

A Citrus Polymethoxy Flavonoid, Nobiletin Inhibits Sebum Production and Sebocyte Proliferation, and Augments Sebum Excretion in Hamsters

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Acne vulgaris is characterized by excess sebum production, and apart from all-*trans* retinoic acid (atRA) or 13-*cis* retinoic acid (13-*cis*RA), there are few effective agents for acne therapy that directly suppresses sebaceous lipogenesis. In this study, we demonstrated that topical application of a citrus polymethoxy flavonoid, nobiletin, to hamster auricles decreased skin surface triacylglycerols (TG) level and the size of sebaceous glands along with inhibition of diacylglycerol acyltransferase (DGAT)-dependent TG synthesis and sebocyte proliferation. The inhibitory actions were similar to that observed with atRA and 13-*cis*RA in hamster sebocytes. The antilipogenic and antiproliferative actions of nobiletin were also reproduced in UVB (5.4 kJ/m²)-irradiated hamsters, which showed aberrant enhancement of sebum accumulation and sebaceous enlargement. Furthermore, nobiletin, but not 13-*cis*RA, augmented sebum excretion along with increases in intracellular cAMP level, protein kinase A (PKA) activation, and apoptosis-independent phosphatidylserine (PS) externalization in cell membrane. These phenomena were reproduced by forskolin and inhibited by a PKA inhibitor, H-89. These results provide early evidence that nobiletin is an effective candidate for acne therapy through mechanisms that include the inhibition of DGAT-dependent TG synthesis and sebocyte proliferation, and the progression of apoptosis-independent and PS-externalization-dependent sebum excretion by PKA activation.

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INTRODUCTION

Acne vulgaris is an inflammatory disease in sebaceous glands and pilosebaceous units in the skin, which often occurs in pubertal young individuals, under androgen influence (Bojar and Holland, 2004). Acne is characterized by: (i) excess sebum production and enlargement of sebaceous glands that are dependent on androgen; (ii) comedogenesis that is closely associated with hypercornification of the follicular wall epidermis; and (iii) local inflammatory events such as an increase in levels of proinflammatory cytokines and the formation of papules and pustules (Pawin *et al.*, 2004). In addition, *Propionibacterium acnes* (*P. acnes*), a Gram-positive anaerobe microbial species, is considered

to play a principal role in the development of acne vulgaris by causing inflammation and hypercornification in ductal and infundibular epidermis (Bojar and Holland, 2004).

Retinoic acids such as tretinoin (all-*trans* retinoic acid (atRA)) and isotretinoin (13-*cis* retinoic acid (13-*cis*RA)) have been topically and/or systemically used for acne therapy (Krautheim and Gollnick, 2004; Chivot, 2005). Both atRA and 13-*cis*RA have caused a decrease of sebum production and sebaceous gland enlargement by inhibiting lipogenesis and cell proliferation in humans, rats, and hamsters (Zouboulis *et al.*, 1991; Kim *et al.*, 2000; Sato *et al.*, 2001; Nelson *et al.*, 2006). In addition, topical and/or systemic application of antibiotics such as erythromycin, tetracycline, and doxycycline has been reported to be effective in the treatment of acne, because they inhibit the proliferation of *P. acnes* in the follicles and thereby decrease the level of free-fatty acids on the skin surface (Toyoda and Morohashi, 1998; Krautheim and Gollnick, 2004; Sapadin and Fleischmajer, 2006). Antibiotic treatments have also been reported to decrease the inflammatory reactions in acne lesions (Akamatsu *et al.*, 2003; Sapadin and Fleischmajer, 2006). However, the use of these RAs and antibiotics in acne therapy has been limited in acceptance because of adverse effects, such as skin irritation and scaling, teratogenicity, and the induction of bacterial resistance (Eady *et al.*, 1994; Toyoda and Morohashi, 1998; Krautheim and Gollnick, 2004). Therefore, novel anti-acne agents with not only anti-

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Abbreviations: atRA, all-*trans* retinoic acid; 13-*cis*RA, 13-*cis* retinoic acid; PKA, protein kinase A; TG, triacylglycerols; DGAT, diacylglycerol acyltransferase

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lipogenic and anti-inflammatory actions, but also with no or less propensity for causing side effects, may be more beneficial for the remission and/or cure of acne.

Flavonoids from medicinal plants exert various pharmacological effects *in vivo* and *in vitro*, including serving as antioxidants, free radical scavenging, anti-inflammatory activity, and have antitumorigenic and antitumor metastatic activities (Robak and Gryglewski, 1988; Kawaii *et al.*, 1999; Sato *et al.*, 2002; Lin *et al.*, 2003). In addition, Seki and Morohashi (1993) reported that wogonin, a flavonoid derived from Japanese–Chinese traditional herbal medicines, inhibits lipogenesis in hamster sebaceous glands. We previously reported that nobiletin (5, 6, 7, 8, 3', 4'-hexamethoxy flavone), a major component in the juice of *Citrus depressa* Hayata (*Rutaceae*), inhibits UVB-induced production of an inflammatory mediator, prostaglandin E₂, in human keratinocytes, as well as hyperkeratinization in the epidermis of mice (Tanaka *et al.*, 2004). Furthermore, nobiletin has been reported to inhibit the gene expression and production of proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor α in lipopolysaccharide-stimulated mouse macrophages (Lin *et al.*, 2003). Therefore, we hypothesized that the anti-inflammatory agent, nobiletin, may be effective for remission of acne vulgaris.

In this study, we examined the effects of nobiletin on sebum production and excretion as compared with RAs *in vivo* and *in vitro*. Our results showed that nobiletin is a novel anti-acne agent with unique therapeutic actions that not only inhibits lipogenesis and cell proliferation in sebaceous glands, but also facilitates the depletion of sebaceous lipids by increasing sebum excretion.

