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3,4-Dihydroxytoluene Inhibits Epidermal Growth Factor-induced Cell Transformation in JB6 P+ Mouse Epidermal Cells by Suppressing Raf-1

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3,4-Dihydroxytoluene의 Raf-1 신호전달체계 억제를 통한 암예방 효능

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국문요약

이전 연구들에서 rutin과 quercetin을 포함한 여러 flavonoids의 암예방 활성이 보고되었으나, rutin의 경우 섭취 시 체내에서 HVA, HPAA, DHT라는 대사체로 변형되어 흡수된다. 그러나, 이들 대사체와 관련한 암예방 효능 및 그 분자생물학적 작용기작에 대한 연구 결과는 보고된 바가 없어, 본 연구에서 이를 규명하였다. DHT는 EGF로 유도된 세포 변형을 억제하였으며, AP-1 전사인자의 활성 또한 억제하였다. DHT는 Raf-1 효소 활성을 효과적으로 저해하므로서 MEK 및 ERK의 인산화를 억제하였으며, Raf-1과 ATP는 비경쟁적으로 직접 결합하여 Raf-1 효소 활성을 저해한다는 사실을 밝혀내었다. 이와는 대조적으로, rutin은 EGF로 유도된 세포 변형, AP-1 활성, ERK 신호전달체계, Raf-1 효소 활성을 억제하지 못하였다. 이상의 연구결과는 DHT의 암예방 활성이 발암과정과 밀접한 연관이 있는 Raf-1 효소 활성을 억제하여 세포 변형을 억제하는 것과 관련되어 있다는 것을 제시한다.

검색어: DHT, 세포 변형, EGF, Raf-1, 화학적 암예방

Introduction

A number of epidemiological investigations and clinical trials have reported the chemopreventive activities of flavonoids, which are consumed regularly in the diet (Surh YJ 2003; Langner & Rzeski 2012). Rutin (3-O-rhamnosylglucosyl-quercetin, Fig. 1A) and quercetin are major flavonoids found in many food sources, including fruits, vegetables, and teas (Garcia-Lafuente et al. 2009). Many studies have reported the biological effects of rutin and quercetin, such as their antioxidative (Merk et al. 1991; Sharma et al. 2013), anti-inflammatory (Potapovich et al. 2011; Vanwert et al. 2012), and anti-carcinogenic (Lin et al. 2012; Alonso-Castro et al. 2013; Choi et al. 2014) effects. Therefore, rutin and quercetin have been proposed as chemopreventive agents that protect against inflammation and cancer.

Rutin is initially hydrolyzed to the aglycone quercetin by intestinal microflora (Jaganath et al. 2009; Pashikanti et al. 2010). Subsequently, quercetin is degraded into homovanillic acid (HVA, Fig. 1A), 3-hydroxyphenylacetic acid (HPAA, Fig. 1A), and 3,4-dihydroxytoluene (DHT, Fig. 1A). Bioavailability studies suggest that these metabolites are active compounds that exhibit the health-benefiting activities of rutin or quercetin (Ito et al. 1981; Glasser et al. 2002). However, less is known about the biological activities of the rutin metabolites. DHT (also known as 4-methylcatechol) has received attention among rutin metabolites due to its suppressive effects on inflammation (Su et al. 2014) and cancer cells (Morita et al. 2003; Payton et al. 2011). Thus, it could be a potent chemopreventive agent against carcino-

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genesis; however, its underlying molecular mechanisms and molecular target(s) remain unclear.

Carcinogenesis is a multi-stage process consisting of initiation, promotion, and progression stages (Surh YJ 2003; Shu et al. 2010). Tumor promotion is a reversible long-term process in which the rates of cell replication and/or changes in gene expression increase. It occurs rarely compared to the initiation stage and requires sequential exposure to tumor promoters, such as epidermal growth factor (EGF) (Dong et al. 1994; Young et al. 1999). Tumor promoters can lead to progression, the final stage of neoplastic transformation, which is related to tumor growth, invasion, and metastasis. Therefore, the promotion stage, and possibly the progression stages, may be more critical steps to prevent cancer than the other stages.

