Augmentation of Lipogenesis by 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 in Hamster Sebaceous Glands: Identification of Cytochrome P-450-mediated 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 Production

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Prostaglandins (PGs) play important roles in the regulation of cutaneous cell functions under physiological and pathological conditions. In this study, we examined the involvement of PGs in sebocyte lipogenesis using non-steroidal anti-inflammatory drugs *in vivo* and *in vitro*. Hamster auricle sebocytes spontaneously differentiated to accumulate intracellular triacylglycerol (TG), under which the relative levels of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) to $PGF_{2\alpha}$ and PGE_2 increased. 15d-PGJ_2 was found to augment the formation of lipid droplets, which was because of an increase of TG synthesis by diacylglycerol acyltransferase (DGAT). Furthermore, sebocytes constitutively produced cyclooxygenase 2 (COX-2), but not COX-1, *in vivo* and *in vitro*. When sebocytes were treated with COX inhibitors such as indomethacin, diclofenac, or NS-398, the production of $PGF_{2\alpha}$ and PGE_2 decreased. The production of 15d-PGJ_2, however, was increased in these inhibitor-treated sebocytes. In addition, indomethacin, diclofenac, and NS-398 augmented the synthesis of TG along with the increase in DGAT activity. Similarly, topical administration of indomethacin to hamster auricles caused the development of sebaceous glands with the augmentation of sebum deposition *in vivo*. Furthermore, indomethacin and NS-398-augmented 15d-PGJ_2 production and TG synthesis were suppressed by a non-selective cytochrome P-450 (CYP) inhibitor, SKF-525A. A ligand activator of peroxisome proliferation activating receptor γ (PPARγ), troglitazone-induced synthesis of TG, however, was not altered even in the presence of SKF-525A. These results suggest that 15d-PGJ_2 is a crucial stimulator of sebocyte lipogenesis by augmenting DGAT-mediated synthesis of TG. In addition to the COX-2-dependent pathway of PG synthesis, our findings suggest a sebocyte-specific pathway of 15d-PGJ_2 production by CYP, the activity of which may be evoked by inhibiting COX-2.

Key words: cyclooxygenase/cytochrome P-450/15-deoxy-$\Delta^{12,14}$-PGJ_2/lipogenesis/sebaceous glands
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By secreting sebum, sebaceous glands participate in the formation of a thin lipid layer on the skin surface as a physiological barrier (Thody and Shuster, 1989; Fluhr *et al*, 2003). Sebaceous gland cells (sebocytes) differentiate to accumulate abundant cytoplasmic lipids, and, using a holocrine mechanism, lead to the secretion of lipids as sebum (Sawaya *et al*, 1988; Akamatsu *et al*, 1992; Zouboulis *et al*, 1994; Rosenfield *et al*, 1998). An excess secretion of sebum causes sebaceous-gland disorders such as acne vulgaris and seborrhea, which are the most common skin diseases (Harris *et al*, 1983; Piérard *et al*, 1987; Zouboulis *et al*, 1998).

Recently, *in vitro* cultures of sebocytes have been established from sebaceous glands of humans (Xia *et al*, 1989; Fujie *et al*, 1996; Zouboulis *et al*, 1999; Thiboutot *et al*, 2003) and rats (Laurent *et al*, 1992). Plewig and Luderschmidt (1977) reported that hamster sebaceous glands are similar to human glands with regard to size, response to androgens, and turnover time. Ito *et al* (1998) described a culture method for hamster sebocytes from the auricles, and demonstrated that the proliferation of hamster sebocytes in response to androgens is similar to that of human sebocytes. Therefore, like human sebocytes, the cultured hamster sebocytes are a useful tool for studying the functions of sebaceous glands *in vitro*.

These *in vitro* cell culture models have stimulated the elucidation of cellular properties of sebocytes, and studies on the pathological and physiological regulation of sebum synthesis by hormones (Xia *et al*, 1989; Rosenfield *et al*, 1998; Zouboulis *et al*, 1999; Thiboutot *et al*, 2003). Sebum secretion and synthesis have been reported to be augmented by androgens such as testosterone and 5α-dihydrotestosterone (Akamatsu *et al*, 1992; Zouboulis *et al*, 1994; Rosenfield *et al*, 1998; Sato *et al*, 2001a), and by insulin (Deplewski and Rosenfield, 1999) in humans, rats, and hamsters. In contrast, estrogen (Ebling and Skinner, 1983) and all-*trans* and 13-*cis* retinoic acids (Hommel *et al*, 1996; Orfanos and Zouboulis, 1998; Zouboulis *et al*, 1999; Sato *et al*, 2001a) have been reported to suppress the formation of intracellular lipid droplets *in vivo* and *in vitro*. We reported

Abbreviations: 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; COX, cyclooxygenase; CYP, cytochrome P450; DGAT, diacylglycerol acyltransferase; EGF, epidermal growth factor; PG, prostaglandin; PPAR, peroxisome proliferation-activating receptor; TG, triacylglycerol

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that epidermal growth factor (EGF), basic fibroblast growth factor, transforming growth factor-α, keratinocyte growth factor, and 1α,25-dihydroxyvitamin D_3 suppress lipogenesis in hamster sebocytes (Sato *et al*, 2001a; Akimoto *et al*, 2002).