RESULTS

Effects of topical application of nobiletin and atRA on sebaceous and epidermal morphology, and sebum levels on the skin surface

We first examined the effects of nobiletin (1 and 2%) (Tanaka *et al.*, 2004) on sebaceous glands and epidermis in hamsters by comparing the effects with those produced by atRA (0.2%), a well-known anti-acne agent (Krautheim and Gollnick, 2004; Chivot, 2005). As shown in Figure 1, when auricles were treated once a day with 50 μ l of 2% nobiletin or 0.2% atRA for 14 days, the size of sebaceous glands (arrow heads) in nobiletin- (panel b) or atRA-treated hamsters (panel c) was found to decrease overall compared with that in the hamsters receiving vehicle only (panel a, arrow heads). Although the topical treatment of atRA was found to increase epidermal thickness (Figure 1c vs 1a, asterisks), there was no change in the nobiletin-treated epidermis (Figure 1b vs 1a, asterisks). In addition, thin-layer chromatographic analysis of sebum on the skin surface showed that levels of triacylglycerols (TG), a major sebum component, decreased in both 2% nobiletin- and atRA-treated hamsters (Table 1).

Inhibition of DGAT-dependent TG synthesis and cell proliferation by nobiletin and RAs in hamster sebocytes

To clarify the molecular mechanisms of the anti-acne actions of nobiletin, we examined the regulation of TG synthesis by

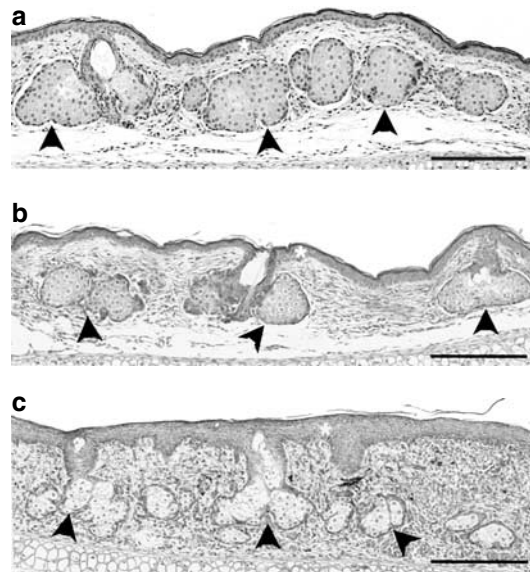


Figure 1. Effects of nobiletin and atRA on hamster sebaceous glands.

Auricles of hamsters were treated once a day (b) with 2% nobiletin (2.5 μ mol) or (c) 0.2% atRA (0.3 μ mol) in 95% ethanol and 5% glycerol, and with the vehicle solution alone (a) for 14 days. After the treatments, auricle tissues were fixed and stained with hematoxylin and eosin as described in the text. Arrowheads, sebaceous glands. Asterisks, epidermis. Bar, 200 μ m.

Table 1. Decrease in the skin surface level of TG in nobiletin- and atRA-treated hamsters

Treatments	Concentrations	Relative amounts of TG on the skin surface (% of vehicle treated hamsters)
Vehicle	—	100
Nobiletin	1%	108.2 \pm 7.4
	2%	66.2 \pm 22.5*
AtRA	0.2%	35.0 \pm 29.3*

atRA, all-*trans* retinoic acid; TG, triacylglycerols.

TG on the skin surface of nobiletin (1 or 2%)- or atRA (0.2%)-treated hamsters was extracted and quantified by thin layer chromatographic analysis as described in the text.

The relative amounts of skin surface TG were expressed by taking vehicle-treated hamsters as 100%.

Data are indicated as mean \pm SD from three individual extracts.

*Significantly different from vehicle-treated hamsters ($P < 0.05$).

this flavonoid and RAs in insulin-differentiated hamster sebocytes, in which TG synthesis and intracellular lipid droplet formation were augmented (Figure 2) (Akimoto *et al.*, 2005). Nobiletin was found to dose-dependently decrease the level of intracellular TG, as did 13-*cis*RA (Figure 2a) and atRA as well (data not shown, Sato *et al.*, 2001). In addition, nobiletin, 13-*cis*RA, and atRA were found to decrease diacylglycerol acyltransferase (DGAT) activity in the differentiated sebocytes (Figure 2b). Furthermore, the proliferation of hamster sebocytes was found to be inhibited by nobiletin, 13-*cis*RA, and atRA in a dose-dependent manner (Table 2).

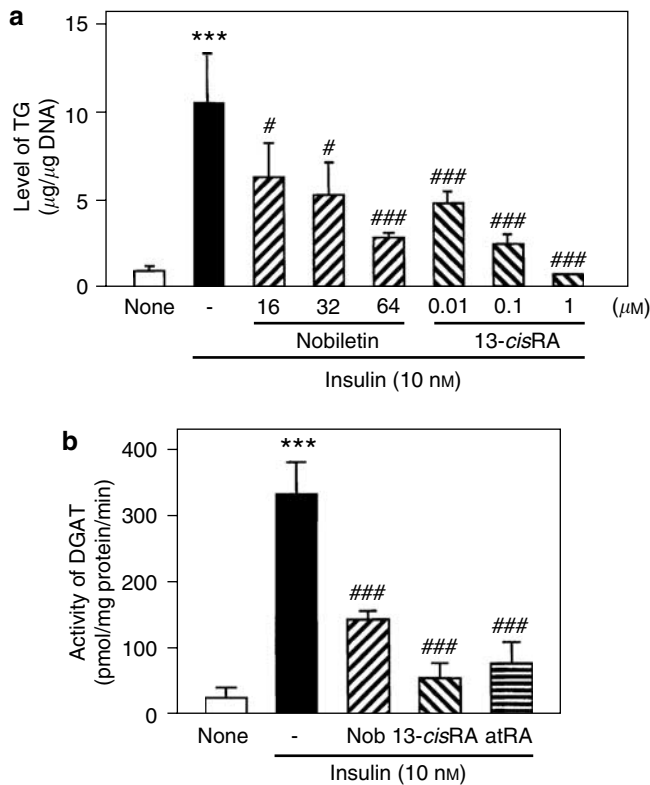


Figure 2. Nobiletin and RAs decrease the production of TG and DGAT activity in insulin-treated hamster sebocytes. (a) Confluent hamster sebocytes at the third passage were treated every 3 days with nobiletin (Nob) (16–64 µM) or 13-cisRA (0.01–1 µM) in the presence of insulin (10 nM) for 12 days, and then the intracellular levels of TG were measured as described in the text. (b) Confluent hamster sebocytes at the third passage were treated with nobiletin (Nob) (64 µM), 13-cisRA (1 µM), or atRA (1 µM) in the presence of insulin (10 nM) for 6 days. The harvested cells were subjected to the measurement of DGAT activity as described in the text. Data are indicated as mean ± SD of three dishes. ***Significantly different from untreated cells (none) ($P < 0.001$). # and ### significantly different from insulin-treated cells ($P < 0.05$ and 0.001, respectively).

