Influence of Vehicle, Distant Topical Delivery, and Biotransformation on the Chemopreventive Activity of Apigenin, a Plant Flavonoid, in Mouse Skin

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Purpose. This study was designed to (a) establish a short-term in vivo system to evaluate topical formulations of apigenin, (b) determine whether apigenin should be topically delivered to the local skin tissue or systemic circulation, (c) investigate if biotransformation was involved in apigenin's chemopreventive activity.

Methods. The effect of topical applied apigenin in acetone/DMSO (A/D, 9:1) on the promotion of skin tumorigenesis was studied. The influence of apigenin in DMSO, A/D (4:1), and propylene glycol/DMSO (PG/D, 4:1) on 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ornithine decarboxylase (ODC) activity was compared. Distant topical delivery of apigenin was conducted on abdominal skin and ODC activity was monitored in dorsal skin. Potential glucuronidation/sulfation of apigenin in intact skin was assessed by measuring isolated apigenin before and after enzyme hydrolysis with glucuronidase/sulfatase. The epidermal extracts from apigenin-treated SENCAR mice were analyzed for metabolites by HPLC.

Results. Apigenin (5μmol) in A/D did not significantly reduce skin tumor incidence in contrast to previous data with DMSO. Inhibition of TPA-induced ODC by apigenin in three vehicles was in the order of DMSO > A/D > PG/D. TPA-induced ODC in dorsal skin was not inhibited by apigenin delivered from abdominal skin. The quantity of apigenin recovered from epidermal extract was not different before and after β-glucuronidase/sulfatase treatment. Metabolites were not observed in the HPLC profiles of epidermal extracts from apigenin-treated mice.

Conclusions. (a) The short-term TPA-induced ODC was validated for evaluating topical formulations of apigenin. (b) Topical delivery of apigenin should target the local skin tissue. (c) Glucuronidation/sulfation appeared not to be involved in apigenin's chemopreventive activity.

KEY WORDS: apigenin; flavonoid; solvent effect; two-stage tumorigenesis; ornithine decarboxylase; flavonoid metabolism.

INTRODUCTION

Apigenin, 4',5,7-trihydroxyflavone (Figure 1), is a natural flavonoid from vegetables and fruits. Compared with other flavonoids such as quercetin, apigenin was relatively nontoxic and nonmutagenic (1). Topical treatment with apigenin significantly inhibited skin carcinogenesis by UV in SKH-1 hairless mice (2) and by dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) in SENCAR mice (3). Although apigenin strongly absorbs UV light (4), the inhibition of UV carcinogenesis was not by simply absorbing UV light or decreasing DNA damage (2). Apigenin was a potent inhibitor of epidermal ODC, a tumor promotion marker, induced by UV light (2) or TPA (3). It was found to arrest cell cycle in the G_2-M phase in mouse keratinocytes (5). These results suggest that apigenin is a promising skin cancer preventive agent. With the increase of skin cancer incidence in recent years, prevention at multiple levels with chemopreventive agents such as apigenin is of great interest.

In our previous skin tumorigenesis studies, apigenin was applied topically to animals either in dimethylsulfoxide (DMSO) (3) or in acetone/DMSO (A/D, 9:1) (2). Obviously, these vehicles will not be acceptable for human application. Thus, development of efficient and pharmaceutically acceptable topical formulations is critical for apigenin's application in skin cancer prevention in humans. The current study was designed to answer several important questions related to topical formulation development for apigenin.

