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Cutaneous Biology

Procyanidin B-2, extracted from apples, promotes hair growth: a laboratory study

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Summary

Background We have previously reported that several selective protein kinase C (PKC) inhibitors, including procyanidin B-2, promote hair epithelial cell growth and stimulate anagen induction.

Objectives We discuss the hypothesis that the hair-growing activity of procyanidin B-2 is related to its downregulation or inhibition of translocation of PKC isozymes in hair epithelial cells.

Methods We examined the effect of procyanidin B-2 on the expression of PKC isozymes in cultured murine hair epithelial cells as well as PKC isozyme localization in murine dorsal skin at different stages in the hair cycle.

Results We observed that procyanidin B-2 reduces the expression of PKC-α, -βI, -βII and -η in cultured murine hair epithelial cells and also inhibits the translocation of these isozymes to the particulate fraction of hair epithelial cells. Our immunohistochemical analyses demonstrated that PKC-α, -βI, -βII and -η are specifically expressed in the outer root sheaths of both anagen and telogen hair follicles. The hair matrix at the anagen stage showed no positive staining for these PKC isozymes. Moderate to intense staining for PKC-βI and -βII in the epidermis and hair follicles was observed in a telogen-specific manner; however, expression of PKC-α and -η during the telogen stage was not conspicuous. Gö 6976, an inhibitor of calcium-dependent (conventional) PKC, proved to promote hair epithelial cell growth.

Conclusions These results suggest that PKC isozymes, especially PKC-βI and -βII, play an important role in hair cycle progression and that the hair-growing mechanisms of procyanidin B-2 are at least partially related to its downregulation of PKC isozymes or its inhibition of translocation of PKC isozymes to the particulate fraction of hair epithelial cells.

Key words: baldness, hair cycle, hair follicles, procyanidin B-2, protein kinase C

Protein kinase C (PKC) is a major signal transduction pathway in many tissues and cells, and is known to play a key role in cell proliferation, differentiation and regulation.[1] PKC was first identified and characterized by Nishizuka *et al.* in 1977 as a serine threonine kinase.[2,3] Up to now, at least 12 isozymes have been isolated. PKC is now classified into three major subgroups: (i) contains conventional PKC (α, βI, βII and γ), which is calcium and diacylglycerol dependent; (ii) comprises novel PKC (δ, ϵ, η and θ) whose activity is calcium independent but diacylglycerol dependent; and (iii) includes atypical PKC (ζ, λ, ι and μ) whose activity is calcium and diacylglycerol independent.[4] PKC is known to act as a differentiation signal in epidermal keratinocytes.[5] However, the role of PKC in hair follicle tissue has not been elucidated. It has been reported that PKC acts as a negative hair-growing factor[6–9] and that several selective PKC inhibitors, including procyanidins, exhibit hair-growing activity.[9]

There is, however, only limited information on PKC isozyme expression in hair follicles. In human hair follicles, expression of PKC-α, -β, -δ and -ζ has been confirmed in cultured outer root sheath keratinocytes;[10] and in immunohistochemical studies, expression of PKC-α in mice[11] and -η in humans[12,13] has been confirmed in outer root sheaths of hair follicles.

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This report describes our investigation of the supposed mechanisms of action of hair-growing activity possessed by procyanidin B-2 from the viewpoint of whether it modulates the expression or translocation of PKC isozymes in hair epithelial cells. We also examined the changes in PKC isozyme expression in murine hair follicles and epidermis in relation to hair cycle progression. In this report, we discuss the hypothesis that the hair-growing activity of procyanidin B-2 is related to its downregulation or inhibition of translocation of PKC isozymes in hair epithelial cells.

Materials and methods

Materials

Procyanidin B-2 [epicatechin-(4β → 8)-epicatechin] (Fig. 1) was obtained from apples according to the method described in a previous report.[14] Polyclonal antibodies against PKC-α, -βI, -βII and -η, –α, -βI and -βII: rabbit antihuman; –η: rabbit antimouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The secondary antibody used was biotinylated goat antirabbit immunoglobulin purchased from DAKO (Glostrup, Denmark). Streptavidin–horseradish peroxidase conjugate was purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, U.K.). Gö 6976 was purchased from Calbiochem-Novabiochem (San Diego, CA, U.S.A.).

Isolation and culturing of murine hair epithelial cells

Murine hair epithelial cells were isolated from 4-day-old C3H/HeNCrj mice (Charles River Japan, Kanagawa, Japan) and cultured in MCDB 153 medium according to the method described in another report.[15]

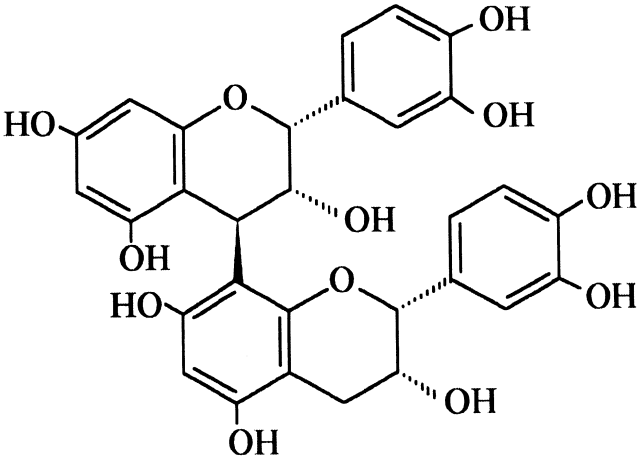

Figure 1. The structure of procyanidin B-2.

