anti-mouse IgG (1:1000), depending on the origin of the primary antibody. Immunoreactive proteins were detected by chemiluminescence (ECL, Amersham Life Science, Arlington Heights, IL) and integrated optical density of bands was quantitated by phosphoimaging and with ImageQuant software on a Storm 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA) as described previously (19).

Confluent microscopy
Exponentially growing C6 glioma cells were seeded in slide chambers. After exposure to 0.25% stripped FBS-containing medium for 2 days, cells were either treated with 20 μM RV in the presence or absence of the cPKC inhibitor, GCP or the MEK inhibitor, PD for 24 h. In other sets of experiments, U87MG cells and GL.261 cells were treated with 20 μM RV, 10⁻⁷ M T₄ or both for 24 h. Cells were fixed with 4% formaldehyde in acetone for 30 min and then permeabilized in 0.06% Triton X-100 for 30 min. The cells were incubated with monoclonal antibody to COX-2 followed by Alexa-488-labeled goat anti-mouse antibody and the signal revealed using the Histostain SP kit as recommended by the manufacturer (Zymed–Invitrogen, Carlsbad, CA). Nuclear staining with propidium iodide was also employed. Cells were examined under ×250 magnification.

Radiolabeled thymidine incorporation
Aliquots of cells were incubated with 1 μCi [³H]-thymidine (final concentration, 13 nM) in a 24-well culture tray for 16 h in the presence or absence of reagents as indicated. Cells were then washed twice with cold phosphate-buffered saline, after which 5% trichloroacetic acid (1 ml) was added and the plate was kept at 4°C for 30 min. The precipitate was then washed twice with cold ethanol, and 1 ml of 20% sodium dodecyl sulfate was added to each well. The trichloroacetic acid-precipitable radioactivity was quantitated in a liquid scintillation counter.

Quantitation of results and statistical analysis
Immunoblot densities were measured with a Storm 860 Phosphorimager followed by analysis with ImageQuant software (Molecular Dynamics). Student’s t-test, with P < 0.05 as the threshold for significance, was used to evaluate results from three or more experiments.

Results
Thyroid hormone and RV, both ligands of integrin αVβ3, have competitive actions in C6 glial cells
In studies of rat glioma C6 cell proliferation, T₄ treatment increased radiolabeled thymidine incorporation (Figure 1A). In contrast, RV inhibited significantly the effect of 10⁻⁷ M T₄, but does not block the effect of the stilbene.

Fig. 1. Effects of RV, T₄ and tetrac on cell proliferation and apoptosis. (A) The effect of 10⁻⁷ M T₄ and 1–50 μM RV on [³H]-thymidine incorporation was measured as described in Materials and Methods. T₄ increased thymidine incorporation by C6 glial cells. This effect was inhibited by RV in a concentration-dependent manner (P < 0.05 with 10 μM and P < 0.01 with 50 μM). (B) C6 cells were treated with 10 μM RV in the presence or absence of T₄ (10⁻⁷ and 10⁻⁸ M). RV-induced apoptosis (nucleosome ELISA) was inhibited significantly by co-incubation with 10⁻⁸ to 10⁻⁷ M T₄ (P < 0.01). (C) The effect of T₄ on RV-induced apoptosis was examined in the presence and absence of tetrac. The inhibition by T₄ of apoptosis induced by RV (lane 7 compared with lane 5, P < 0.05) was blocked by the addition of tetrac (lane 8 versus lane 7, P < 0.01), indicating that tetrac blocks the inhibitory effect of T₄, but does not block the effect of the stilbene.
Fig. 2. RV activates ERK1/2 and cPKC via binding to integrin αVβ3 in C6 glioma cells. (A) Cells were treated with 10 μM RV for 4 h, in the presence or absence of 5–500 nM RGD peptide or 500 nM arginine–glycine–glutamate (RGE) peptide (24 h). Concentration-dependent inhibition of RV-induced cPKC and ERK1/2 activation was seen with the RGD peptide (P < 0.01), but no inhibition was seen with arginine–glycine–glutamate peptide. Lamin-B and β-actin immunoblots are provided in this and subsequent figures as controls for gel loading of nucleoproteins and cytosolic proteins, respectively. The graphs in this and subsequent figures represent the mean ± SD of band intensities normalized to a value of 1 in untreated cells, in three separate experiments. (B) C6 cells were treated with PMA (100 ng/ml) for 24 h prior to addition of RV (10 μM) for 4 h. PMA treatment, alone, caused a significant increase in nuclear accumulation of nuclear factor-κB subunits, both p65 and p50 (P < 0.01). RV did not cause nuclear factor-κB (NFκB) accumulation, but did cause a significant increase in ERK1/2 activation and nuclear translocation (P < 0.01), which was reduced by 24 h pre-treatment with PMA (P < 0.01). The levels of cytosolic phosphorylated PKCα/β in cells treated with RV were not seen in cells depleted of PKC with PMA treatment. (C) Cells were treated with RV (10 μM, 4 h), in the presence or absence of the ERK1/2 activation inhibitor, PD98059 (PD, 3 or 30 μM, 4.5 h) or the PKCα/β inhibitor, CGP41251 (CGP, 10 or 100 nM, 4.5 h). ERK1/2 activation associated with RV treatment, shown in nuclear fractions, was inhibited by both PD and CGP. RV-induced phosphorylation of PKCα/β was inhibited by CGP but not by PD (P < 0.05, comparing RV-treated cell fractions with corresponding samples treated with RV and either inhibitor).
(1–50 μM) caused apoptosis and thus suppressed thymidine uptake. Further, the growth-stimulatory effect of T4 on C6 cells was progressively diminished by increased concentrations of RV (Figure 1A). In studies of C6 cell apoptosis, T4 alone had no appreciable effect, whereas RV treatment produced apoptosis as expected (nucleosome ELISA, Figure 1B). The deaminated T4 analog tetrac did not inhibit the effect of apoptosis of RV, alone (Figure 1C, lane 6), but did inhibit T4 suppression of RV-induced apoptosis (comparing lanes 7 and 8). This finding suggests that T4 and RV interact with the integrin at discrete sites and that tetrac only binds to the T4-binding site.