Inhibitory actions of nobiletin on UVB-induced abnormal sebaceous lipogenesis in hamsters

We considered that *in vivo* and *in vitro* acne models might be useful to further evaluate the pharmacological efficacy of nobiletin for acne therapy, but there are few established acne models. As UVB has been reported to exhibit aberrant functional and morphological changes in sebaceous and pilosebaceous units, for example, sebaceous hyperplasia, augmentation of sebum synthesis and accumulation, and hypercornification of follicular keratinocytes (Lesnik *et al.*, 1992; Suh *et al.*, 2002; Akitomo *et al.*, 2003), we employed UVB-irradiated hamsters and sebocytes to estimate the anti-acne action of nobiletin against sebum production. As shown in Figure 3, oil red O staining showed that sebum accumulation was augmented in sebaceous glands and follicular ducts in UVB (5.4 kJ/m²)-irradiated hamsters (panels b vs a). When nobiletin was topically applied to the skin of auricles after each UVB-irradiation for 7 days, the aberrant sebum accumulation in sebaceous glands and follicular ducts was found to be depressed (Figure 3c vs 3b). The level

Table 2. Inhibition of cell proliferation by nobiletin and RAs in hamster sebocytes

Treatments	Concentrations (µM)	Cell proliferation ($\times 10^3$ d.p.m./well)	% Of untreated cells
None	-	2.03 ± 0.20	100.0
Nobiletin	16	1.10 ± 0.25**	54.2
	32	0.36 ± 0.07***	17.7
	64	0.16 ± 0.03***	7.9
13-cisRA	0.01	1.50 ± 0.13**	73.9
	0.1	0.60 ± 0.11***	29.5
	1	0.49 ± 0.02***	24.1
atRA	0.01	1.86 ± 0.39	91.6
	0.1	1.02 ± 0.20**	50.2
	1	0.76 ± 0.10**	37.4

atRA, all-trans retinoic acid; 13-cisRA, 13-cis retinoic acid. Hamster sebocytes at the third passage in 24-well multiplates were treated every 3 days with nobiletin (16–64 µM), 13-cisRA (0.01–1 µM), or atRA (0.01–1 µM) for 12 days, and then [³H]thymidine (1 kBq/well) was added to the cells for the last 3 h of the treatments as described in the text. Data are indicated as mean ± SD of four wells. ** and *** significantly different from untreated cells (none) ($P < 0.01$ and 0.001, respectively).

of TG on the skin surface was found to increase in the UVB-irradiated hamsters (2.1-fold), and the augmentation was inhibited by nobiletin-treatment (Figure 4a). Moreover, the DGAT activity was time-dependently increased by UVB irradiation for 24 hours (data not shown), and the augmented enzymic activity was dose-dependently suppressed by nobiletin in the differentiated hamster sebocytes (Figure 4b), suggesting that nobiletin inhibited the aberrant sebum production by decreasing DGAT activity in UVB-irradiated hamster sebaceous glands.

Nobiletin induces sebum excretion in a cAMP/PKA-dependent manner

Although RAs have been reported to directly inhibit sebum production *in vivo* and *in vitro* (Zouboulis *et al.*, 1991; Kim *et al.*, 2000; Sato *et al.*, 2001; Nelson *et al.*, 2006), there is little evidence whether or not abundantly accumulated sebum in sebaceous glands might be excreted to the skin surface by anti-acne agents. Therefore, we examined the effect of nobiletin and RAs on sebum excretion in the differentiated hamster sebocytes by measuring the extracellular TG level. As shown in Figure 5a, nobiletin was found to dose-dependently augment the TG level in the culture medium. The extracellular TG level was also time-dependently augmented by nobiletin for 24 hours, and the increased level of TG was sustained for up to 48 hours (data not shown), indicating that nobiletin depleted the accumulated sebum along with the inhibition of TG synthesis in sebaceous glands. However, there was no change in the extracellular TG level in both 13-cisRA- and atRA-treated sebocytes (Figure 5b and data not shown, respectively). Furthermore, we investigated the involvement of intracellular cAMP in the nobiletin-induced sebum excretion, because cAMP has been reported

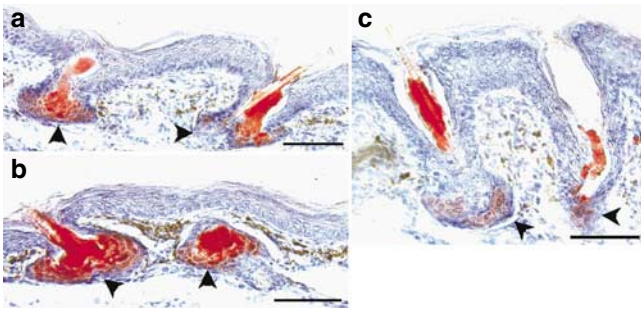


Figure 3. Inhibition of sebum accumulation by nobiletin in sebaceous glands and ducts from the UVB-irradiated hamsters. Auricles of hamsters were treated once a day with 2% nobiletin (2.5 μmol) in 95% ethanol and 5% glycerol or the vehicle solution alone after each UVB irradiation at 5.4 kJ/m^2 for 7 days. After the treatments, the tissues were fixed and stained with oil red O, indicating lipid accumulation in sebaceous glands and ducts. The tissues were also counterstained with hematoxylin. (a-c), vehicle-, UVB plus vehicle-, and UVB plus nobiletin-treated hamsters, respectively. Arrowheads, sebaceous glands. Bars, (a and b) 200 μm and (c) 100 μm .