Immunoblot analysis (Western blotting)

The cultured murine hair epithelial cell pellet was: (i) sonicated in five 10-second bursts in Buffer A [20 mmol L^{-1} Tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7·5), 2 mmol L^{-1} ethylenediamine tetraacetic acid, 10 mmol L^{-1} ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0·25 mol L^{-1} sucrose, 2 mmol L^{-1} phenylmethylsulphonyl fluoride, 10 μg mL^{-1} leupeptin and 10 mmol L^{-1} 2-mercaptoethanol; final concentrations], and (ii) centrifuged at 100 000 × ***g*** for 60 min (4 °C). The supernatants were concentrated to 1/10 volume using an ultrafilter (MW 30 000 cutting, UFP2 TTK, Millipore, MA, U.S.A.). The fraction is referred to as a cytosol fraction. The pellets were then: (i) dissolved in Buffer B [Buffer A + 0.5% (w/v) polyoxyethylene (10) octylphenyl ether (Triton X-100®]; (ii) sonicated in five 10-s bursts; and (iii) centrifuged at 100 000 × ***g*** for 60 min (4 °C). The supernantants were concentrated to 1/10 volume using an ultrafilter (MW 30 000 cutting UFP2 TTK, Millipore). The fraction is referred to as a particulate fraction. Protein concentrations were determined spectrophotometrically using a DC-Protein Assay kit (Bio-Rad, Hercules, CA, U.S.A.). Sodium dodecyl sulphate–polyacrylamide gel electrophoresis was performed according to the method of Laemmli.[16] The proteins in the gel were electroblotted on to a nitrocellulose membrane (Schleicher, Schuell & Keene, NH, U.S.A.) using a submarine transfer apparatus (Trans-Blot Cell®, Bio-Rad) for 3 h at 60 V per 320 cm^2. The membranes were incubated with diluted polyclonal antibodies (× 500 dilution by the blocking solution) against PKC isozymes (-α, -βI, -βII and -η; Santa Cruz Biotechnology). The membranes were then incubated with biotinylated goat antirabbit immunoglobulin [× 3000 dilution by phosphate-buffered saline (PBS)-T] and with streptavidin–horseradish peroxidase conjugate (× 1000 dilution with PBS-T). Detection of immunoreactive protein was achieved by chemiluminescence using the ECL Western blotting detection system (Amersham Pharmacia Biotech) and recorded by exposure of X-ray film (RX-U, Fuji Photo Film, Tokyo, Japan). Protein bands were identified as PKC by their molecular weight, comigration with their standard proteins (PKC-α, -βI, -βII and -η; human recombinant; Calbiochem-Novabiochem) and lack of staining by the secondary antibody when the primary antibody

was omitted. Quantitative analysis of PKC-isozyme expression was performed by densitometry (CS-9000, Shimadzu, Kyoto, Japan).

Colorimetric assay for cell proliferation by MTT

The degree of cell growth was determined by means of an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The details of the procedure are described in another report.[15]

Immunohistochemical staining

Paraffin-embedded tissue sections of C3H/HeSlc mouse (male, Japan SLC, Shizuoka, Japan) back skin were incubated with the primary polyclonal antibody against PKC-α, -βI, -βII and -η [Santa Cruz Biotechnology; diluted 1 : 100 using 5% (w/v) skim milk and 0·1% (w/v) Tween® 20 in PBS] after incubation with 10% (v/v) normal goat serum. Next, they were treated with biotinylated secondary antibody (goat antirabbit), incubated with streptavidin–horseradish peroxidase conjugate, and reacted with 3-amino-9-ethylcarbazole (AEC) solution (Histostain-SP™ Kit, Zymed, San Francisco, CA, U.S.A.) and hydrogen peroxide. Next, the specimens were counterstained with haematoxylin. Negative controls were obtained by omission of primary antibody.

Results

Procyanidin B-2 intensively promotes hair epithelial cell growth

We examined the growth-promoting activity on murine hair epithelial cells by procyanidin B-2, and confirmed that procyanidin B-2 shows a high growth-promoting activity of more than 300% (30 μmol L^{-1}) relative to controls (= 100%) in a 5-day culture of hair epithelial cells (Fig. 2).