Prostaglandins (PGs) play important roles in the regulation of cellular functions under physiological and pathological conditions. Cyclooxygenase (COX)/prostaglandin endoperoxide H synthase, two types of which, COX-1 and COX-2, have been characterized in mammalians, is a rate-limiting enzyme complex for the production of PGs (Hruza and Pentland, 1993; Narumiya *et al*, 1999; Tiano *et al*, 2002). It has been reported that PGE_2 participates in epidermal repair such as wound healing by its regulation of proliferation and differentiation of keratinocytes (Pentland and Needleman, 1986; Unemori *et al*, 1994; Sato *et al*, 1997). Sebocytes are classified as epithelial cells, and morphologically and functionally differentiate to form intracellular lipid droplets (Rosenfield *et al*, 1998; Deplewski and Rosenfield, 1999). Therefore, it seems that, like keratinocytes, PGs may participate in the regulation of biological functions of sebaceous glands. It remains unclear, however, whether PGs are associated with the development of sebaceous glands and sebocyte lipogenesis.

In this study, we investigated the possible involvement of PGs in the formation of intracellular lipid droplets in hamster sebaceous glands *in vivo* and *in vitro*. Our findings show that 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) plays a crucial role in the augmentation of lipid-droplet formation by increasing triacylglycerol (TG) synthesis in hamster sebocytes, and that the synthesis of 15d-PGJ_2 is mediated by COX-2 and cytochrome P-450 (CYP)-dependent pathways.

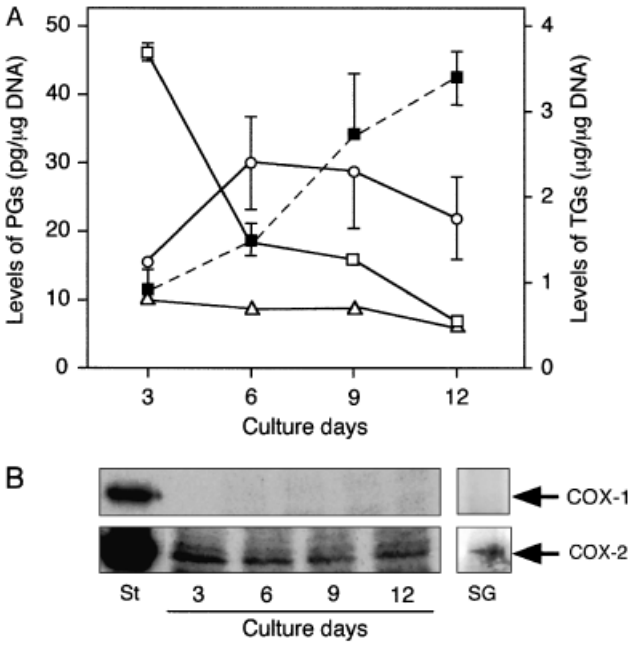

Figure 1
Levels of prostaglandins (PG) during sebocyte differentiation *in vitro*. Hamster sebocytes at the 2nd passage were cultured for 12 d, with replacement of fresh culture medium every 3 d. (*A*) The harvested culture medium at each indicated period was subjected to measurement of PGE_2 (*open triangles*), $PGF_{2\alpha}$ (*open squares*), and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (*open circles*). Levels of intracellular triacylglycerol (TG) (*closed squares*) were time dependently augmented during cell culture. Data are shown as mean ± standard deviation of triplicate dishes. (*B*) The harvested cells at each indicated period and sebaceous glands (SG) were subjected to western blot analysis for cyclooxygenase (COX)-1 and COX-2. St indicates human COX-1 (*upper panel*) and COX-2 (*lower panel*). Three independent experiments were reproducible, and typical data are shown.