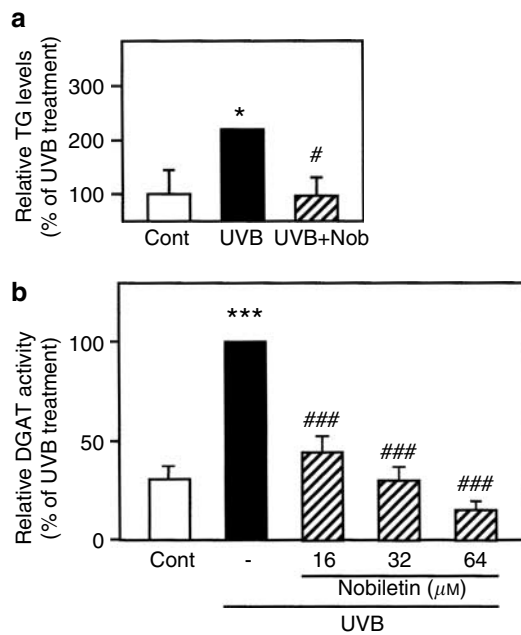


Figure 4. Suppression of sebum production and DGAT activity by nobiletin in UVB-irradiated hamsters and differentiated sebocytes. (a) Levels of TG on the skin surface of UVB- and/or nobiletin-treated hamsters as described in Figure 3 were measured by automatic thin-layer chromatography as described in Table 1. Cont, vehicle-treated hamsters; UVB, UVB (5.4 kJ/m^2) plus vehicle-treated hamsters; and UVB + Nob, UVB (5.4 kJ/m^2) plus nobiletin (64 μM)-treated hamsters. (b) Confluent hamster sebocytes at the third passage were treated every 3 days with insulin (10 nM) to complete the sebocytic differentiation as described in the text. The differentiated cells were irradiated with UVB (0.6 kJ/m^2) and/or nobiletin (16–64 μM) for 24 hours, and then DGAT activity in the cells was measured as described in Figure 2. Data are indicated as mean \pm SD of three dishes. * and ***significantly different from (a) vehicle-treated hamsters (Cont) or (b) differentiated control cells (Cont) ($P < 0.05$ and 0.001, respectively). # and ###significantly different from UVB-irradiated hamsters (a) or sebocytes (b) ($P < 0.05$ and 0.001, respectively).

to play a role as a possible second messenger for the pharmacological actions of nobiletin (Nagase *et al.*, 2005). As shown in Figure 6a, nobiletin was found to increase the intracellular cAMP level within 20 minutes in the differentiated hamster sebocytes. Thereafter, the cAMP level slightly decreased for 20–60 minutes, but was still significantly higher than that in the untreated cells. In addition, cAMP levels were dose-dependently increased in the nobiletin treatment (Figure 6b). Moreover, nobiletin was found to activate protein kinase A (PKA) in the differentiated hamster sebocytes (Figure 6c). Similar sebum excretion and PKA activation was reproduced by forskolin in the sebocytes (Figures 5c and 6c, respectively). However, there was no change in the intracellular cAMP level and PKA activity in the 13-*cis*RA-treated sebocytes (Figure 6b and c, respectively).

Non-apoptotic PS exposure is associated with the nobiletin-induced sebum excretion

Sebum secretion (or excretion) from sebaceous glands to follicular ducts or the skin surface has been reported to be regulated by a holocrine mechanism, which may include sebocyte apoptosis (Wróbel *et al.*, 2003). We, therefore, examined whether or not nobiletin-induced sebum excretion might be associated with sebocyte apoptosis. Flow cytometric analysis showed that nobiletin treatment augmented the number of Annexin V-FITC-positive sebocytes, whereas there was no change in the 13-*cis*RA and aTRA-treated cells

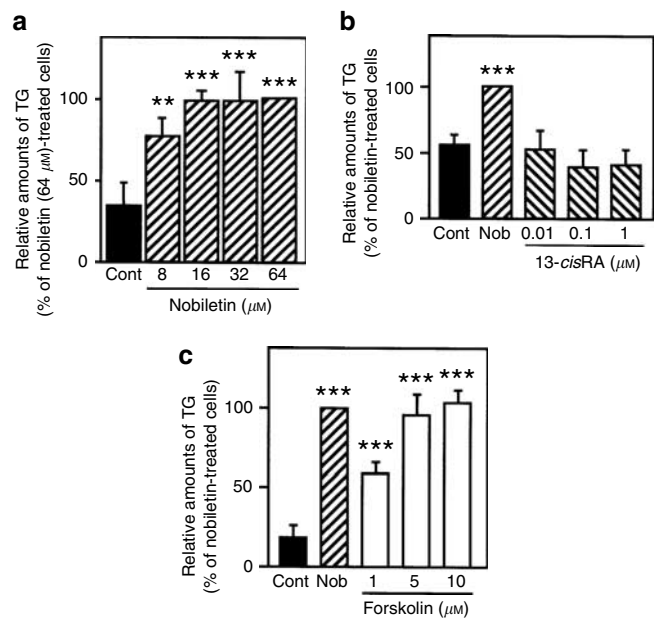


Figure 5. Augmentation of extracellular TG levels by nobiletin, 13-*cis*RA, and forskolin in the differentiated hamster sebocytes. The differentiated sebocytes at the third passage as described in Figure 4 were treated with nobiletin (Nob) (a, 8–64 μM and b and c, 64 μM), (b) 13-*cis*RA (0.01–1 μM), and (c) forskolin (1–10 μM) for 24 hours. The extracellular levels of TG were measured as described in the text. The relative amounts of TG were expressed by taking 64 μM nobiletin (Nob)-treated cells as 100%. Data are indicated as mean \pm SD of three dishes. ** and ***significantly different from the insulin-differentiated cells (Cont) ($P < 0.01$ and 0.001, respectively).