Procyanidin B-2 decreases the levels of PKC-α, -βI, -βII and -η in both the cytosol and particulate fraction of cultured murine hair epithelial cells

We examined the effect of procyanidin B-2 on the expression of PKC isozymes in cultured murine hair epithelial cells using western blotting. The calcium concentration of the culture medium was raised from 0.03 mmol L^{-1} to 0.5 mmol L^{-1} on day 3 during the 7-day culture period. The hair epithelial cells were

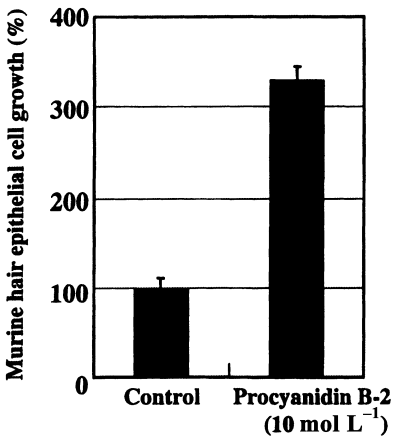

Figure 2. Procyanidin B-2 markedly promotes growth of hair epithelial cells. Growth-promoting activity for hair epithelial cells relative to controls (= 100%) is shown. Procyanidin B-2 was added to the culture during the last 5 days. For the control, a medium without procyanidin B-2 was used. Results are represented as the mean ± SD ($n = 6$).

incubated in media containing 10 μmol L^{-1} of procyanidin B-2 for the final 96 h of the 7-day culture period. Intense staining for PKC-α and -βI was observed in the cytosol fraction of cultured hair epithelial cells; only weak staining for PKC-βII and -η was observed in the cytosol fraction of cultured hair epithelial cells. Intense staining for PKC-α, -βI, -βII and -η was observed in the particulate fraction of cultured hair epithelial cells. We observed decreases in the levels of PKC-α, -βI, -βII and -η in the cytosol fraction of hair epithelial cells cultured in media containing 10 μmol L^{-1} of procyanidin B-2: (i) α = from 72·9% (procyanidin B-2 = 0 μmol L^{-1}) to 34·2% (procyanidin B-2 = 10 μmol L^{-1}) ($P < 0·02$, two-sample t-test); (ii) βI = from 34·6% to 30·6%; (iii) βII = from 4·1% to 0·9%; and (iv) η = from 8·6% to 2·6%. (The level of overall expression of each PKC isozyme in the controls, i.e. procyanidin B-2 = 0 μmol L^{-1}, is represented as 100%.) We observed decreases in the levels of PKC-α, -βI, -βII and -η in the particulate fraction of hair epithelial cells cultured in media containing 10 μmol L^{-1} of procyanidin B-2: (i) α = from 27·1% (procyanidin B-2 = 0 μmol L^{-1}) to 12·5% (procyanidin B-2 = 10 μmol L^{-1}); (ii) βI = from 65·4% to 48·3% ($P < 0·05$, two-sample t-test); (iii) βII = from 95·9% to 58·5% ($P < 0·05$, two-sample t-test); and (iv) η = from 91·4% to 12·9% ($P < 0·05$, two-sample t-test). (The level of overall expression of each PKC isozyme in the controls, i.e. procyanidin B-2 = 0 μmol L^{-1}, is represented as 100%.) As a result of the addition of 10 μmol L^{-1} of procyanidin B-2, the

overall expression of PKC-α, -βI, -βII and -η in hair epithelial cells decreased: (i) α = from 100% (procyanidin B-2 = 0 μmol L^{-1}) to 46·7% (procyanidin B-2 = 10 μmol L^{-1}) ($P < 0{\cdot}02$, two-sample *t*-test); (ii) βI = from 100% to 78·9% ($P < 0{\cdot}02$, two-sample *t*-test); (iii) βII = from 100% to 59·4% ($P < 0{\cdot}002$, two-sample *t*-test); (iv) η = from 100% to 15·5% ($P < 0{\cdot}05$, two-sample *t*-test). (The level of overall expression of each PKC isozyme in the controls, i.e. procyanidin B-2 = 0 μmol L^{-1}, is represented as 100%.) (Figs 3 and 4).

Immunohistochemical study of protein kinase C-α, -βI, -βII and -η in murine hair follicles at different stages in the hair cycle

In 3-day-old dorsal skin in the anagen stage, only weak staining for PKC-α was observed in the outer root sheath keratinocytes below the bulge area (data not shown). In 3·5-week-old and 4·5-week-old dorsal skin, weak staining for PKC-α was observed in the basal and spinous layer of the epidermis. In the hair follicles of 3·5-week-old dorsal skin in the telogen stage, moderate staining for PKC-α was observed in the infundibulum of the outer root sheath keratinocytes (Fig. 5a). In the hair follicles of 4·5-week-old dorsal skin in the anagen stage, intense staining for PKC-α was observed in the bulge area of the outer root sheath keratinocytes, but no staining for PKC-α was observed in the hair matrix (Fig. 5b).