Neoplastic transformation is closely related to activator protein-1 (AP-1), which is a crucial cell proliferation and inflammation transcription factor (Dong et al. 1994; Huang et al. 1997). In response to a wide variety of tumor promoters, such as EGF and 12-O-tetradecanoylphorbol-13-acetate (TPA), AP-1 is activated through upregulation of the mitogen-activated protein (MAP) kinase signaling pathway (Bode & Dong 2005; Lee & Lee 2006). One of the three members of the Raf/MEK/extracellular signal-regulated protein kinase (ERK) pathway, plays a central role regulating cell proliferation and transformation (Cheepala et al. 2009). In particular, overexpression of activated Raf-1 is associated with cell transformation and tumor development (Kern et al. 2011; Kern et al. 2013). These findings indicate that Raf-1 is an important carcinogenesis target and that inhibiting it could be an effective cancer prevention and therapeutic strategy. Here, we report that DHT, but not rutin, may potently inhibit Raf-1 and subsequently suppress downstream signaling and neoplastic transformation.

Materials and Methods

1. Chemicals

Rutin, HVA, HPAA, DHT, and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Eagle's minimum essential medium (MEM) was purchased from Cellgro (Manassas, VA, USA). Penicillin-streptomycin and 0.5% trypsin-EDTA were obtained from Gibco/Invitrogen (Auckland, NZ). EGF was purchased from BD Bioscience (San Jose, CA, USA). Antibodies against phosphorylated Raf-1 (Ser338) and total Raf-1, phosphorylated MEK1/2 (Ser217/221), and total MEK1/2 were purchased from Cell Signaling Biotechnology (Danvers, MA, USA). Antibodies against phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ADP-Glo Kinase assay kit, G418, and the luciferase assay substrate were obtained from Promega (Fitchburg, WI, USA). CNBr-Sepharose 4B and a chemiluminescence detection kit were purchased from Amersham Biosciences (Piscataway, NJ, USA). The protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2. Cell culture

The JB6 P+ mouse epidermal cell line was cultured in a monolayer at 37℃ in a 5% CO_2 incubator with the AP-1 luciferase reporter plasmid and maintained in MEM containing 5% FBS and penicillin-streptomycin. The JB6 P+ cells were stably transfected with 5% FBS containing penicillin-streptomycin and 200 μg/mL G418.

3. Cell proliferation assay

JB6 P+ cells were seeded (10^3 cells/well) in 96-well plates in MEM with 5% FBS at 37℃ in a 5% CO_2 incubator to estimate cell proliferation. After culturing for the indicated times, 20 μL CellTiter 96 Aqueous One Solution (Promega) was added to each well, and the cells were incubated for 1 h at 37℃ in a 5% CO_2 incubator. Absorbance was measured at 492 and 690 nm.

4. Anchorage-independent transformation assay

The effects of rutin, HVA, HPAA, and DHT on EGF-induced cell transformation were investigated in JB6 P+ cells. Cells (2.4×10^4 cell/mL) were exposed to EGF with rutin, HVA, HPAA, or DHT (10 or 20 μM) in 1 mL 0.33% basal medium Eagle's (BME) agar containing 10% FBS or in 3 mL 0.5% BME agar containing 10% FBS. The cultures were maintained at 37℃ in a 5% CO_2 incubator for 10 days, at which time the cell colonies were counted under a Nikon phase-contrast microscope (Tokyo, Japan) with the aid of NIS-Elements 3.0 and Image-Pro Plus software programs (v. 5, Media Cybernetics, Silver Spring, MD, USA).