We first studied the effect of apigenin in acetone/DMSO (A/D, 9:1) on skin tumorigenesis compared with previous DMSO data, and the effect of apigenin in three model vehicles, DMSO, A/D(4:1), and propylene glycol/DMSO(PG/D, 4:1), on TPA-induced ODC. These studies established a correlation between the short-term ODC model and the long-term skin tumorigenesis model so that the TPA-induced ODC was validated for evaluating topical formulations of apigenin. Secondly, we delivered apigenin from a distant site of skin (abdomen) and determined the effect of apigenin on TPA-induced ODC activity in the dorsal epidermis. This allowed us to elucidate whether topical delivery of apigenin should target the local skin tissue or the systemic circulation for skin cancer prevention. Thirdly, we probed the existence of apigenin glucuronides, sulfates or any other metabolites in intact skin from apigenin-treated mice to see if biotransformation played a role in apigenin's chemopreventive activity.

MATERIALS AND METHODS

Chemicals

Apigenin was obtained as a salt with 93.3% purity from Fluka Chemical Co., Tonkonkama, NY. It was changed to the phenol form and purified through recrystallization in acidic methanol to more than 99% pure by HPLC. TPA was obtained from L. C. Service Co., Woburn, MA, acetone and DMSO were purchased from Mallinckrodt, Inc., Paris, KY. Propylene glycol, β-glucuronidase/sulfatase, and enzyme standard substrates were purchased from Sigma Chemical Company, St. Louis, MO.

Animals

Female SENCAR mice (5–7 weeks old) were obtained from Frederick Cancer Research Facility, Frederick, MD, and maintained on Wayne LaBlox Diet unless indicated below. The mice were kept five per cage under standard conditions (12-h light/12-h dark cycle, humidity at 40 ± 5%, temperature at 21 ± 2°C and 10 air changes/h). The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23).

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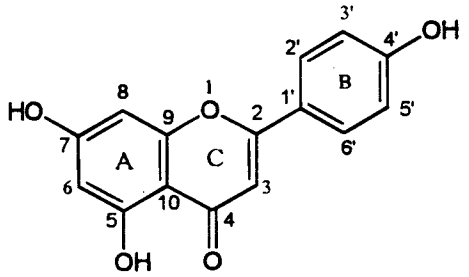

Fig. 1. Chemical structure of apigenin (4',5,7-trihydroxyflavone, $C_{15}H_{10}O_5$, mol wt 270.23).

Two-stage Skin Tumorigenesis Study

Female SENCAR mice were fed and treated as described previously (3). At 8 weeks of age, a 2 × 2 cm area of dorsal skin on each mouse was shaved with a clipper and only those mice not showing signs of hair regrowth 2 days later were used. The animals were grouped into two treatment categories: (i) with DMBA plus TPA treatment (30 mice for each group), (ii) without DMBA plus TPA treatment (15 mice for each group). Each category included a vehicle control group and an apigenin group. In the first category, animals received a single initiating dose of 10 nmol DMBA in 0.2 mL acetone topically. One week later, animals began to receive twice weekly applications of 3.2 nmol TPA in 0.2 mL acetone. Thirty minutes before each TPA treatment, the animals were treated topically with 0.2 mL A/D (9:1) with (apigenin group) or without (vehicle control) 5 μmol apigenin freshly prepared. TPA and apigenin treatment continued for 20 weeks and animals were held for 30 weeks after DMBA initiation. In the second category, animals were treated with vehicle or apigenin only.

ODC Studies

Female SENCAR mice (8 weeks old) were shaved 48 hr prior to topical treatment with 0.2 mL DMSO, A/D (4:1), or PG/D (4:1) with or without 10 μmol apigenin on the back (4 cm^2). One hour later, the mice were treated with 8.5 nmol TPA in 0.2 mL acetone. Six hours following TPA treatment, the mice were killed, the dorsal skin was removed, and epidermis was separated by heat shock in 57°C water for 30 seconds followed by immersing in an ice bath for 5 seconds. The epidermis was homogenized in homogenizing buffer containing protease inhibitors. After centrifugation at 15,000 rpm for 15 minutes, the epidermal extract (supernate) was assayed for ODC activity using DL-[1-^{14}C]-ornithine as the substrate as described previously (3).