In 3-day-old dorsal skin in the anagen stage, only weak staining for PKC-βI was observed in the bulge area of the outer root sheath keratinocytes (data not shown). In 3·5-week-old dorsal skin in the telogen stage: (i) intense staining for PKC-βI was observed in the infundibulum of the outer root sheath keratinocytes and the hair germ, and (ii) moderate staining for PKC-βI was observed in the basal layer of the epidermis and the outer root sheath keratinocytes below the sebaceous gland in the hair follicles (Fig. 5c). In 4·5-week-old dorsal skin in the anagen stage: (i) scattered staining for PKC-βI was observed in the basal layer of the epidermis and the infundibulum of the outer root

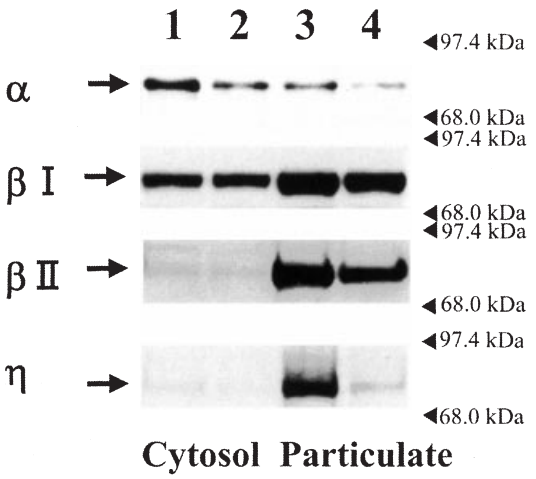

Figure 3. Procyanidin B-2 decreases the levels of protein kinase C (PKC)-α, -βI, -βII, and -η in both the cytosol and particulate fractions of cultured murine hair epithelial cells. Western blotting analytical results are shown for PKC-α, -βI, -βII and -η in cytosol and particulate fractions extracted from cultured murine hair epithelial cells. Procyanidin B-2 (10 μmol L^{-1}) was added to the culture medium during the final 96 h of the 7-day culture period. Procyanidin B-2 dissolved in purified water was added at a rate of 1% (v/v) to the culture medium. The calcium concentration of the culture medium was raised from 0.03 mmol L^{-1} to 0.5 mmol L^{-1} on day 3 during the 7-day culture period. The data show the cytosol fraction of the control (lane 1), the cytosol fraction of 10 μmol L^{-1} procyanidin B-2 (lane 2), the particulate fraction of the control (lane 3), and the particulate fraction of 10 μmol L^{-1} procyanidin B-2 (lane 4). Specific immunoreactive 80 kDa bands for PKC-α, -βI, -βII and -η were detected. Typical results are shown in three independent experiments performed.

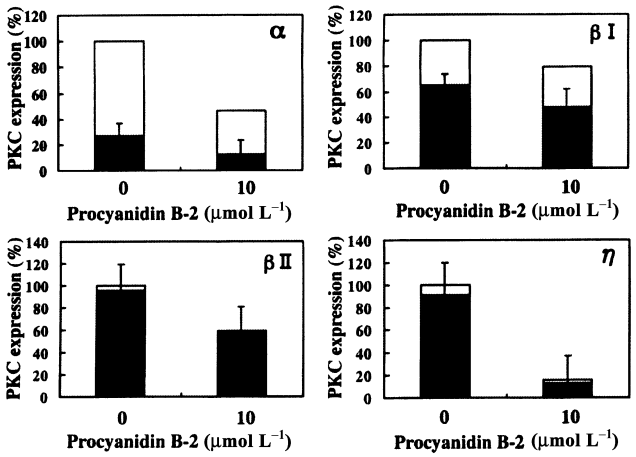

Figure 4. Densitometric analysis of the Western blotting results. Procyanidin B-2 decreases the levels of protein kinase C (PKC)-α, -βI, -βII and -η in both the cytosol and particulate fraction of cultured murine hair epithelial cells; and also suppresses the overall expression of PKC-α, -βI, -βII and -η in cultured murine hair epithelial cells. Procyanidin B-2 (10 μmol L^{-1}) was added to the culture medium during the final 96 h of the 7-day culture period. The calcium concentration of the culture medium was raised from 0.03 mmol L^{-1} to 0.5 mmol L^{-1} on day 3 during the 7-day culture period. (a) PKC-α, (b) PKC-βI, (c) PKC-βII, (d) PKC-η. Clear bar, cytosol fraction; solid bar, particulate fraction; overall = cytosol fraction (clear bar) + particulate fraction (solid bar). The level of overall expression of each PKC isozyme in the controls (procyanidin B-2 = 0 μmol L^{-1}) is represented as 100. Values are represented as mean (for cytosol fractions and overall) or mean ± SD (for particulate fractions) of three independent experiments.

sheath keratinocytes; (ii) intense staining for PKC-βI was observed in the bulge area of the outer root sheath keratinocytes; (iii) weak staining for PKC-βI was observed in the outer root sheath keratinocytes below the bulge area; and (iv) no staining for PKC-βI was observed in the hair matrix (Fig. 5d).

In 3-day-old dorsal skin in the anagen stage, only weak staining for PKC-βII was observed in the bulge area of the outer root sheath keratinocytes (data not shown). In 3·5-week-old dorsal skin in the telogen stage: (i) intense staining for PKC-βII was observed in the basal layer of the epidermis, in the infundibulum of the outer root sheath keratinocytes and in the hair germ, and (ii) weak staining for PKC-βII was observed in the outer root sheath keratinocytes below the sebaceous gland (Fig. 5e). In 4·5-week-old dorsal skin in the anagen stage: (i) scattered staining for PKC-βII was observed in the basal layer of the epidermis and the infundibulum of the outer root sheath keratinocytes; (ii) intense staining for PKC-βII was observed in the bulge area of the outer root sheath keratinocytes; (iii) weak staining for PKC-βII was observed in the outer root sheath keratinocytes below the bulge area; and (iv) no staining for PKC-βII was observed in the hair matrix (Fig. 5f).