Results

15d-PGJ_2 stimulates the differentiation of sebocytes with increasing intracellular levels of TG We first examined the relationship between PG levels and lipogenesis during the differentiation of hamster sebocytes. As intracellular lipid droplets accumulate in sebocytes mostly consist of TG (Sato *et al*, 2001a), we evaluated sebocytic differentiation by measuring intracellular levels of TG. As shown in Fig 1*A*, levels of TG (*closed squares*) were found to increase in cell culture time dependently . The level of $PGF_{2\alpha}$ (*open squares*) was somewhat higher than that of PGE_2 (*open triangles*) and 15d-PGJ_2 (*open circles*) on day 3 in culture, but was found to decrease time dependently for up to 12 d (to $<$10 pg per μg DNA). The level of PGE_2 was considerably lower ($<$10 pg per μg DNA). 15d-PGJ_2 levels were augmented for 6 d and this augmented level (20–30 pg per μg DNA) was sustained for up to 12 d, indicating that 15d-PGJ_2 is predominant in differentiated hamster sebocytes. It was found that hamster sebocytes constitutively produce COX-2 with the same molecular weight as the human COX-2 protein and its production slightly decreased for up to 12 d (Fig 1*B*, *lower panel*). Furthermore, COX-2 was found to be expressed in sebaceous glands (see SG, Fig 1*B*, *lower panel*). There was no detectable COX-1 in either sebaceous glands or cultured sebocytes (see Fig 1*B*, *upper panel*).

To clarify whether 15d-PGJ_2 might induce the differentiation of sebocytes, we investigated the effect of exogenous 15d-PGJ_2 on the formation of lipid droplets in hamster sebocytes. When 15d-PGJ_2 (1–10 μM) was added in the sebocyte culture, the formation of intracellular lipid droplets (Fig 2*B vs A*) and TG levels (Fig 2*C*) increased in a concentration-dependent manner. As shown in Table I, 15d-PGJ_2-induced augmentation of TG production was found to be associated with an increase in the activity of diacylglycerol acyltransferase (DGAT), a rate-limiting enzyme of TG synthesis ($p < 0.001$).

Effects of COX inhibitors on the production of PGs in hamster sebocytes To clarify the possible involvement of 15d-PGJ_2 in increasing sebocyte lipogenesis, we investigated the effects of COX inhibitors on the production of TG and the formation of intracellular lipid droplets in cultured hamster sebocytes. As shown in Fig 3, levels of PGE_2 (*panel A*) and $PGF_{2\alpha}$ (*panel B*) were significantly diminished ($p < 0.001$) by non-selective COX inhibitors, indomethacin (10 μM) and diclofenac (10 μM), and by a COX-2-specific inhibitor, NS-398 (20 μM). The level of 15d-PGJ_2 in the medium was found to increase in sebocytes treated with indomethacin ($p < 0.01$), diclofenac ($p < 0.01$), and NS-398 ($p < 0.001$) (Fig 3*C*). In contrast, there was no change in the level of PGD_2 between untreated and indomethacin (10

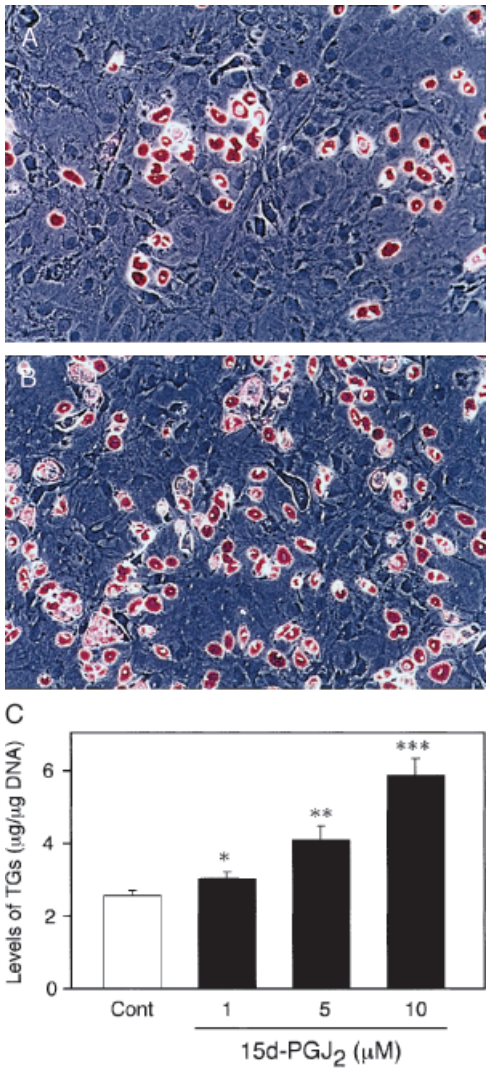

Figure 2
Exogenous 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) augments the formation of lipid droplets by increasing intracellular levels of triacylglycerol (TG) in hamster sebocytes. Oil-red-O staining of cultured hamster sebocytes at the 2nd passage shows that the accumulation of lipid droplets is spontaneously observed in untreated cells (*A*) and augmented by addition of 15d-PGJ$_2$ (10 μM) for 12 d (*B*). Intracellular levels of TG (*C*) also increase in a concentration-dependent manner using 15d-PGJ$_2$ (1–10 μM). Four independent experiments were reproducible, and typical data are shown. Data are shown as mean ± standard deviation of triplicate dishes. *, **, and *** indicate results that were significantly different from untreated control cells (Cont) at p levels of <0.05, <0.01, and <0.001, respectively. Magnifications in panels *A* and *B*: × 200.