To study the influence of distant topical delivery, animals were treated with apigenin (5 μmol in 0.1 mL DMSO) on the abdomen skin and DMSO (0.1 mL) on the dorsal skin (2 cm^2 area). One hour later, the dorsal skin was treated with 4.25 nmol TPA in 0.1 mL acetone. Six hours following TPA treatment, ODC activity in the dorsal epidermis was assayed as described above. For the positive control, animals were treated with DMSO on the abdomen and apigenin in DMSO on the dorsal skin. For the negative control, both dorsal skin and abdominal skin were treated with DMSO only. ODC activity in dorsal epidermis was assessed for both the positive and negative controls.

Enzyme Hydrolysis Studies

Apigenin, 5 μmol in 0.1 mL DMSO or A/D, was topically applied to the shaved dorsal skin of female SENCAR mice (8–10 weeks old). Six hours later, the mice were killed and the apigenin-treated area was washed with 5 × 100μl 0.1% Tween 20 solution to remove the drug on the skin surface. The apigenin-treated skin area was cut, subcutaneous fat was removed, and epidermis was separated by heat-shock as described earlier. The epidermis was homogenized in absolute ethanol to extract apigenin. After evaporating the solvent, the residue was dissolved in acetate buffer (0.1 M, pH 5) and the aqueous samples were incubated at 37°C for 16 hours with or without β-glucuronidase and sulfatase. Apigenin in the solution was recovered by ethyl ether extraction and quantitated by HPLC using an Alltima C18 column (2.1×250mm, Alltech, Deerfield, IL) and a variable wavelength UV detector (Model 164, Beckman Instruments, Fullerton, CA). The column was eluted with 0.1% TFA in H_2O/acetonitrile (52:48) at a flow rate of 0.3 mL/min and detected at 337 nm.

Parallel with the enzyme hydrolysis, the activity of β-glucuronidase and sulfatase were assayed in epidermal extract aqueous solution (samples) or acetate buffer (controls) using standard substrates (phenolphthalein glucuronide for β-glucuronidase and p-nitrocatechol sulfate for sulfatase).

RESULTS

Vehicle Effect on Apigenin's Inhibition of Skin Tumorigenesis

The effect of apigenin in A/D (9:1) on tumorigenesis was studied in DMBA initiated mice by comparing mice treated with apigenin in A/D with mice treated with A/D only before TPA treatment (Figure 2). The vehicle and apigenin groups without DMBA and TPA treatment are not shown because the animals did not develop tumors. With 5 μmol apigenin in A/

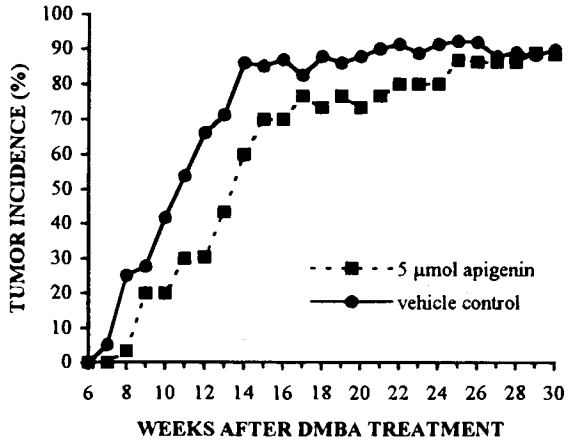

Fig. 2. Skin papilloma incidence in apigenin-treated and vehicle control groups. The mice of both groups received a single initiating dose of 10 nmol DMBA in 0.2 mL acetone on dorsal skin. One week after initiation these animals received twice weekly application of TPA (3.2 nmol in 0.2 mL acetone) for 20 weeks post initiation, 0.2 mL acetone/DMSO(9:1) with or without 5 μmol apigenin was applied 30 min prior to each TPA treatment. The two groups were not significantly different by Log-rank lifetest ($p > 0.1$).