RV-stimulated ERK1/2 and cPKC activation in C6 cells is inhibited by an integrin αVβ3 recognition site RGD peptide

To illustrate the role of integrin αVβ3 and consequent intracellular signaling in RV action, we measured the effect of RGD and arginine–glycine–glutamate–glutamate peptides on the activation of ERK1/2 and of cPKC by RV. While control arginine–glycine–glutamate–glutamate peptide did not alter ERK1/2 or cPKC phosphorylation by the stilbene, increasing concentrations of RGD peptide (5–500 nM) suppressed these effects (Figure 2A). This finding is consistent with displacement of RV from an integrin-binding site at or near the RGD recognition domain.

Fig. 3. Activation of the ERK1/2 pathway is required for RV-induced COX-2 nuclear accumulation in C6 cells. (A) Cells were treated with RV (10 μM) for 24 h, with or without PD98059 (PD, 30 μM) or with PD for the last 12 h of RV treatment. Nuclear content of COX-2 increased with RV treatment, and this effect was inhibited by PD (P < 0.05), particularly after 24 h of inhibitor treatment. On the other hand, the accumulation of cytosolic COX-2 in RV-treated cells was significantly enhanced by PD treatment (P < 0.05) for 12 or 24 h. (B) C6 cells were treated with 10 μM RV in the presence or absence of CGP41251 (CGP) or PD98059 (PD) for 24 h and then examined by confocal microscopy. RV caused nuclear accumulation of COX-2, indicated by the appearance of a yellow color due to superimposition (co-localization) of COX-2 (green) and nuclear (red) images. In the presence of CGP (two middle panels) and PD (two panels at right), nuclear COX-2 was not evident, although cytosolic COX-2 is still seen in some cells. Cells were viewed at ×250 magnification. Images by differential interference contrast are shown at the lower left of each group of four images, in order to clarify outlines of cells and nuclei.
In cells depleted of PKC by treatment with PMA (100 mg/ml) for 24 h, RV-induced ERK1/2 activation and phosphorylation of PKCα/β were both inhibited (Figure 2B). There was no nuclear factor-kB activation in RV-treated cells, although PMA alone increased both p50 and p65 subunit accumulation in nuclei (Figure 2B). RV-induced ERK1/2 activation and nuclear translocation were inhibited by the ERK1/2 activation inhibitor PD (Figure 2C). Treatment of cells with the cPKC activation inhibitor, CGP, resulted in similar suppression of ERK1/2 phosphorylation; this inhibitor also partially inhibited phosphorylation of PKCα/β, although PD did not inhibit this latter effect. These results indicate that ERK1/2 activation in RV-treated C6 cells is dependent on cPKC activation as well as on MEK activation.