5. Luciferase assay for AP-1 transactivation

Confluent monolayers of JB6 P+ cells stably transfected with the AP-1 luciferase reporter plasmid were trypsinized, and 5×10^3 viable cells suspended in 100 μL MEM with 5% FBS were added to each well of a 96-well plate. The plates were incubated

at 37℃ in a humidified atmosphere of 5% CO_2. When the cells reached 80 – 90% confluence, they were starved by culture in MEM with 0.1% FBS for an additional 24 h. The cells were then treated for 1 h with rutin or DHT (0 – 20 μM) and exposed to 10 ng/mL EGF for 4 h. After treatment, the cells were disrupted with 100 μL lysis buffer, and luciferase activity was measured using a Glomax luminometer (Promega).

6. Western blotting

Cells (1×10^5 cell/mL) were cultured in a 6 cm dish for 24 h and then starved in MEM with 0.1% FBS for an additional 24 h to eliminate the influence of FBS on the activation of kinases. The cells were treated with rutin or DHT (10 or 20 μM) for 1 h before being exposed to 10 ng/mL EGF for 30 min. The harvested cells were disrupted, and the supernatant fractions were boiled for 5 min. Protein concentrations were determined using a dye-binding protein assay kit (Bio-Rad Laboratories), as described in the manufacturer's manual. Lysate proteins (20 – 40 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membranes (Millipore, Milford, MA, USA). After blotting, the membranes were incubated with specific primary antibodies at 4℃ overnight. After hybridization with the appropriate horseradish-peroxidase-conjugated secondary antibody, the protein bands were visualized with a chemiluminescence kit.

7. *In vitro* Raf-1 kinase assay

The *in vitro* ADP-Glo kinase assay was performed according to the manufacturer's instructions. In brief, each reaction contained 1× reaction buffer (200 mM Tris, 100 mM $MgCl_2$, 0.5 mg/mL BSA [pH 7.5]), ADP-Glo Reagent, and a detection buffer. The inactive MEK1/2 substrate peptide (0.05 μM) was included for the Raf-1 assay. A 10 μL aliquot of Raf-1 kinase was added to a 96-well plate with 5 μL rutin or DHT, and 1× kinase buffer (as a control) and incubated for 15 min at room temperature. After Raf-1 reacted with rutin or DHT, 10 μL the ATP-MEK1/2 mixture was added and incubated at 30℃ for 30 min. Then, 25 μL ADP-Glo Reagent was added to all wells. The plate was shaken well and incubated at room temperature for 40 min. A 50 μL aliquot of kinase detection reagent was added to all wells. The plate was shaken well and incubated at room temperature for 60 min. Luminescence was measured on a Glomax luminometer with 0.5 s integration time. Each experiment was performed three times.

8. *In vitro* and *ex vivo* Raf-1 pull-down assay

Recombinant Raf-1 (50 ng) or the JB6 P+ cellular supernatant fraction (1,000 μg) was incubated with rutin- or DHT-Sepharose 4B (or Sepharose 4B as a control) beads (100 μL of a 50% slurry) in reaction buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/mL bovine serum albumin, 0.02 mM PMSF, and 1× protease inhibitor mixture). After incubation with gentle rocking overnight at 4℃, the beads were washed five times with buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF), and proteins bound to the beads were analyzed by Western blot.

9. ATP and DHT competition assays

Briefly, recombinant Raf-1 (50 ng) was incubated with 100 μL DHT-Sepharose 4B or 100 μL Sepharose 4B in the reaction buffer described above for 12 h at 4℃, and ATP was added at 10 or 100 μM to a final volume of 500 μL for 30 min. The samples were washed, and proteins were detected by Western blot.