D, the tumor incidence was not significantly different from that of vehicle control (87% vs. 92% at week 26) ($p > 0.1$ by Log-rank lifetest). This result was in contrast to our previous data with apigenin delivered in DMSO at the same dose (37% inhibition) (3). Furthermore, the tumor multiplicity was not different between the apigenin group and the vehicle control ($p > 0.5$ by t-Test). The numbers of tumors per tumor bearing mouse were 6.6 ± 1.0 (Mean ± SE) for apigenin group and 6.4 ± 0.9 for A/D control at week 20 post DMBA initiation. The body weights of the mice were not different between vehicle (29–32 g) and apigenin groups (28–37 g).

Vehicle Effect on Apigenin's Inhibition of TPA-induced ODC

The effect of vehicle on the inhibition of TPA-induced epidermal ODC by apigenin is compared among DMSO, A/D (4:1), and PG/D (4:1) in Table I. Apigenin at 10 μmol dose inhibited TPA-induced ODC in the order of DMSO > A/D > PG/D (82, 38, and 3% inhibition over vehicle control). Earlier experiments showed that TPA-induced ODC activity was not different when the animals were treated with DMSO, A/D (4:1) or PG/D (4:1) compared with TPA treatment alone. In addition, the three solvent control groups in this study had similar TPA-induced ODC activity ($p > 0.1$ by Anova). In another experiment, it was found that apigenin in DMSO was twice as effective as apigenin in A/D at both 4:1 and 9:1 ratios in inhibition of TPA-induced ODC. The reason we used 4:1 ratio in this ODC experiment instead of 9:1 was that apigenin was easier to dissolve in A/D (4:1) and the solution could be stored for several days without precipitation. This will benefit future applications of this vehicle for more quantitative percutaneous absorption studies.

Effect of Distant Topical Delivery on Apigenin's Inhibition of TPA-induced ODC

This experiment was designed to determine if the inhibition of TPA-induced ODC activity by apigenin was through systemic or local action. We applied apigenin on the abdomen skin of mice to see its effect on TPA-induced ODC in dorsal skin (Table II). Apigenin treatment on the abdomen skin did not inhibit TPA-induced ODC activity on the dorsal skin relative to vehicle control ($p > 0.4$ by t-Test), while apigenin treatment on the dorsal skin (positive control) inhibited 74% of the ODC activity. In this study, the drug treatment area on the back or abdomen skin was limited to one half of the area used in other ODC studies (2 cm^2 vs. 4 cm^2). Thus, the two treatment areas on mouse skin (dorsal and abdomen) could be kept at a large enough distance to prevent possible lateral diffusion of the drug to the other site of skin. The mice were caged individually.

Metabolism of Apigenin in Mouse Skin

The purpose of this study was to investigate whether biotransformation of apigenin in skin was involved in its chemoprevention activity. Our metabolism study was conducted at 5–7 hr following apigenin treatment to replicate the period of time between apigenin treatment and ODC assay. Epidermal extracts from apigenin-treated mice were incubated with β-glucuronidase and sulfatase to hydrolyze any existing apigenin glucuronide or sulfate. The apigenin quantities recovered were then compared with those of controls that were incubated without the enzymes. The results are summarized in Table III. Relative to controls, apigenin recoveries were 101% and 100% for samples incubated with glucuronidase/sulfatase at 16.7/0.83 unit/mL and 33.3/1.66 unit/mL, respectively. Apigenin quantities recovered were not different from samples with or without enzyme treatment ($p > 0.9$ by Anova). To make sure that the enzymes were active under our conditions, both β-glucuronidase and sulfatase activities were assayed with standard substrates in parallel with the enzyme hydrolysis experiments. The results showed that the enzymes at the same two doses were able to hydrolyze the standard substrates in epidermal extract (samples) or in acetate buffer (control). The data presented in Table III are from mice treated with apigenin in DMSO. Similar results were observed with A/D (4:1) as the vehicle. HPLC profiles of these epidermal extracts at different ratios of elution solvent mixture (water and acetonitrile), different flow rates, or different detection wavelengths did not show additional peaks