In these three stages examined (3-day-old, 3·5-week-old and 4·5-week-old), intense staining for PKC-η was observed in the granular layer of the epidermis. In the hair follicles of 3-day-old dorsal skin in the anagen stage, scattered staining for PKC-η was observed in the outer root sheath keratinocytes (data not shown). In the hair follicles of 3·5-week-old dorsal skin in the telogen stage, weak staining for PKC-η was observed in the whole outer root sheath keratinocytes (Fig. 5g). In the hair follicles of 4·5-week-old dorsal skin in the anagen stage, intense staining for PKC-η was observed in the bulge area of the outer root sheath keratinocytes, but no staining for PKC-η was observed in the hair matrix (Fig. 5h).

Gö 6976 promotes the growth of hair epithelial cells at the optimum concentration

It is reported that Gö 6976 selectively inhibits PKC-α,[17] -βI[17] and -μ.[18] We examined the effects of Gö 6976 on hair epithelial cell growth. Our results confirmed that Gö 6976 promotes cultured murine hair epithelial cell growth at about 120% relative to controls over the Gö 6976 concentration range of 0·1–1·0 nmol L^{-1}. Hair epithelial cell growth was inhibited above a Gö 6976 concentration of 10 nmol L^{-1} (Fig. 6).

Discussion

Procyanidin B-2 inhibits protein kinase C

Procyanidin B-2 is a polyphenol compound classified as a proanthocyanidin, whose structure comprises epicatechin dimmers linked in the 4β → 8 connection mode. It is commonly found in plant species such as apples and grape seeds. We have previously reported and also confirmed in this report that procyanidin B-2 intensively promotes murine hair epithelial cell growth at a rate of about 300% relative to controls (Fig. 2) and stimulates anagen induction *in vivo*;[14] and its hair-growing mechanisms are speculated to be linked to its selective PKC inhibition.[9] Procyanidin B-2 has been shown to inhibit PKC in enzyme assay systems using rat brain PKC, showing an IC_{50} (= 50% inhibiting constant) value of 8·6 μmol L^{-1}.[19]

Protein kinase C regulates cell differentiation and proliferation

PKC has been suggested as a potential mediator for signal transduction in cell regulation in relation to differentiation, proliferation and apoptosis.[1,20] PKC is known to act as a differentiation signal in epidermal keratinocytes. It has been reported that in epidermal keratinocytes, PKC activation is essential in differentiation in the course of keratinization.[5] It has been reported that PKC-α,[21–24] -β[25] and -η[12,26] are assumed to play a role in epidermal keratinocyte differentiation. It is known that PKC exerts a negative influence on the growth of some types of cells such as vascular smooth muscle cells (rat[27]), a breast adenocarcinoma cell line (MCF-7, human[28]), a colon cancer cell line (HT-29, human[29]), bone marrow-derived mast cells (mouse[30]) and a mammary epithelial cell line (HC11, mouse, PKC-α and -βI[31]). However, there is as yet limited information on the role of PKC in hair follicles.

Procyanidin B-2 decreases the level of protein kinase-α, -βI, -βII and -η in murine hair epithelial cells

We examined for the first time the effects of procyanidin B-2, a known specific PKC inhibitor, on PKC isozyme expression and translocation in hair epithelial cells. Our results indicate that procyanidin B-2 decreases the levels of PKC-α, -βI, -βII and -η in both the cytosol and particulate fraction of cultured murine hair epithelial cells. It is known that the distribution of PKC undergoes changes during cell activation. It is thought that procyanidin B-2 affects the intracellular