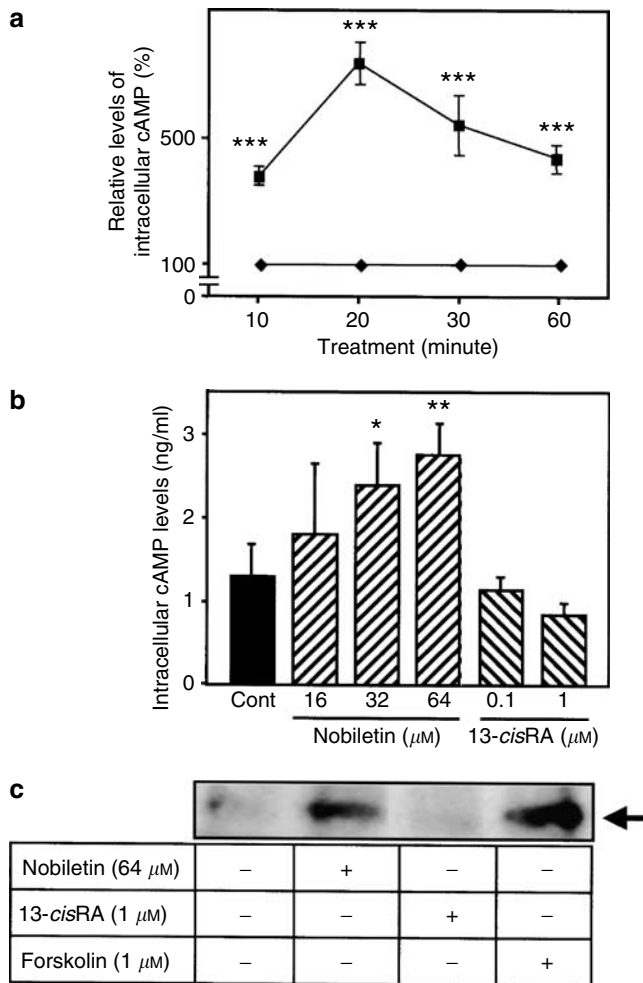


Figure 6. Nobiletin increases the intracellular levels of cAMP and PKA activity in differentiated hamster sebocytes. The differentiated hamster sebocytes as described in Figure 4 were treated (a) with nobiletin (Nob) (64 μM) for 10–60 minutes, and (b) with nobiletin (16–64 μM), or 13-cisRA (0.1 and 1 μM) for 10 minutes. The harvested cells were subjected to the measurement of intracellular cAMP as described in the text. (a) The relative amounts of intracellular cAMP in nobiletin treatment (closed squares) were expressed taking each control cell (closed diamonds) as 100% at indicated time points. Data are indicated as mean \pm SD of three dishes. ***Significantly different from control cells ($P < 0.001$). (b) The amounts of intracellular cAMP in nobiletin- or 13-cisRA-treated cells were measured and data are indicated as mean \pm SD of three dishes. * and **significantly different from untreated cells (Cont) ($P < 0.05$ and 0.01, respectively). (c) The differentiated hamster sebocytes at the third passage as described in Figure 4 were treated with nobiletin (64 μM), 13-cisRA (1 μM), or forskolin (1 μM) for 30 minutes. The harvested cells were subjected to Western blot analysis for PKA-dependent phosphorylated protein (arrow) as described in the text.

(Figure 7a–c), indicating that nobiletin, but not RAs, caused phosphatidylserine (PS) externalization in the differentiated sebocytes. Similar PS exposure to the cell surface was observed in the forskolin-treated sebocytes (Figure 7d). However, no DNA ladders were detected in the nobiletin-treated sebocytes for 24 hours (inserted panel in Figure 7a, lane 2) and further for up to 72 hours (data not shown), whereas staurosporine, a broad-spectrum protein kinase

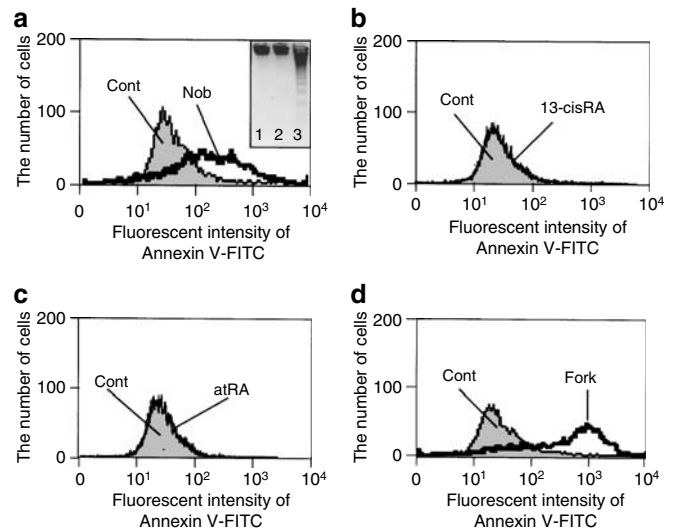


Figure 7. Augmentation of the cell surface exposure of phosphatidylserine by nobiletin in the differentiated hamster sebocytes. The differentiated hamster sebocytes as described in Figure 4 were treated with (a) nobiletin (Nob) (64 μM), (b) 13-cisRA (1 μM), (c) atRA (1 μM), and (d) forskolin (Fork) (1 μM) for 24 hours, and then stained with Annexin V-FITC for 5 minutes as described in the text. The number of Annexin V-positive cells was measured by flow cytometric analysis. Cont, differentiated control cells. Inserted panel in (a) DNA fragmentation in the differentiated hamster sebocytes treated without (lane 1) or with nobiletin (64 μM) (lane 2), or with staurosporine (5 μM) (lane 3) for 24 hours.

inhibitor and well-known apoptosis inducer (Bertrand *et al.*, 1994; Wróbel *et al.*, 2003), exhibited the DNA degradation for 24 hours (inserted panel in Figure 7a, lane 3). Furthermore, the nobiletin-induced PS externalization was found to be inhibited by a PKA inhibitor, H-89, in the differentiated sebocytes (Figure 8a and b). Moreover, under the same culture conditions, the nobiletin-augmented extracellular TG level was found to decrease on treatment with H-89 in a dose-dependent manner (Figure 8c).

DISCUSSION

Regarding lipid metabolism by flavonoids, quercetin, a common dietary flavonoid, has been reported to show an inhibitory activity on TG synthesis by decreasing DGAT activity in cultured human intestinal CaCo-2 cells (Casaschi *et al.*, 2002). Cha *et al.* (2001) reported that a citrus flavonoid, hesperetin, inhibits the activity of hepatic microsomal phosphatidate phosphohydrolase, which is a rate-limiting enzyme for TG synthesis in hepatocytes, and thereby causes the reduction of hepatic TG content in rats fed diets with 1% orotic acid. In this study, we demonstrated that topical application of nobiletin depressed the skin surface TG level. In addition, we learned that the decrease in DGAT activity in differentiated hamster sebocytes was associated with the suppression of TG production. Similarly, topical application of atRA also decreased the levels of skin surface TG as well as sebaceous gland size. As was true for nobiletin, both atRA and 13-cisRA inhibited DGAT activity in differentiated hamster sebocytes. As TG is a major component of sebum