Table I. Effect of Apigenin in Different Vehicles on Epidermal ODC Activity Induced by TPA[a]

Treatment	n	ODC activity[b] (nmol CO_2/30 min/mg protein)	%Inhibition[c]
DMSO	3	5.4 ± 0.7	—
DMSO + apigenin	3	1.0 ± 0.3	82
A/D(4:1)	3	4.0 ± 0.6	—
A/D(4:1) + apigenin	3	2.5 ± 0.3	38
PG/D(4:1)	4	4.0 ± 0.2	—
PG/D(4:1) + apigenin	4	3.9 ± 0.4	3

[a] Animals were treated topically with 0.2 mL solvent with or without 10 μmol apigenin on 4 cm^2 dorsal skin 1 hr before TPA treatment (8.5 nmol in 0.2 mL acetone). A/D = acetone/DMSO. PG/D = propylene glycol.
[b] Data are expressed as mean ± standard error.
[c] Percentage of inhibition over vehicle control. The three apigenin groups are significantly different ($p < 0.005$) while the three solvent control groups are not significantly different ($p > 0.1$) by Anova.

Table II. Inhibition of TPA-induced ODC in the Back Skin by Apigenin Treated on Back or Abdomen Skin of Mice[a]

Treatment on skin		n	ODC activity[b] (nmol CO_2/30 min/mg protein)	%Inhibition[c]
Dorsal	Abdomen			
DMSO	DMSO	3	3.0 ± 0.9	—
DMSO	Apigenin	3	2.8 ± 1.2	6
Apigenin	DMSO	3	0.8 ± 0.5	74

[a] Animals were treated with 0.1 mL DMSO with or without 5 μmol apigenin on dorsal skin and abdomen skin (2 cm^2 area). One hour later, animals were treated with TPA (4.25 nmol in 0.1 mL acetone) on the dorsal skin. ODC activity in dorsal skin was assayed 6 hr after TPA treatment.
[b] Data are expressed as mean ± standard error. The two apigenin groups are significantly different ($p < 0.05$) while the apigenin group treated on the abdomen is not significantly different from the solvent control group ($p > 0.4$) by t-Test.
[c] Percentage of inhibition over vehicle control.

Table III. Apigenin Recovered After Enzyme Hydrolysis of the Epidermal Extracts from Apigenin Treated Mice[a]

Glucuronidase/Sulfatase unit/mL		n	Apigenin Recovery[b] μg	%
0	0	8	1.26 ± 0.04	—
16.7	0.83	5	1.27 ± 0.02	101
33.3	1.66	8	1.26 ± 0.11	100

[a] Animals were treated topically with 5 μmol apigenin in 0.1 mL DMSO for 6 hr. The epidermis was extracted with ethanol. After removing the solvent, the residue was incubated in acetate buffer with the enzymes at 37°C for 16 hr. Apigenin recovered was assayed by HPLC.

[b] The μg data are expressed as mean ± standard error, and there is no difference among the three groups by Anova ($p > 0.9$). % = percentage of recovery over vehicle control.

compared with the profile of blank epidermal extract spiked with apigenin.

DISCUSSION

We previously conducted two experiments demonstrating the inhibition of DMBA/TPA skin tumorigenesis by apigenin in DMSO (3). In the first experiment, tumor incidence was reduced to 16.7% from 76.7% of the control with apigenin in DMSO at 20 μmol dose and the numbers of tumors per effective mouse were reduced to 0.37 from 4.33. In the second experiment, tumor incidence was reduced to 39.3% and 58.6% from 93.3% of the control, and the numbers of tumor per effective mouse were reduced to 1.8 and 2.5 from 7.5 with apigenin in DMSO at 20 μmol and 5 μmol dose, respectively. In contrast, treatment with apigenin in A/D prior to TPA did not inhibit skin carcinogenesis (Figure 2). Because of the strength of our earlier observations, we did not include apigenin in DMSO in the current tumorigenesis study. In addition, the tumor incidence of the solvent controls in the present study and previous studies was the same (92% vs. 93% at week 26), indicating that these experiments are comparable.