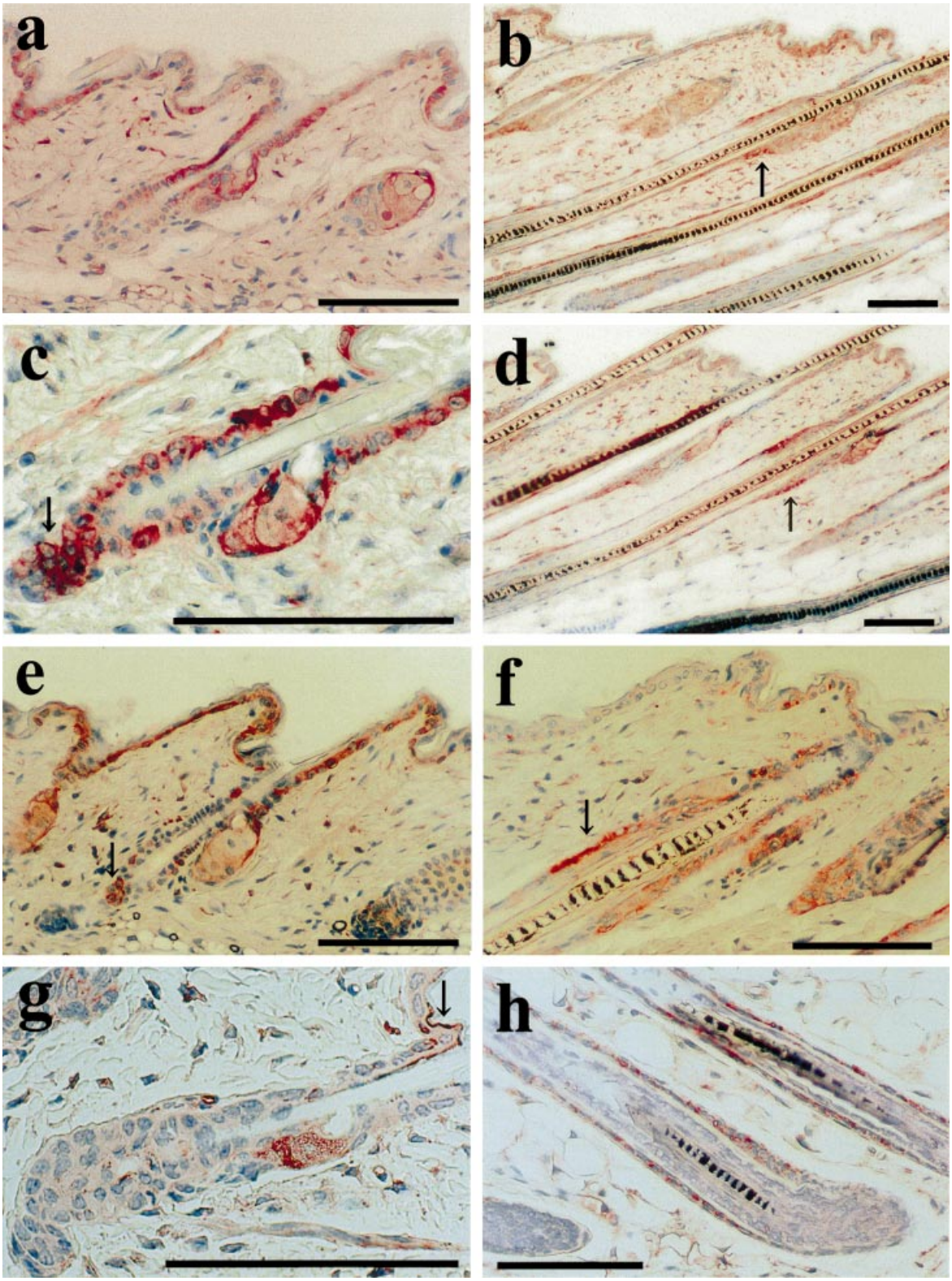
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Figure 5. Immunohistochemical staining for PKC-α, -βI, -βII and -η in murine dorsal skin at different stages in the hair cycle: (a) 3·5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-α; (b) 4·5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-α (arrow indicates the bulge area of hair follicle); (c) 3·5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-βI (arrow indicates the hair germ of hair follicle); (d) 4·5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-βI (arrow indicates the bulge area of hair follicle); (e) 3·5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-βII (arrow indicates the hair germ of hair follicle); (f) 4·5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-βII (arrow indicates the bulge area of hair follicle); (g) 3·5-week-old C3H mouse dorsal skin (telogen stage) stained for PKC-η (arrow indicates the granular layer of epidermis); (h) 4·5-week-old C3H mouse dorsal skin (anagen stage) stained for PKC-η. Bar = 100 μm.

localization of PKC-α, -βI, -βII and -η and modulates interactions with membranes, the cytoskeleton, and with distinct subcellular compartments, followed by initiation of cellular reactions such as mitogenesis.

The localization of protein kinase C isozymes in skin

As for the localization of PKC isozymes in skin, expression of PKC-α, -β, -γ, -δ, -ϵ, -η, -ζ and -μ in murine epidermis has been reported: (i) α: C57BL/6 mice;[11] (ii) α, β and γ: C57BL/6 mice and Sencar mice;[32] (iii) η: CD-1 mice;[33] (iv) η and ζ: NMRI mice;[34] (v) α, β, δ and ϵ: CD-1 mice;[35] (vi) α, βII, δ, ϵ and ζ: CD-1 mice;[36] (vii) α, β, γ, δ, ϵ and ζ: Sencar mice;[37,38] and (ix) α, β, δ, ϵ, η and ζ: CD-1 mice;[39] (x) α, δ, ϵ, η, ζ and μ: NMRI mice.[40] In murine cultured epidermal keratinocytes, expression of PKC-α, -β, -δ, -ϵ, -η and -ζ has been reported: (i) α, δ, ϵ, η and ζ: BALB/c mice;[41,42] (ii) α, δ, η and ζ: BALB/c mice;[43] and (iii) α, β, δ and ζ: CD-1 mice.[44] Abundant localization of PKC-β in the Langerhans cells in mice has also been reported (C57BL/6 mice;[45] CD-1 mice[46]). Wang and Smart[11] observed the expression of PKC-α in the outer root sheaths of murine hair follicles. Little is known about the localization of PKC isozymes in murine hair follicles.