Activated ERK is associated in nuclei with COX-2 in C6 cells treated with RV; this effect is required for apoptosis

We have shown that activated ERK1/2 is essential for RV-induced COX-2 expression (19). Treatment of C6 glioma cells with RV caused nuclear accumulation of COX-2, and inhibition of ERK1/2 activation with PD for 24 h blocked this RV effect (Figure 3A). However, treatment of C6 cells with PD for only the last 12 h of a 24-h RV exposure did not inhibit COX-2 expression but partially inhibited nuclear accumulation of COX-2 and there was increased accumulation of COX-2 in the cytosol. COX-2 levels increased in the cytosol of cells treated with PD alone and in cells exposed to RV and PD; this suggests that activation of ERK1/2 is required for nuclear accumulation of COX-2 in the presence of RV.

In confocal microscopy studies, we have also demonstrated ERK1/2 activation-dependent nuclear accumulation of COX-2 (green) in RV-treated cells (Figure 3B). Untreated cells demonstrate a minimal amount of COX-2 in cytosol. Cells treated with RV show increased cellular COX-2, and in particular increased nuclear COX-2, indicated by a yellow color formed by superimposition of the COX-2 green color on a red nucleoprotein stain. Inhibitors of cPKC activation and pERK1/2 activation both blocked nuclear COX-2 accumulation in the presence of RV. These results confirm that RV-induced cPKC activation is upstream of ERK1/2 activation and that inhibition of either cPKC or ERK1/2 activation will inhibit ERK1/2-dependent nuclear COX-2 accumulation in RV-treated C6 cells.

Demonstration of the interrelationships between COX-2 activity, pERK1/2 accumulation and Ser-15 phosphorylation of p53

In the presence of the specific COX-2 inhibitor, NS-398 (0.1–10 nM), RV-induced nuclear pERK1/2 content was unaffected (Figure 4). However, the formation of co-immunoprecipitated nuclear complexes of COX-2 and pERK1/2 in RV-treated cells was inhibited by NS-398, but not by the non-specific COX inhibitor, indomethacin. NS-398 did not affect pERK1/2 activation per se confirming that RV-induced ERK1/2 activation is upstream COX-2 expression. On the other hand, NS-398 reduced RV-induced Ser-15–p53 phosphorylation (Figure 4). Apoptosis induced by RV was also inhibited by NS-398, but not affected by indomethacin (Figure 4). These results also appear to implicate RV-inducible nuclear COX-2 accumulation in the ERK1/2-dependent activation (phosphorylation) of p53 that leads to cancer cell apoptosis.

Thyroid hormone blocks RV action by disrupting COX-2–pERK1/2 complexing in C6 glial cells

Thyroid hormone inhibits RV-induced apoptosis in C6 cells (Figure 1), although the two agonists share the same plasma membrane receptor and some of the same signaling pathways. At what point in the signaling sequence does the hormone block the action of RV? We have demonstrated that T4 stimulates ERK1/2 activation and cell proliferation in different glioma cell lines (1). In contrast, RV (10 µM) also stimulates ERK1/2 activation (Figures 2 and 4), but causes nuclear COX-2 accumulation and apoptosis. We therefore examined whether T4 blocks RV-induced nuclear COX-2 accumulation. Studies of confocal microscopy using two different glioma cell lines, human glioblastoma U87MG cells (Figure 5A, left panel) and rat glioma GL261 cells (Figure 5A, right panel) indicate that RV caused nuclear COX-2 accumulation and that this effect was blocked by T4. This inhibitory effect of T4 on RV-induced nuclear COX-2 accumulation is also shown in immunoblots of nuclear fractions with a parallel decrease in the pre-apoptotic protein, BcLx-s (Figure 5B).
The thyroid hormone analog, tetrac, is known to inhibit T4 binding to plasma membranes and purified integrin αVβ3 (5) and to inhibit membrane-associated effects of T4 on signal transduction pathways (20,22). Results also indicate that tetrac reversed the inhibitory effect of T4 on RV-induced apoptosis (Figure 1C). We therefore examined the actions of tetrac on the inhibitory effect of T4 on nuclear accumulation of pERK1/2, COX-2 and pSer15–p53 in C6 cells treated with RV. T4 treatment alone caused nuclear accumulation of pERK1/2 and pSer15–p53, but not that of COX-2 (Figure 6, lane 3). Tetrac partially blocked the hormone effects (lane 4). RV stimulated nuclear accumulation of pERK1/2, COX-2 and pSer15–p53, as well as the pro-apoptotic protein BcLx-s (Figure 6, lane 5). Tetrac did not inhibit the action of RV on these parameters (lane 6 compared with lane 5). When RV and T4 were added together to cells (Figure 6, lane 7),
The stibene effects on COX-2, pSer15-p53 and BcLx-s were inhibited, although pERK1/2 activation remained. In contrast, when tetrac was added to both RV and T₄, the inhibitory effects of T₄ on RV action were blocked (lane 8), but the changes with RV were similar to those shown in lane 5 with RV, alone, and with RV and tetrac (lane 6).