μM)-treated sebocytes (0.49 ± 0.06 *vs* 0.54 ± 0.09 pg per μg DNA, respectively). Neither COX-1 nor COX-2 production was altered in the COX inhibitor-treated cells (see Fig S1). Moreover, in primarily cultured hamster skin fibroblasts, these three inhibitors were found to inhibit the production of PGE$_2$, PGF$_{2\alpha}$, and 15d-PGJ$_2$ (data not shown).

Augmentation of lipogenesis and development of sebaceous glands by COX inhibitors Because of our finding that 15d-PGJ$_2$ production was increased in sebocytes even when COX inhibitors were present, we examined whether the inhibitors could augment lipogenesis in sebaceous glands. As shown in Fig 4, the production of TG was found to increase concentration dependently in the presence of indomethacin (1–10 μM), diclofenac (0.1–10 μM), and NS-398 (5–20 μM). The formation of intracellular lipid droplets also increased (data not shown). There were no significant changes in the levels of free fatty acids and cholesterol compared with those of TG in the lipids (data not shown). The selective augmentation of TG production by indomethacin, diclofenac, and NS-398 was found to be associated with an increase in DGAT activity in hamster sebocytes (Table I). When auricles from 5-wk-old male golden hamsters were topically treated with 2% (wt/vol)

Table I. Increase of DGAT activity by 15d-PGJ$_2$ and COX inhibitors in hamster sebocytes

Treatments	μM	DGAT activity (pmol per min per μg DNA)
None	—	564.4 ± 14.6
15d-PGJ$_2$	10	961.7 ± 18.1[a]
Indomethacin	10	1142.0 ± 36.9[a]
Diclofenac	10	1120.3 ± 39.5[a]
NS-398	20	869.7 ± 63.2[a]

[a]$p<0.001$, between untreated and COX inhibitors or 15d-PGJ$_2$-treated cells.
DGAT, diacylglycerol acyltransferase; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$; COX, cyclooxygenase.

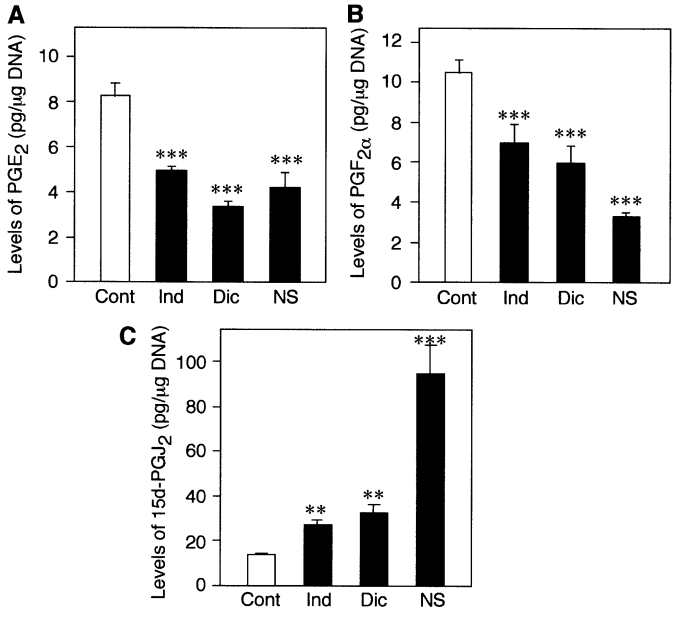

Figure 3
Regulation of prostaglandin (PG) production by cyclooxygenase (COX) inhibitors in hamster sebocytes. Hamster sebocytes at the 2nd passage were treated every 3 d with indomethacin (Ind) (10 μM), diclofenac (Dic) (10 μM), and NS-398 (NS) (20 μM) for 12 d. Culture media were harvested at 12 d and subjected to measurement of PGE$_2$ (*A*), PGF$_{2\alpha}$ (*B*), and 15-deoxy-$\Delta^{12,14}$-PGJ$_2$ (15d-PGJ$_2$) (*C*). Four independent experiments were reproducible, and typical data are shown. Data are shown as mean ± standard deviation of triplicate dishes. ** and *** indicate significant differences from untreated control cells (Cont) with p values of <0.01 and <0.001, respectively.