In our experiments, positive staining was observed in the basal (PKC-α), spinous (PKC-α) (Fig. 5b) and granular (PKC-η) (Fig. 5h) layers of the epidermis in the anagen stage, consistent with other reported results (α;[11] η[33]). We observed the expression of PKC-α in the hair follicles (Fig. 5a,b) consistent with other reported results.[11] For PKC-βII, scattered staining was observed in the basal layers of the epidermis in the anagen stage (4·5 weeks old) (Fig. 5f) consistent with reported results for Langerhans cells (β;[45] βII[46]). PKC-β is also known to be involved in murine melanogenesis.[47] However, too little is known to enable PKC-β to be discussed separately as βI or βII with respect to its presence in skin. Concerning the existence of PKC-β in primary cultured murine epidermal keratinocytes, positive[44] and negative[41,43] reports exist. We also confirmed the expression of PKC-β in primary cultured murine hair epithelial cells from the support data in an experiment using a reverse transcriptase–polymerase chain reaction (RT–PCR). We obtained at high frequency a RT–PCR product identical to PKC-β[48] from the cDNA of primary cultured murine hair epithelial cells in an experiment using a set of primers with sequence CGGGGTACCGTXATGGAG and CCGGAATTCCCACCAGTC (data not shown). Consequently, we have confirmed the expression of PKC-α, -βI, -βII and -η in murine hair epithelial cells.

Speculations for the role of protein kinase C in hair cycle progression

We observed the expression of PKC-α, -βI, -βII and -η in the outer root sheaths of both anagen and telogen hair follicles (Tables 1–4): PKC-α, -βI, -βII and -η were specifically expressed with the highest intensity in the bulge area of the outer root keratinocytes of the 4·5-week-old anagen hair follicles; no expression was observed of PKC-α, -βI, -βII or -η in the hair matrix

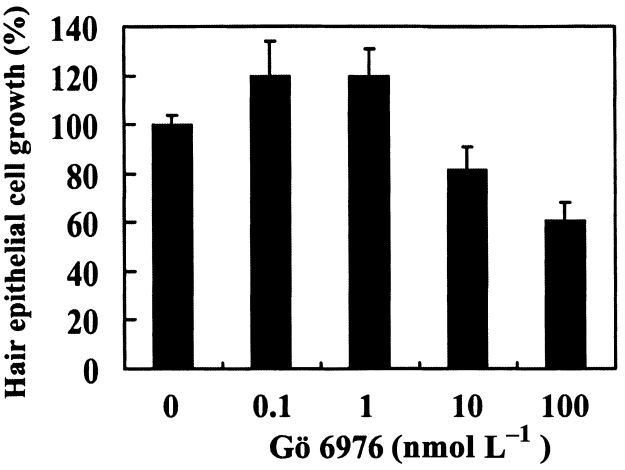

Figure 6. Gö 6976 promotes the growth of cultured murine hair epithelial cells. Growth-promoting activities relative to controls (= 100%) are shown. Gö 6976 dissolved in dimethyl sulphoxide was added at a rate of 1% (v/v) to the culture medium during the 5-day culture period. For the control, we used a medium to which dimethyl sulfoxide was added at the same rate of 1% (v/v). Results are represented as mean ± SD ($n = 6$) carried out with primary cultures prepared from 50 neonatal mice. These results were confirmed in an additional experiment.

Table 1. The localization of PKC-α in murine dorsal skin at different stages in the hair cycle

Age Hair cycle stage	3 days Anagen	3·5 weeks Telogen	4·5 weeks Anagen
Epidermis			
Granular layer	None	None	None
Spinous layer	None	Weak	Weak
Basal layer	None	Weak	Weak
Hair follicle			
Outer root sheath			
Infundibulum[a]	None	Moderate	None
Below the sebaceous gland[b]	–	Weak	–
Bulge area[c]	None	–	Intense
Below the bulge area[c]	Weak	–	Moderate
Hair germ[b]	–	None	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

Table 2. The localization of PKC-βI in murine dorsal skin at different stages in the hair cycle

Age Hair cycle stage	3 days Anagen	3·5 weeks Telogen	4·5 weeks Anagen
Epidermis			
Granular layer	None	None	None
Spinous layer	None	None	None
Basal layer	None	Moderate	Scatterable
Hair follicle			
Outer root sheath			
Infundibulum[a]	None	Intense	Scatterable
Below the sebaceous gland[b]	–	Moderate	–
Bulge area[c]	Weak	–	Intense
Below the bulge area[c]	None	–	Weak
Hair germ[b]	–	Intense	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

Table 3. The localization of PKC-βII in murine dorsal skin at different stages in the hair cycle