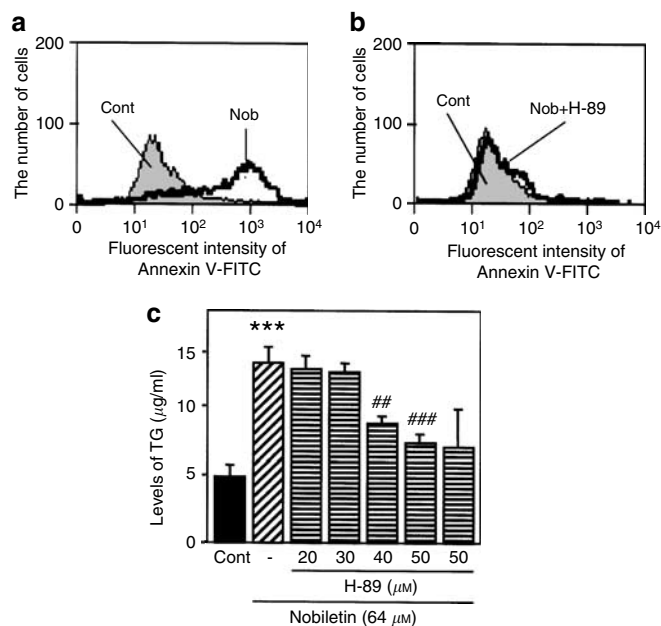


Figure 8. Inhibition of nobiletin-induced cell surface exposure of phosphatidylserine and TG excretion by a PKA inhibitor, H-89, in the differentiated hamster sebocytes. (a and b) The differentiated hamster sebocytes as described in Figure 4 were pretreated with or without H-89 (50 μM) for 1 hour, and then treated with nobiletin (Nob) (64 μM) for another 24 hours. The harvested cells were stained by Annexin V-FITC for 5 minutes, and the number of Annexin V-positive cells was then measured by flow cytometric analysis as described in the text. Three independent experiments were highly reproducible and typical data are shown. (c) The cells were treated with nobiletin (64 μM) and H-89 (20–50 μM) as described above, and then the harvested culture media were subjected to measurement of TG. Data are indicated as mean \pm SD of three dishes. ***Significantly different from the differentiated control cells (Cont) ($P < 0.001$). ##, ###significantly different from the nobiletin (64 μM)-treated cells ($P < 0.01$ and 0.001 , respectively).

(Sato *et al.*, 2001), and a nutritional element for *P. acnes* leading to the development of acne vulgaris (Bojar and Holland, 2004; Pawin *et al.*, 2004), these results strongly suggested that, like RAs, nobiletin might be an effective agent for acne therapy by virtue of directly inhibiting TG production both *in vivo* and *in vitro*. Moreover, the topical application of atRA, but not nobiletin, augmented epidermal thickness in the hamsters. Taken together with our previous studies (Minagawa *et al.*, 2001; Tanaka *et al.*, 2004) and a recent report by Rittie *et al.* (2006), we suggest the possibility that acne therapy with nobiletin may decrease or avoid adverse effect, at least scaling seen with RA treatment.

Many investigators have reported experimental and clinical evidence regarding the pathophysiology of acne and acne therapy (Bojar and Holland, 2004; Krautheim and Gollnick, 2004; Schaller *et al.*, 2005; Trivedi *et al.*, 2006), but there are few *in vivo* acne models for the estimation of the potential efficacy of novel anti-acne agents. Sebaceous glands in acne lesions have been characterized not only by excess lipogenesis but also by sebaceous enlargement owing to accumulating sebum (Pawin *et al.*, 2004). In addition, hyperkeratinization of the follicular wall has been reported to participate in microcomedo development at the primary

site (Bojar and Holland, 2004). We hypothesized that UVB-irradiation might mimic aberrant sebum production and sebaceous enlargement seen in acne pathology *in vivo* (Lesnik *et al.*, 1992; Suh *et al.*, 2002; Akitomo *et al.*, 2003). Indeed, the augmentation of skin surface lipid levels and sebum accumulation in sebaceous glands and ducts, and sebaceous enlargement were all observed in our *in vivo* model of UVB-irradiated hamsters. In addition, the topical application of nobiletin prevented these aberrant changes in sebaceous glands in the UVB-irradiated hamsters. Furthermore, we determined that the suppression of TG production by nobiletin was associated with a decrease in the UVB-augmented DGAT activity in the differentiated hamster sebocytes. We also demonstrated that nobiletin suppressed sebocyte proliferation, suggesting that nobiletin may interfere with UVB-induced sebaceous gland enlargement. Taken together with our previous reports that nobiletin exerts anti-inflammatory actions (Lin *et al.*, 2003; Tanaka *et al.*, 2004), we propose that nobiletin is a useful candidate as a novel anti-acne agent with antilipogenic, antiproliferative, anticomedogenic, and anti-inflammatory actions against sebaceous glands, pilosebaceous units, and infiltrated inflammatory cells in acne lesions.

As sebaceous glands in acne lesions accumulate abundant sebum along with the augmentation of lipogenesis (Pawin *et al.*, 2004), it seems to be important to stimulate promptly the excretion of accumulated sebum in follicular ducts or on the skin surface in concomitant with the inhibition of sebaceous lipogenesis. In this study, we found early evidence that nobiletin positively excreted TG to culture medium in the differentiated hamster sebocytes within 24 hours, whereas there was no TG excretion in either 13-*cis*RA- or atRA-treated cells. In addition, the nobiletin-mediated TG excretion was initiated by a transient increase in intracellular cAMP level and, thereafter, PKA activation. Similar TG excretion was reproduced in the forskolin-treated differentiated sebocytes. However, neither 13-*cis*RA nor atRA augmented the intracellular cAMP level and PKA activity in the hamster sebocytes. To date, some reports have shown an increase in intracellular cAMP level by plant flavonoids such as quercetin, fisetin, and genistein in adipocytes from rats (Kuppusamy and Das, 1994; Szkudelska *et al.*, 2000) and mice (Harmon and Harp, 2001). Recently, Nagase *et al.* (2005) reported that nobiletin in combination with forskolin augments PKA activity by increasing cAMP level in PC12D cells, a rat pheochromocytoma cell line. Taken together, it is suggested that the transient increase in cAMP level and the sequential PKA activation are involved in the nobiletin-mediated sebum excretion.