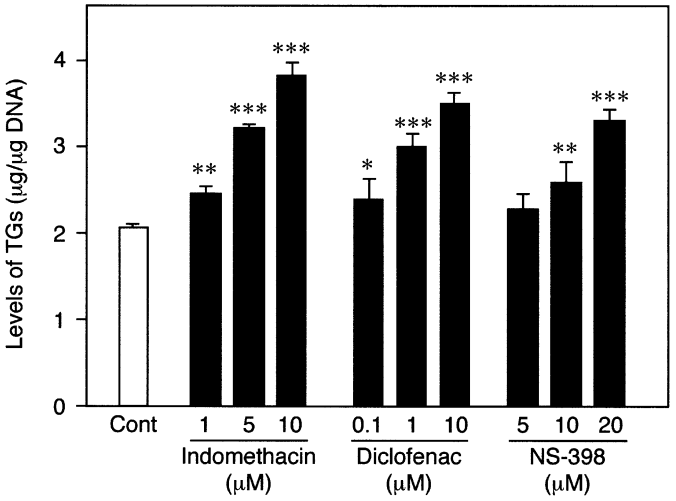

Figure 4
Effects of cyclooxygenase (COX) inhibitors on levels of triacylglycerol (TG) in hamster sebocytes. Intracellular levels of TG were concentration-dependently augmented in indomethacin (1–10 μM)-, diclofenac (0.1–10 μM)-, and NS-398 (5–20 μM)-treated hamster sebocytes at the 2nd passage. Four independent experiments were reproducible and typical data are shown. Data are shown as mean ± standard deviation of triplicate dishes. *, **, and *** indicate significant differences from untreated control cells (Cont) with p values of <0.05, <0.01, and <0.001, respectively.

indomethacin for 14 d, the development of sebaceous glands (*arrow heads*) with the deposition of sebum (*asterisks*) was observed (Fig 5*B vs A*).

Involvement of a novel CYP-mediated pathway of 15d-PGJ_2 synthesis in sebocyte lipogenesis Based on our finding that indomethacin, diclofenac, and NS-398 cause the selective augmentation of 15d-PGJ_2 production in hamster sebocytes, a COX-independent pathway may be involved in the increase of 15d-PGJ_2 production. As it has been reported that arachidonic acid is metabolized not only by COX but also by CYP (Laniado-Schwartzman *et al*, 1988; Capdevila *et al*, 2000), we investigated the effect of a CYP inhibitor on the production of 15d-PGJ_2 and on the synthesis of intracellular TG in COX inhibitor-treated sebocytes. As shown in Fig 6*A*, a non-selective CYP inhibitor, SKF-525A (10 and 20 μM), suppressed indomethacin and NS-398-induced production of 15d-PGJ_2. These COX inhibitor-augmented levels of intracellular TG were diminished by SKF-525A (Fig 6*B*). SKF-525A did not influence the basal levels of 15d-PGJ_2 and TG in hamster sebocytes (Fig 6).

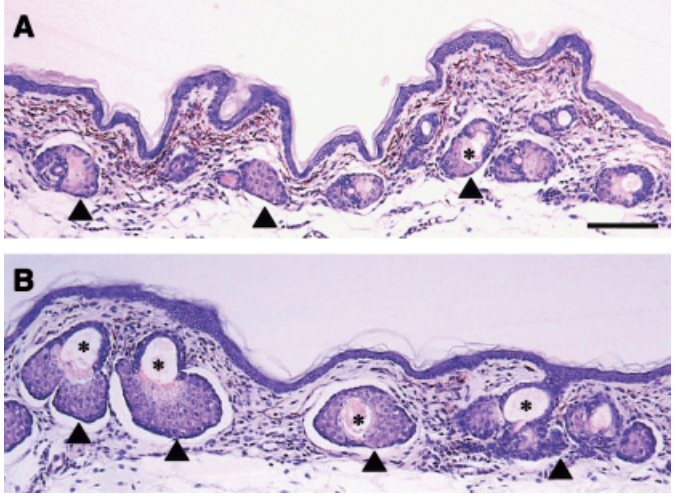

Figure 5
Development of sebaceous glands by topically administering indomethacin *in vivo*. Histochemical staining of hamster auricles shows that topical treatment of indomethacin for 2 wk (*B*) leads to the development of sebaceous glands (*arrow heads*), and augments the deposition of sebum (*asterisks*) compared with vehicle-treated auricles (*A*). Three independent experiments were reproducible, and typical data are shown. *Scale bar*, 300 μm.