Age Hair cycle stage	3 days Anagen	3·5 weeks Telogen	4·5 weeks Anagen
Epidermis			
Granular layer	None	None	None
Spinous layer	None	None	None
Basal layer	None	Intense	Scatterable
Hair follicle			
Outer root sheath			
Infundibulum[a]	None	Intense	Scatterable
Below the sebaceous gland[b]	–	Weak	–
Bulge area[c]	Weak	–	Intense
Below the bulge area[c]	None	–	Weak
Hair germ[b]	–	Intense	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

Table 4. The localization of PKC-η in murine dorsal skin at different stages in the hair cycle

Age Hair cycle stage	3 days Anagen	3·5 weeks Telogen	4·5 weeks Anagen
Epidermis			
Granular layer	Intense	Intense	Intense
Spinous layer	None	Weak	None
Basal layer	None	Weak	None
Hair follicle			
Outer root sheath			
Infundibulum[a]	Scatterable	Weak	Weak
Below the sebaceous gland[b]	–	Weak	–
Bulge area[c]	Scatterable	–	Intense
Below the bulge area[c]	Scatterable	–	Moderate
Hair germ[b]	–	None	–
Hair matrix[c]	None	–	None
Dermal papilla	None	None	None

[a]For anagen and telogen, [b]for telogen, [c]for anagen.

cells in the anagen stage. From the fact that the hair matrix is assumed to be in a highly proliferative state in the anagen stage, it is speculated that PKC-α, -βI, -βII and -η at least are not involved in promoting hair epithelial cell growth. The basal layer of the epidermis and the hair follicles, especially the infundibulum of the outer root sheath keratinocytes and the hair germ, were moderately or intensely stained for PKC-βI and -βII in a telogen-specific manner. We present for the first time the hypothesis that dynamic changes, such as increased expression of PKC-βI and -βII in the epidermis and hair follicles, act to induce and maintain the telogen stage of the hair cycle. This hypothesis is supported by the result that Gö 6976, which is an inhibitor of calcium-dependent PKC inhibitor, promotes murine hair epithelial cell growth (Fig. 6); this is also supported by the results of an experiment using a PKC-βI-overexpressing murine epidermal keratinocyte cell line (3PC cells) whose results suggest that PKC-βI has a growth inhibitory effect on epidermal keratinocytes.[49]

Results of experiments using several PKC inhibitors or activators suggest that PKC acts as a negative hair-growing factor.[6–9] Li *et al*. examined the levels of PKC-α and -δ in BALB/c mice back skin in the course of hair growth induced by diphencyprone[50] and hair plucking;[51] and concluded that the downregulation of PKC-α

expression in skin appears to cause anagen induction in the hair cycle progression. Cyclosporin A is an immunosuppressive agent known to cause hirsutism. It is reported that cyclosporin A downregulates the expression of PKC-α and -β[52] and inhibits the activation and translocation of PKC-β to the plasma membrane[53] in human lymphocytes. We examined the effect of cyclosporin A on PKC expression and translocation in murine hair epithelial cells and observed that cyclosporin A reduced the levels of PKC-α, -βI, -βII and -η in the particulate fraction of cultured murine hair epithelial cells.[54] In addition, calphostin C, a selective PKC inhibitor, known to possess hair-growing activity, has been reported to inhibit translocation of PKC-βII in lymphocytes to cytoplasmic aggregates of spectrin, a major cytoskeleton component.[55]

As the hair-growing mechanism of procyanidin B-2, the involvement of its inhibitory effects on the expression of one or more of these PKC isozymes (α, βI, βII and η) in keratinocytes in both skin and hair was considered likely.

Gö 6976, a specific inhibitor of protein kinase C-α and -βI, promotes hair epithelial cell growth

Gö 6976 is reported to inhibit PKC-μ ($IC_{50} = 20$ nmol L^{-1}) in addition to PKC-α ($IC_{50} = 2{\cdot}3$ nmol L^{-1}) and -βI ($IC_{50} = 6{\cdot}2$ nmol L^{-1}). Growth-inhibiting effects on hair epithelial cells were observed above a Gö 6976 concentration of 10 nmol L^{-1}, while Gö 6976 promotes hair epithelial cell growth at Gö 6976 concentrations of between $0{\cdot}1$ nmol L^{-1} and 1 nmol L^{-1} (Fig. 6). The growth-inhibiting action caused by Gö 6976 at a higher dose range of above 10 nmol L^{-1} may well derive from its inhibiting action on PKC-μ, as PKC-μ is a PKC isozyme that is known to play a role in promoting epidermal keratinocyte growth.[56] Further investigations into other selective PKC inhibitors, which possess a diverse spectrum of PKC isozyme inhibiting activities, will provide useful information about which PKC isozymes are most involved in the regulation of progression of the hair cycle.

Conclusions

The results of the experiments reported in this paper suggest that procyanidin B-2, a compound that possesses hair-growing activity, causes modulation of the expression and translocation of PKC isozymes (α, βI, βII and η) in hair epithelial cells. Our results, combined with those of other investigations, suggest a possible link between the hair-growing activity possessed by procyanidin B-2 and its downregulation or inhibition of translocation of PKC isozymes in hair epithelial cells in addition to its PKC inhibiting activity. It is highly probable that PKC plays a key role in hair cycle regulation.

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