Further, development of the immunoprecipitable nuclear complex of inducible COX-2 and activated ERK1/2 in RV-treated cells (Figure 6, lane 5) was inhibited by co-incubation with RV and T₄ (lane 7), whereas T₄ alone did not induce this nuclear association (lane 3). With the addition of tetrac, the inhibitory effect of T₄ on COX-2 and pERK1/2 complex formation was reversed (Figure 6, lane 8). These results suggest that the nuclear immunoprecipitable complex formation between pERK1/2 and COX-2 plays a crucial role in RV-induced apoptosis. Further, this complex formation can be disrupted by thyroid hormone, leading to inhibition of RV-induced pro-apoptotic protein BcLx-s accumulation (Figure 6, lane 7) and apoptosis (Figure 1).

As indicated above, we know that both thyroid hormone and RV have receptor sites on integrin αβ3 and that both hormone and stibene activate ERK1/2. The antagonism by T₄ of the action of RV on signaling events that promote apoptosis and the reversal by tetrac of this hormone effect, but not the activity of RV, point to the existence of discrete hormone- and stibene-binding sites on integrin αβ3.

Discussion

RV is a stibene that has antitumor properties in gliial cells (8). Using a variety of human cancer cell lines, we have demonstrated that RV is capable of inducing p53-dependent apoptosis via ERK1/2-mediated serine phosphorylation at residue 15 of the oncogene suppressor protein (10–13). This is the case even when the cancer cells contain mutated p53, as long as residue 15 remains a serine (12). We have recently shown, however, that this clinically desirable action of RV may be antagonized by estrogen in estrogen-responsive (ERα-positive) tumor cells, such as MCF-7 breast cancer cells (10).

We have also determined that the growth of gloma cells in vitro is importantly thyroid hormone dependent (1). We have shown previously that both T₄ and RV are ligands of integrin αβ3 (5,9) and both activate ERK1/2 via the integrin. Both receptors are at or near the RGD recognition site on the integrin, but our current findings do not disclose direct competition between thyroid hormone and RV for a single site on integrin αβ3. That is, tetrac, a specific inhibitor of binding of T₄ to the integrin and of downstream actions of the hormone initiated at the integrin (1,5) did not block the actions of RV.

We have identified a step downstream of ERK1/2 in the transduction of the RV and thyroid hormone signals at which a divergence occurs that appears to account for the different biological effects of T₄ on cell growth and of RV leading to apoptosis. RV increases nuclear abundance of COX-2, whereas T₄, alone, has no effect on nuclear COX-2 (Figures 5 and 6). However, the hormone decreases formation of nuclear complexes between pERK1/2 and COX-2 in stibene-treated cells (Figure 6) and also inhibits RV-induced p53 phosphorylation. How this signal transduction step (nuclear complexing of COX-2 and activated ERK1/2, upstream of activation of p53) is differentially affected by T₄ and RV is not yet clear. Among the possible explanations is that the pools of activated ERK1/2 that result from the transduction of stibene and thyroid hormone signals are discrete (23). For example, the pro-apoptotic response of C6 cells to RV treatment was blocked in vitro by physiological concentrations of thyroid hormone. In the intact animal test model, normal circulating levels of thyroid hormone may blunt an apparent response to the stibene. Since tetrac does not block stibene action, however, a potential therapeutic combination of tetrac—to block endogenous T₄ action at the integrin—and RV would allow an unopposed pro-apoptotic effect of the stibene at its integrin receptor to be obtained.

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