Induction of ODC has been used extensively as a biological marker to evaluate agents with tumor promotion or antitumor promotion activity (6). Thus, we employed TPA-induced ODC to assess the impact of vehicle on apigenin's inhibition of tumor promotion. The greater inhibitory effect with DMSO than with A/D on TPA-induced ODC paralleled the results in the long-term two-stage tumorigenesis studies. This parallelism validates the short-term TPA-induced ODC as a reliable in vivo system to evaluate apigenin topical formulations for chemoprevention.

Since no difference between vehicle controls was observed in the ODC or tumorigenesis studies, the vehicle impact we found on apigenin's effect in the two models must be caused by the interactions between vehicles and apigenin. One possible consequence of such interactions is that the three vehicles delivered apigenin to the target site with different efficiency. In vivo and in vitro percutaneous absorption of apigenin in the three model vehicles was studied to address this possibility (7).

Another possible consequence is that different vehicles might influence the metabolism of apigenin in the skin differently. Acetone is a well known inducer of many metabolic enzymes (8). It might induce the metabolism of apigenin in skin. However, our preliminary metabolism studies did not show any evidence of apigenin glucuronide, sulfate, or other metabolites in apigenin-treated mouse skin with A/D or DMSO.

For our goal of developing a practical and effective topical delivery system for apigenin to improve its cancer prevention activity, it is important to determine if apigenin should be delivered into the skin or through the skin into blood circulation. Our approach to answer this question was to determine if the inhibitory effect of apigenin on TPA-induced ODC is through systemic or local action. The results of this study support the local effect hypothesis. This suggests that the topical delivery systems of apigenin should deliver apigenin into local skin tissues, not transdermally into blood circulation, for its cancer prevention activity.

Although the metabolism of apigenin and many other flavonoids has been studied in internal organs of animals, metabolism of flavonoids in skin has not been reported (9). It was reported that the major metabolites found in rat urine and feces following oral administration of apigenin were glucuronide (72%) and sulfate (23%) (10). Glucuronidation and sulfation reactions have been observed in rat and mouse skin for compounds such as aminophenol and steroids (11). Accordingly, we focused our initial metabolism studies on glucuronidation and sulfation pathways. Our enzyme hydrolysis experiments did not detect the existence of apigenin glucuronide or sulfate. Furthermore, no evidence of metabolites was observed from the HPLC profiles of epidermal extracts from apigenin-treated mice. These results suggest that apigenin biotransformation in mouse skin may not be involved in its chemopreventive activity. This notion has been confirmed by more intensive studies with [G-^{3}H]-apigenin in a closed in vitro diffusion system using mouse skin (7) and in cell culture using mouse epidermal cells (C50) (5).

In summary, (a) The influence of vehicle on inhibition of TPA-induced ODC was correlated with vehicle effect on two-stage tumorigenesis, thus, TPA-induced ODC was validated for evaluating apigenin topical formulation; (b) apigenin's chemopreventive activity appeared not to be through systemic action, thus, topical delivery of apigenin should target the local skin tissue; and (c) glucuronidation/sulfation appeared not to be involved in apigenin's chemopreventive activity.

With the answers to the basic questions addressed in this paper, studies to directly examine the effects of these vehicles on the percutaneous absorption of apigenin are warranted. Characterization of apigenin percutaneous absorption in these vehicles tested in chemoprevention studies is also necessary for configuring the percutaneous absorption properties of apigenin for its optimal chemoprevention activity.

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