Sebum secretion is generally considered to be regulated by a holocrine mechanism, which may contain sebaceous apoptosis (Wróbel *et al.*, 2003). However, there is little evidence dealing with the regulation of sebum holocrine secretion, and especially the question of whether or not sebocyte apoptosis may be requisite for the holocrine mechanism. In this study, flow cytometric analysis using Annexin V-FITC revealed that nobiletin facilitated cell surface exposure of PS in the differentiated hamster sebocytes.

However, neither 13-*cis*RA nor atRA induced the PS externalization, in keeping with the previous report by Wróbel *et al.* (2003). Although Annexin V is an indicator for cell apoptosis induction (Koopman *et al.*, 1994; Wróbel *et al.*, 2003), there was no DNA ladder detected in the nobiletin-treated sebocytes, even for as long as 72 hours. In addition, we confirmed that the PS exposure was reversible by changing the fresh culture medium for another 24 hours after pretreating the cells with nobiletin for 24 hours in the differentiated sebocytes (data not shown). Furthermore, we also confirmed that there were no propidium iodide-positive signals in the nobiletin-treated sebocytes by immunocytochemical staining and flow cytometric analyses, whereas the staurosporine (5 μM)-treated cells were stained with propidium iodide (data not shown). Thus, nobiletin-induced sebum excretion may be associated with the PS translocation to the plasma membrane outer leaflet in sebocytes, which may differ from the holocrine mechanism and cell death related to sebaceous apoptosis.

Extracellular exposure of PS has been reported to be involved in physiological events such as myotube formation (van den Eijnde *et al.*, 2001) and sperm capacitation (Gadella and Harrison, 2002), which are independent of apoptosis. A recent report by Elliott *et al.* (2005) showed that apoptosis-independent PS externalization modulates several functions, including Na^+ and Ca^{2+} uptake through P2X₇ cation channel, and reversal of the activity of P-glycoprotein, the multidrug transporter, in T lymphocytes. Furthermore, Smith *et al.* (2002) reported that the cAMP analog, 8-Br-cAMP, induces PS externalization and lipid efflux via ABCA1 in RAW264.7. Regarding mechanisms of PS externalization in sebocytes, we suggest that the activation of cAMP-dependent PKA is at least a principal signal pathway for PS exposure leading to sebum excretion. Further experiments are needed to clarify how cAMP/PKA activation causes the PS externalization and whether nobiletin could modulate functions of aminophospholipid transporters such as floppases and scramblase (Zhou *et al.*, 1997; Bevers *et al.*, 1999).

In conclusion, we demonstrated that nobiletin inhibited sebaceous lipogenesis and enlargement and hyperkeratinization in the epidermis and follicular wall of hamsters, both *in vivo* and *in vitro*. Furthermore, we have supplied the early evidence that nobiletin facilitates the excretion of sebum accumulated in differentiated sebocytes via a molecular mechanism that includes non-apoptotic PS externalization through a cAMP/PKA-dependent pathway. Taken together with our previous studies of the anti-inflammatory actions of nobiletin (Lin *et al.*, 2003; Tanaka *et al.*, 2004), this early evidence shows that nobiletin is likely to be a unique anti-acne agent with not only antilipogenic and antiproliferative actions for sebocytes, but also the acceleration of sebum excretion to deplete lipids in sebaceous glands.

MATERIALS AND METHODS

Histochemical analysis

Auricles of 5-week-old male golden hamsters were topically treated once a day for 14 days with a 50- μl solution of 1 and 2% nobiletin (1.25 and 2.5 μmol) or 0.2% atRA (0.3 μmol) (Sigma Chemical,

St Louis, MO) in 95% ethanol and 5% glycerol, or with the same volume of vehicle alone according to our previous report (Tanaka *et al.*, 2004) with some modifications. After the treatments, tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and their sections were subjected to Mayer's hematoxylin-eosin staining (Wako Pure Chemicals, Osaka, Japan). After terminating the staining, tissue secretions were washed with ethanol and xylene and then viewed with a light microscope furnished with a digital camera (Olympus Optical, Tokyo, Japan). Epidermal thickness in 10 randomly chosen areas per section was measured under the microscope at 100-fold magnification. The animals had free access to food and water according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Sciences.

Oil red O staining

Skin from hamster auricles was snap-frozen in liquid nitrogen. The frozen tissue sections (8 μm thickness) were incubated in 60% isopropanol after washing with distilled H₂O. Tissue sections were stained with 0.3% oil red O (Sigma) in a solution of isopropanol and distilled H₂O (3:2, vol:vol) at 37°C for 15 minutes, and were then viewed with a light microscope furnished with a digital camera. Sections were also counterstained with Mayer's hematoxylin solution (Wako).

Cell culture and treatments

Hamster sebocytes were established from sebaceous glands of auricles of 5-week-old male golden hamsters as described previously (Sato *et al.*, 2001). Sebocytes (2.35×10^4 cells/cm²) in DMEM/Ham's F12 medium (1:1) (DMEM/F12) (Invitrogen, Carlsbad, CA) supplemented with 6% heat-denatured fetal bovine serum (JRH Bioscience, Tokyo, Japan), 2% human serum (ICN Biochemicals, Costa Mesa, CA), 0.68 mM L-glutamine (Invitrogen), and recombinant human epidermal growth factor (10 nM) (Progen Biotechnik GmbH, Heidelberg, Germany) were plated onto 35 or 100-mm diameter culture dishes (Becton Dickinson, Tokyo, Japan) for 24 hours to achieve complete cell adhesion. For the portion of the study dealing with sebum synthesis, the cells were treated every 3 days with nobiletin (16–64 μM), 13-*cis*RA (0.01–1 μM) (Sigma), or atRA (0.01–1 μM) in the presence or absence of insulin (10 nM) (Sigma), in DMEM/F12 supplemented with heat-denatured fetal bovine serum, human serum, and L-glutamine for up to 12 days according to previous studies (Akimoto *et al.*, 2005 and Iwata *et al.*, 2005). For the measurement of sebum excretion, the cells were pretreated with insulin (10 nM) in DMEM/F12 supplemented with heat-denatured fetal bovine serum, human serum, and L-glutamine to accumulate intracellular lipid droplets (termed, differentiated sebocytes), and then treated with nobiletin, 13-*cis*RA, forskolin (Sigma), and/or a PKA inhibitor, H-89 (BIOMOL, Plymouth Meeting, PA), in the medium without serum for up to 72 hours. In this series of experiments, hamster sebocytes were used at up to the third passage level.