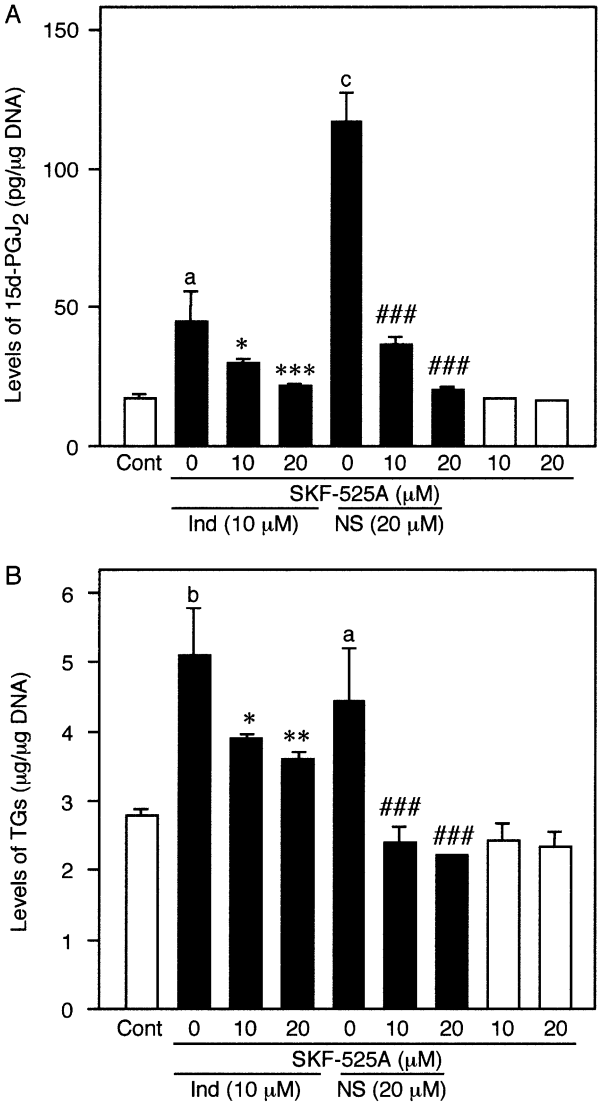

Figure 6
A cytochrome P-450 (CYP) inhibitor suppresses indomethacin- and NS-398-augmented production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) and triacylglycerol (TG) in hamster sebocytes. Hamster sebocytes at the 2nd passage were treated with indomethacin (Ind) (10 μM) and NS-398 (NS) (20 μM) in the presence of SKF-525A (10 and 20 μM) for 12 d. Culture media and cells harvested at 12 d were subjected to measurement of 15d-PGJ_2 (*A*) and intracellular TG (*B*). Three independent experiments were reproducible, and typical data are shown. Data are shown as mean ± standard deviation of triplicate dishes. a, b, and c indicate significant differences from untreated control cells (Cont) with p values of <0.05, <0.01, and <0.001, respectively. *, **, and *** indicate significant differences from indomethacin (Indo) alone with p values of <0.05, <0.01, and <0.001, respectively. ### indicates significant differences from NS-398 (NS) alone with p values of <0.001.

Table II. No effect of CYP inhibitor on troglitazone-augmented TG synthesis in hamster sebocytes

Treatments	μM	Levels of TG (μg per μg DNA)
None	—	0.74 ± 0.24
Troglitazone	10	2.37 ± 0.17[a]
Troglitazone + SKF-525A	5	2.13 ± 0.22
	10	2.51 ± 0.30
	20	2.13 ± 0.14

[a]$p < 0.001$, between untreated and troglitazone-treated cells.
CYP, cytochrome P-450; TG, triacylglycerol.

Similar results were obtained when another non-selective CYP inhibitor, methoxsalen, was used (data not shown). As it has been reported that 15d-PGJ$_2$ as well as PGJ$_2$ is an endogenous activator of peroxisome proliferation-activating receptor γ (PPARγ) (Kliewer *et al*, 1995; Yu *et al*, 1995; Shibata et al, 2002; Scher and Pillinger, 2005), and that the activation of PPARγ1 in sebocytes causes an increase in lipogenesis (Rosenfield *et al*, 1999), we examined the effect of SKF-525A on the synthesis of TG in hamster sebocytes treated with a ligand activator of PPARγ, troglitazone. As shown in Table II, troglitazone-augmented TG synthesis was not influenced by SKF-525A (5–20 μM).

Discussion

The proliferation and differentiation of keratinocytes have been reported to be regulated by PGE$_2$, which may be associated with the development of the epidermis and epidermal repair under wound healing (Pentland and Needleman, 1986; Unemori *et al*, 1994; Sato *et al*, 1997). Sebocytes are classified as epithelial cells, and cause terminal differentiation with the intracellular accumulation of lipids (Rosenfield *et al*, 1998; Deplewski and Rosenfield, 1999), it seems that PG might be involved in the regulation of sebaceous development and functions. In this study, the basal level of 15d-PGJ$_2$ was found to increase, whereas that of PGF$_{2\alpha}$ decreased and that of PGE$_2$ did not change during spontaneous differentiation of hamster sebocytes *in vitro*. We demonstrated that the formation of intracellular lipid droplets was augmented by exogenously administering 15d-PGJ$_2$, and was associated with an increase in DGAT-mediated TG biosynthesis in hamster sebocytes. Neufang *et al* (2001) reported that the development of sebaceous glands with sebum deposition is augmented in transgenic mice overexpressing COX-2 in the epidermis, in which the levels of PGF$_{2\alpha}$ and PGE$_2$ are elevated. But, they did not show data for 15d-PGJ$_2$ in their transgenic mice. As a COX-mediated arachidonic acid metabolite, PGH$_2$, is a common precursor for 15d-PGJ$_2$, PGF$_{2\alpha}$, and PGE$_2$ (Narumiya *et al*, 1999; Tiano *et al*, 2002; Helliwell *et al*, 2004; Scher and Pillinger, 2005), it is assumed that levels of not only PGF$_{2\alpha}$ and PGE$_2$ but also 15d-PGJ$_2$ are augmented in the epidermis in the transgenic mice. Taken together, these findings suggest that the increase in levels of 15d-PGJ$_2$ rather than PGF$_{2\alpha}$ and PGE$_2$ could cause augmentation of lipogenesis in sebaceous glands.