10. Docking and modeling

The Raf-1 (PDB: 3OMV) catalytic domain was used for docking simulations. The missing hydrogen atoms and charges on the receptor (Raf-1 catalytic domain) were added using AutoDock tools (ADT) v1.5.4 r29. The DHT ligand was prepared with both Spartan'10 (Wavefunction, Inc., Irvine, CA, USA) and the scripts provided with ADT v1.5.4 r29. AutoDock Vina was used to perform the docking (Trott & Olson 2010). The docking grid sizes and locations were set using ADT and were large enough (70×70×70 Å) to cover the entire receptor structure. The lowest docking energy was retained as the most favorable docking solution among the 20 best solutions. The 20 best docking solutions were manually inspected using COOT and PyMOL to verify and confirm their compatibility with existing knowledge of the receptor (active site location, binding pockets, and conserved amino acid residues potentially involved in binding). Electrostatic calculations were performed with APBS V1.2.1, and the molecular surfaces with electrostatics properties were rendered with both VMD V1.9.1 and PyMOL V1.6 using the PDB2PQR program in conjunction with the CHARMm force field (Oberoi & Allewell 1993).

11. Statistical analysis

Data are expressed as mean and standard deviation, and

Student's *t*-test was used for single statistical comparisons. A $p<0.05$ was considered significant.

Results

1. DHT has no toxic effect on JB6 P+ cells

First, we examined the effects of rutin and rutin metabolites (Fig. 1A) on JB6 P+ cell proliferation using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium assay. The results indicated that rutin, HVA, and HPAA at concentrations up to 20 μM did not significantly affect cell viability 24 h after treatment. DHT, at 10 and 20 μM, suppressed growth of JB6 P+ cells by 20 and 30% (Fig. 1B).

2. DHT inhibits EGF-induced neoplastic transformation in JB6 P+ cells but not rutin, HVA, or HPAA

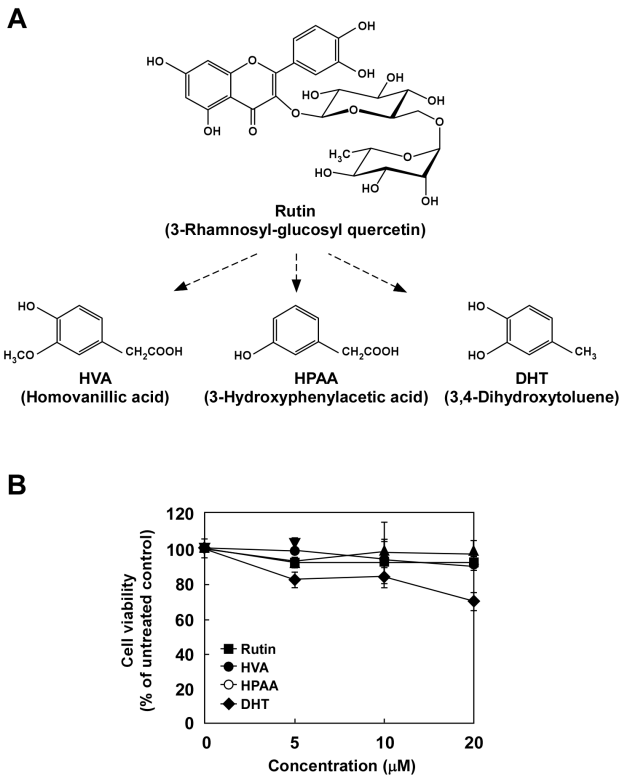

Fig. 1. Toxic effects of rutin and rutin metabolites on JB6 P+ cells. (A) Chemical structures of rutin, homovanillic acid (HVA), 3-hydroxyphenylacetic acid (HPAA), and 3,4-dihydroxytoluene (DHT). (B) Cells proliferation was determined by the MTS assay. Results are expressed as cell viability relative to the untreated control. Data are means ± standard deviations from three independent experiments.