UVB irradiation

UV has been reported to cause abnormal sebum production and sebaceous enlargement (Lesnik *et al.*, 1992; Suh *et al.*, 2002; Akitomo *et al.*, 2003). Therefore, to further evaluate the anti-acne

activity of nobiletin *in vivo*, we established UVB-irradiated hamster sebocyte models *in vivo* and *in vitro*. Briefly, the skin of 3-week-old male golden hamsters was treated, as described above, once a day with nobiletin or vehicle after each UVB irradiation (5.4 kJ/m²) for 7 days (Tanaka *et al.*, 2004). The insulin-differentiated hamster sebocytes were irradiated with UVB (0.6 kJ/m²) (Tanaka *et al.*, 2004), and then cultured for 24 hours. The UVB irradiation was created using a Toshiba FL20S fluorescent sunlamp (Toshiba, Tokyo, Japan), emitting rays between 275 and 375 nm with a peak emission of 313 nm. The radiance was measured by a UV Indicator MI-340 (Eko, Tokyo, Japan) as described previously (Tanaka *et al.*, 2004).

TG measurement

TG levels in sebum from the skin surface of hamster auricles were analyzed by automatic thin-layer chromatography, Iatroscan (Iatron Laboratories, Tokyo, Japan), as described previously (Sato *et al.*, 2001). Briefly, the auricles were wiped with acetone-impregnated cotton, and then the sebum on the skin surface was extracted twice with 50 μ l of acetone for 30 seconds using stainless cups, 1 hour after the wiping. The sebum extracts were subjected to Iatroscan and the amount of TG was calculated using an internal control concomitantly performed using authentic cholesterol acetate (2 μ g) (Doosan Serdary Research Laboratories, Englewood Cliffs, NJ). Otherwise, sonicated cell lysates and the harvested culture media were used to measure the intracellular and extracellular levels of TG using Liquitech TG-II (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. The amount of TG was calculated using an authentic trioleinate-standard solution (0.6 mg/ml). The intracellular DNA content was measured using authentic salmon sperm DNA (6.25–100 μ g/ml) and 3,5-diaminobenzoic acid dihydrochloride (Sigma) as described previously (Sato *et al.*, 2001).

Measurement of DGAT activity

DGAT activity in hamster sebocytes treated with nobiletin, 13-*cis*RA, atRA, or UVB was measured using 1,2-dioleoyl glycerol (Cayman Chemical, Ann Arbor, MI), and [¹⁴C]palmitoyl-CoA (Amersham Biosciences, Tokyo, Japan) as described previously (Iwata *et al.*, 2005).

Cell proliferation assay

When hamster sebocytes in 24-well multiplates were treated every 3 days with nobiletin, 13-*cis*RA, or atRA for a total of 12 days, [³H]thymidine (1 kBq/well) (Amersham Biosciences) was added to the cells for the last 3 hours of the treatments. The radioactivity of [³H]thymidine incorporated into the DNA was measured by a liquid scintillation counter (Aloka, Tokyo, Japan) after lysing the cells, and recorded as the cell proliferative activity.

Measurement of intracellular cAMP levels

Hamster sebocytes were treated with nobiletin or 13-*cis*RA for up to 60 minutes. The harvested cells were subjected to the measurement of intracellular cAMP levels via a cAMP enzyme immunoassay kit (Cayman Chemical) according to the manufacturer's instructions.

Measurement of PKA activity

Cells were treated with nobiletin, 13-*cis*RA, or forskolin for 30 minutes, and then homogenized in Ca²⁺- and Mg²⁺-free phosphate-buffered saline supplemented with 1% Nonidet P-40,

0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride, 5 μ M pepstatin, 10 μ M leupeptin, and 1 mM sodium orthovanadate. After centrifugation at 15,000 \times g for 20 minutes at 4°C, the resultant supernatant was collected as the cytosol fraction and used for the analysis of kinase activity. Briefly, aliquots (20 μ g) of the cytosol protein were analyzed by Western blotting using a specific rabbit antibody against phospho-(Ser/Thr) PKA substrate (Cell Signaling Technology, Danvers, MA) under nonreducing conditions. Immunoreactive phosphorylated PKA substrates were detected with enhanced chemiluminescence Western-blotting detection reagents (Amersham Bioscience) after being complexed with horseradish peroxidase-conjugated antirabbit IgG (Sigma). Relative amounts of the immunoreactive proteins were quantified by densitometric scanning using an Image Analyzer LAS-1000 plus (Fuji Film, Tokyo, Japan).

Flow cytometric analysis

Insulin-differentiated hamster sebocytes were treated with nobiletin, 13-*cis*RA, or forskolin for 24 hours. In addition, the cells were pretreated with a PKA inhibitor, H-89, for 1 hour, and then treated with nobiletin for another 24 hours. After the treatments, the cells were stained with Annexin V-FITC (10 μ g/ml) (Medical Biological Laboratories, Nagoya, Japan) for 5 minutes. The cells were harvested with 0.25% trypsin and 0.02% EDTA in Ca²⁺- and Mg²⁺-free phosphate-buffered saline and then resuspended in the culture medium. The number of Annexin V-positive cells was measured via a FACScan flow cytometer (Becton Dickinson, Tokyo, Japan).

DNA fragmentation assay

Sebocytes treated with nobiletin or staurosporine (1 μ M) (Sigma) were lysed in Tris-EDTA (TE) buffer (50 mM Tris-HCl (pH 7.5) and 10 mM EDTA) containing 0.5% sodium *N*-lauroylsarcosinate, and then incubated with RNase (500 μ g/ml) for 30 minutes at 50°C. After RNase treatment, the cell lysates were incubated for 24 hours at –20°C with proteinase K (500 μ g/ml) for 30 minutes at 50°C, and after which the DNA in the lysates was precipitated with 50% isopropanol solution containing 0.5 M NaCl. Precipitated DNA was resuspended in TE buffer and then electrophoresed on 1% agarose gels. Ethidium bromide-stained gels were photographed using a UV transilluminator as the sole light source.

Statistical analysis

Data are presented as mean \pm SD, and were analyzed by a one-way analysis of variance and by the Fisher test for multiple comparisons. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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