Non-steroidal anti-inflammatory drugs such as indomethacin and diclofenac have been useful in elucidating the involvement of PG in pathological and physiological reactions *in vivo* and *in vitro* by their inhibition of COX activity (Kobayashi *et al*, 1985; Smith *et al*, 1998). In this study, we demonstrated that hamster sebocytes express COX-2 constitutively, but not COX-1, and that indomethacin, diclofenac, and NS-398 inhibit the production of PGE$_2$ and PGF$_{2\alpha}$ in sebocytes, which is dependent on a COX-2 pathway. Unexpectedly, however, there was no change in the level of PGD$_2$ in indomethacin-treated sebocytes. Furthermore, the treatment of hamster sebocytes with indomethacin, diclofenac, and NS-398 caused an increase in 15d-PGJ$_2$ production, suggesting that the production of PGD$_2$ and 15d-PGJ$_2$ in hamster sebocytes is regulated by the COX-independent pathway. In accord with the augmentation of 15d-PGJ$_2$ production, the formation of intracellular lipid droplets and of DGAT-mediated biosynthesis of TG was found to be increased in these COX inhibitor-treated sebocytes. Topical administration of indomethacin to auricles of male golden hamsters resulted in the development of sebaceous glands and the enhancement of sebum accumulation *in vivo*. These findings strongly suggested that hamster sebocytes possess at least two mechanisms for the biosynthesis of PG: (i) a general COX-2-dependent pathway for prostanoid synthesis (Narumiya *et al*, 1999; Tiano *et al*, 2002) and (ii) a novel pathway for 15d-PGJ$_2$ synthesis, which may be stimulated by inhibiting COX-2 activity (Fig 7).

Regarding COX-independent PG production, it has been reported that CYP participates in arachidonic acid metabolism (Laniado-Schwartzman *et al*, 1988; Capdevila *et al*, 2000). Keeney *et al* (1998) reported that CYP2B12 is specifically expressed in rat sebaceous glands. Thiboutot *et al*

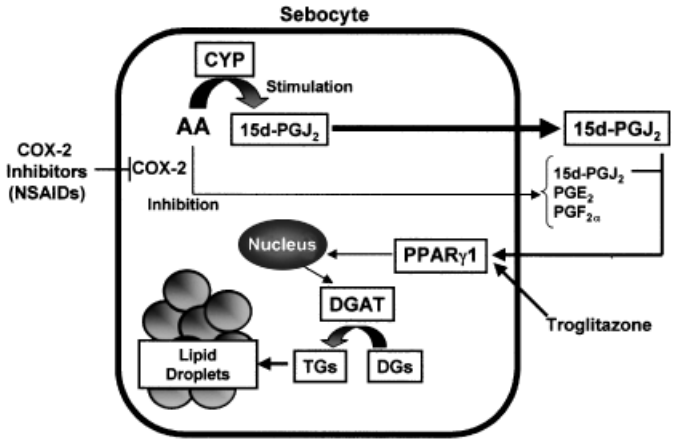

Figure 7
A possible mechanism for sebocyte lipogenesis through a cytochrome P-450-15-deoxy-$\Delta^{12,14}$-PGJ$_2$-peroxisome proliferation activating receptor γ1 (CYP-15d-PGJ$_2$-PPARγ1) pathway. Cyclooxygenase (COX)-2-dependent and -independent pathways of 15d-PGJ$_2$ production are present in hamster sebocytes. Although the basal production of PGs is mediated by a COX-2-dependent pathway, the inhibition of COX-2 activity by non-steroidal anti-inflammatory drugs (NSAIDs) specifically leads to an increase in 15d-PGJ$_2$ production though a CYP-mediated pathway. The basal and the CYP-mediated augmentation of 15d-PGJ$_2$ production result in an increase of diacylglycerol acyltransferase (DGAT)-mediated production of TG through PPARγ1 activation.