Next, we examined the inhibitory effects of rutin and rutin metabolites on EGF-induced neoplastic transformation in JB6 P+ cells. DHT significantly inhibited EGF-induced neoplastic transformation in JB6 P+ cells at 10 and 20 μM based on the number of cell colonies (Fig. 2A). DHT suppressed EGF-induced cell transformation by 78% and 85%, respectively (Fig. 2B). In contrast, rutin, HVA, and HPAA had no effect on EGF-induced neoplastic transformation at any concentration. These results

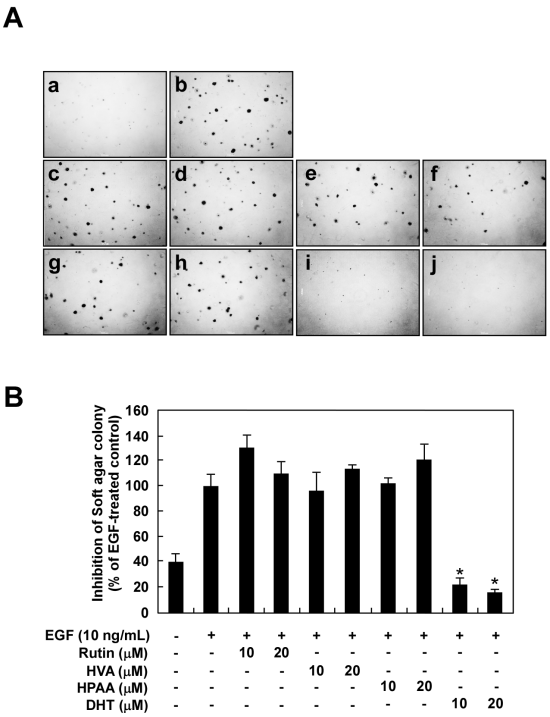

Fig. 2. Comparison of the inhibitory effects of rutin, homovanillic acid (HVA), 3-hydroxyphenylacetic acid (HPAA), and 3,4-dihydroxytoluene (DHT) on epidermal growth factor (EGF)-induced neoplastic transformation in JB6 P+ cells. (A) JB6 P+ cells were treated without or with samples, and colonies were counted 10 days later: (a) untreated control, (b) EGF alone, (c) EGF and 10 μM rutin, (d) EGF and 20 μM rutin, (e) EGF and 10 μM HVA, (f) EGF and 20 μM HVA, (g) EGF and 10 μM HPAA, (h) EGF and 20 μM HPAA, (i) EGF and 10 μM DHT, and (j) EGF and 20 μM DHT. (B) Cell colonies were counted using a microscope with the aid of Image-Pro Plus software. The numbers of colonies in soft agar are presented as percentages relative to EGF-stimulated cells. Data are means ± standard deviations of the number of colonies determined from three independent experiments. Asterisk (*) indicates a significant difference between groups treated with EGF and rutin, HVA, HPAA, or DHT together, and the group treated with EGF alone ($p<0.05$).

suggest that DHT was effective for inhibiting EGF-induced neoplastic cell transformation in JB6 P+ cells.

3. DHT attenuates EGF-induced AP-1 transactivation and phosphorylation of MEK, and ERK, but not Raf-1, in JB6 P+ cells

Activation of AP-1 plays a major role in tumor promoter-induced neoplastic transformation in JB6 P+ cells (Dong et al. 1994; Huang et al. 1997). We measured AP-1 transactivation using JB6 cells stably transfected with the AP-1 luciferase plasmid. DHT effectively inhibited EGF-induced AP-1 transactivation in a dose-dependent manner, but not rutin (Fig. 3A).

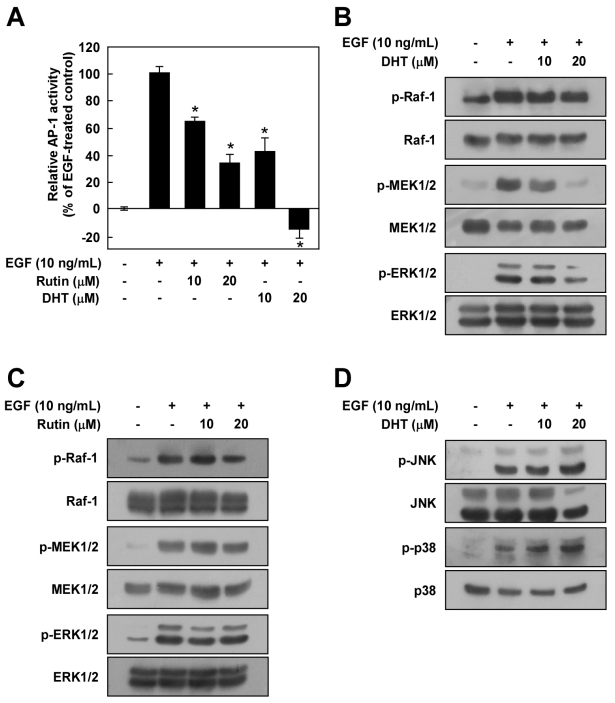

Fig. 3. Effects of rutin or 3,4-dihydroxytoluene (DHT) on epidermal growth factor (EGF)-induced activator protein-1 (AP-1) transactivation and extracellular regulated kinase (ERK) signaling in JB6 P+ cells. (A) JB6 cells stably transfected with an AP-1 luciferase reporter were cultured. Luciferase activity was assayed, and AP-1 activity is expressed as percent inhibition relative to cells treated with EGF alone. Data are means ± standard deviations of AP-1 luciferase activity calculated from three independent experiments. Asterisk (*) indicates a significant difference between groups treated with EGF and rutin or DHT together, and the group treated with EGF alone ($p<0.05$). JB6 P+ cells were treated with the indicated concentrations of DHT (B and D) or rutin (C) for 1 h, stimulated with 10 ng/mL EGF, and harvested 30 min later. The levels of phosphorylated and total Raf-1, MEK1/2, ERK1/2, JNK, and p38 proteins were determined by Western blot. Data are representative of three independent experiments that produced similar results.

The Raf/MEK/ERK signaling pathway is an upstream kinase of AP-1 and is clearly involved in neoplastic transformation (Huang et al. 1998; Bode & Dong 2005). Thus, the effect of DHT or rutin on the Raf/MEK/ERK signaling pathway was investigated. DHT suppressed EGF-induced MEK and ERK1/2 phosphorylation in a dose-dependent manner (Fig. 3C). However, DHT had no effect on EGF-induced Raf-1 phosphorylation. In addition, rutin had no effect on EGF-induced activation of Raf-1, MEK, or the ERK1/2 signaling pathway in JB6 P+ cells (Fig. 3D). Overall, these results confirm that DHT suppressed EGF-induced neoplastic cell transformation by inhibiting AP-1 transactivation and MEK and ERK1/2 phosphorylation in JB6 P+ cells.

4. DHT strongly inhibits Raf-1 kinase activity *in vitro*

Next, we examined the effect of DHT on Raf-1 kinase activity *in vitro* to investigate whether Raf-1 is a molecular target of DHT to inhibit cell transformation. DHT strongly inhibited Raf-1 kinase activity, whereas rutin only slightly suppressed Raf-1 kinase activity (Fig. 4A). DHT (10 μM) reduced Raf-1 kinase activity by 92.5. These results indicate that DHT-inhibited cell transformation involves suppressing Raf-1 activity and subsequently inhibiting downstream signaling pathways.

5. DHT binds with Raf-1 *in vitro* and *ex vivo*

We first performed DHT-binding assays to determine whether DHT directly interacts with Raf-1 *in vitro* and *ex vivo*. Raf-1 was observed on DHT-Sepharose 4B beads (Fig. 4B, left upper panel, lane 3), but not on Sepharose-4B-only beads (Fig. 4B, left upper panel, lane 2). The Raf-1 protein was loaded as a control (Fig. 4B, left upper panel, lane 1). We also observed *ex vivo* DHT and Raf-1 binding in JB6 P+ cell lysates (Fig. 4B, left middle panel, lane 3). However, rutin did not directly interact with Raf-1 *in vitro* or *ex vivo* (Fig. 4B, right upper and middle panels, lane 3). Furthermore, ATP did not compete with DHT for Raf-1 binding (Fig. 4B, left lower panel, lanes 4 and 5). The computer modeling also indicated that DHT docked easily to Raf-1 near the ATP binding site (Fig. 4C). These data reveal that DHT directly binds Raf-1 and subsequently inhibits activation of Raf-1 and downstream signals in an ATP non-competitive manner.

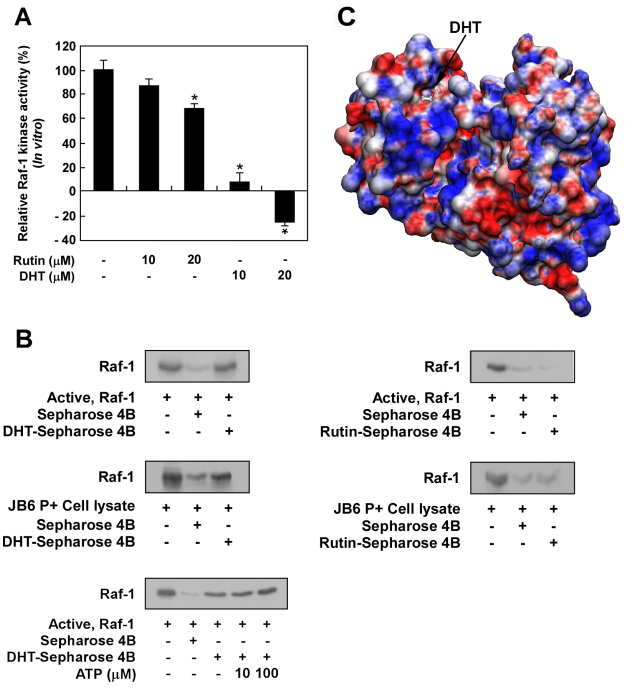

Fig. 4. Effects of rutin or 3,4-dihydroxytoluene (DHT) on Raf-1 kinase activity and direct binding. (A) The *in vitro* Raf-1 kinase assay was performed, and kinase activity is expressed as percent inhibition relative to the untreated control. Data are means ± standard deviations determined from three independent experiments. Asterisk (*) indicates a significant decrease in kinase activity between groups treated with activated Raf-1 and rutin or DHT and the group treated with activate Raf-1 alone (*in vitro* kinase assay; $p<0.05$), EGF and rutin, or DHT together, and the group treated with EGF alone ($p<0.05$). (B) Pull-down and ATP competition assays were performed. *In vitro* and *ex vivo* Raf-1-rutin or -DHT binding was confirmed by immunoblotting using a Raf-1 antibody (B, left panels), first lane (input control); Raf-1 protein standard or whole-cell lysate from JB6 P+ cells, second lane (control). Sepharose 4B was used to pull-down Raf-1 or a JB6 P+ cell lysate precipitated with Sepharose 4B beads, third lane; Raf-1 or a whole JB6 P+ cell lysate was pulled down using rutin- or DHT-Sepharose 4B affinity beads. (C) Modeling and docking of DHT to the Raf-1 kinase domain. Molecular surface of the Raf-1 kinase domain bound with the DHT best docking solution. Electrostatic surface is colored as follows: blue, positive charges; red, negative charges with the units +5/ − 5 kb • T • e_c^{-1}.

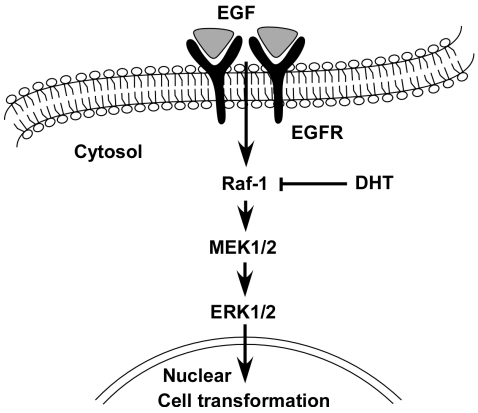

Fig. 5. Simplified depiction of the effects of 3,4-dihydroxytoluene (DHT) on epidermal growth factor (EGF)-induced cell transformation.

Discussion

It has been thought for many years that dietary components such as flavonoids positively impact various diseases (Surh YJ 2003; Langner & Rzeski 2012). Of note, rutin and quercetin have been studied for their substantial potential effects against inflammation (Choi et al. 2014) and cancer (Lin et al. 2012; Alonso-Castro et al. 2013). However, these biological activities of rutin and quercetin may result from rutin metabolites because rutin is metabolized to HVA, HPAA, and DHT (Fig. 1A) in humans (Jaganath et al. 2009; Pashikanti et al. 2010). Therefore, we focused on rutin and rutin metabolites due to their biological and pharmacological potential. A previous study showed the anti-inflammatory properties of DHT in lipopolysaccharide-activated macrophages (Su et al. 2014). DHT is toxic to murine tumor cells (Morita et al. 2003) and induces apoptosis in metastatic melanoma cells (Payton et al. 2011). These results suggest that DHT may have a preventive effect against the development of inflammation and cancer. In the present study, DHT exhibited a much stronger inhibitory effect on EGF-induced neoplastic cell transformation in JB6 P+ cells than that of rutin, HVA, and HPAA (Fig. 2).

Transactivation of AP-1, which is an important transcription factor in tumor promoter-induced transformation and tumor development, is activated during cell transformation by MAP kinase signaling in response to various tumor promoters (Dong et al. 1994; Huang et al. 1997). In this study, DHT inhibited EGF-induced AP-1 activation in a dose-dependent manner, whereas rutin had little effect (Fig. 3A). As a major MAP kinase, the Ras/MEK/ERK signaling pathway acts as an upstream kinase for AP-1 transactivation and is constitutively activated resulting in cell proliferation and transformation (Dong et al. 1994; Huang

et al. 1997; Bode & Dong 2005). DHT, but not rutin, inhibited EGF-induced phosphorylation of MEK, and ERK1/2, but not Raf-1, in JB6 P+ cells (Fig. 3B and C). However, DHT did not inhibit EGF-induced phosphorylation of JNK or p38 in JB6 P+ cells, which are other MAP kinases involved in EGF-induced cell transformation (Antonyak et al. 1998; He et al. 2003) (Fig. 3D).

In vitro kinase, pull-down, and ATP competition assays were performed to determine whether Raf-1 is a DHT molecular target. DHT strongly inhibited Raf-1 activity by directly binding to Raf-1 in an ATP-noncompetitive manner, but not rutin (Fig. 4A and B). Next, we performed a modeling study with docking of DHT to the crystal structure of the Raf-1 catalytic domain to detect the molecular interaction between Raf-1 and DHT. The Raf-1 kinase domain had a large cleft to bind one ATP molecule. About 75% of the best Raf-1 docking solutions were located in a pocket across the nucleotide binding cleft (Fig. 4C). DHT showed strong affinity for Raf-1, and the best docking solution binding energy was −6.2 kcal/mol. A pair of hydrogen bonds involving the Gln386 backbone oxygen and side chain anchored DHT. As a complement, both Phe360 and Phe390 sandwiched the DHT benzene ring. Taken together, our modeling study indicated that DHT suppressed Raf-1 by binding to the neighboring ATP site.

Summary

In summary, the rutin metabolite DHT significantly reduced EGF-induced neoplastic transformation in JB6 P+ cells. This inhibition was mediated mainly by blocking the Raf/MEK/ERK signaling pathway and subsequent suppression of AP-1 activities. DHT attenuated Raf-1 kinase activity by directly binding to Raf-1 *in vitro* and *ex vivo*. Taken together, these results suggest that Raf-1 may be a critical molecular target to suppress DHT-induced neoplastic transformation, which is mainly attributable to the chemopreventive potential of several foods containing rutin.

Acknowledgments

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