(2003) reported that CYP11A and CYP17 are expressed in sebaceous glands and ducts of human facial skin. In this study, our finding that SKF-525A suppressed indomethacin and NS-398-induced production of 15d-PGJ_2 and TG suggests that there is a novel CYP-mediated pathway for the synthesis of 15d-PGJ_2 in hamster sebaceous glands, which may be activated by inhibiting COX-2 activity (Fig 7). There was no change in the basal level of 15d-PGJ_2 in sebocytes treated with CYP inhibitor alone. Taken together with data for hamster sebocytes that constitutively produce COX-2 *in vivo* and *in vitro*, these results suggest the possibility that the basal level of 15d-PGJ_2 may be produced through a COX-2-dependent pathway (Fig 7). Further experiments will be needed to identify the subtype of CYP in hamster sebocytes, and to clarify the mechanism for the production of 15d-PGJ_2 through COX-2- and CYP-dependent pathways during the synthesis of sebum in sebaceous glands.

15d-PGJ_2 is an endogenous activator of PPARγ for lipogenesis in adipocytes (Kliewer *et al*, 1995; Yu *et al*, 1995; Shibata *et al*, 2002; Scher and Pillinger, 2005). Rosenfield *et al* (1999) reported the involvement of PPARγ1 in the augmentation of lipogenesis in rat sebocytes. We also demonstrated that hamster sebocytes constitutively express PPARα and γ1 *in vitro*, whose activation by ligand activators for PPARα and γ augments the formation of lipid droplets and the production of TG (Akimoto *et al*, 2005). Therefore, it is likely that the elevated levels of 15d-PGJ_2 cause the activation of PPARγ1, which in turn augments the synthesis of TG in hamster sebocytes. Moreover, we found that PPARγ1-mediated augmentation of DGAT-dependent production of TG is not influenced by SKF-525A. This suggests that the CYP-mediated augmentation of 15d-PGJ_2 production may occur upstream of the PPARγ1 pathway in sebocyte lipogenesis (Fig 7).

In conclusion, we suggest that 15d-PGJ_2 is a crucial stimulator of sebocyte lipogenesis by augmenting DGAT-mediated synthesis of TG or by decreasing the activity of TG lipase (Dolinsky *et al*, 2004). In addition to the COX-2 pathway, we suggest a CYP-mediated sebocyte-specific pathway for 15d-PGJ_2 synthesis, the activity of which may be evoked by selectively inhibiting COX-2 (Fig 7). Finally, these findings should provide an increased understanding of the functions of sebaceous glands in normal and diseased skin, and could contribute to the development of drugs and clinical strategies for skin diseases such as acne vulgaris.

Materials and Methods

Cell culture and treatments Hamster sebocytes were established from sebaceous glands of auricles of 5-wk-old male golden hamsters as described previously (Ito *et al*, 1998; Sato *et al*, 2001a). Sebocytes (2.35×10^4 cells per cm^2) in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1) (DMEM/F12) (Invitrogen, Carlsbad, California) supplemented with 6% (vol/vol) heat-denatured fetal bovine serum (Asahi Techno Glass, Tokyo, Japan), 2% (vol/vol) human serum (ICN Biochemicals, Costa Mesa, California), 0.68 mM L-glutamine (Invitrogen), and recombinant human EGF (10 ng per mL) (Progen Biotechnik GmbH, Heidelberg, Germany) were plated in 35, 60, or 100 mm diameter culture dishes (Becton Dickinson, Tokyo, Japan) for 24 h to achieve complete cell adhesion. Next, the cells were treated every 3 d with non-selective COX inhibitors, indomethacin and diclofenac (Sigma Chemical, St. Louis, Missouri); a COX-2-specific inhibitor; NS-398 (Calbiochem-Novabiochem, San Diego, California); and ligand activators of PPARγ, 15d-PGJ_2 (Cayman Chemical, Ann Arbor, Michigan) and troglitazone (kindly provided by Sankyo, Tokyo, Japan), in the presence or absence of non-selective CYP inhibitors, SKF-525A or methoxsalen (Sigma), in DMEM/F12 supplemented with heat-denatured fetal bovine serum, human serum, and L-glutamine for up to 14 d. In this series of experiments, hamster sebocytes were used at up to the 3rd passage level.

Oil-red-O staining Cultured hamster sebocytes were washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and fixed with 4% (wt/vol) paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) diluted with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline for 1 h at room temperature. The cells were washed with distilled H_2O and then stained with 0.3% (wt/vol) oil-red O (Sigma) in isopropanol:distilled H_2O (3:2, vol/vol) at 37°C for 15 min. The stained cells were washed with distilled H_2O, and then viewed with a light microscope equipped with a digital camera (Olympus Optical, Tokyo, Japan).

Histochemical staining Auricles of 5-wk-old male golden hamsters were topically treated everyday with 50 μL of 2% (wt/vol) indomethacin in 95% (vol/vol) ethanol and 5% (wt/vol) glycerol, or with the same volume of vehicle for 14 d. After the treatment, the tissues were fixed with 4% (wt/vol) paraformaldehyde, embedded in paraffin, and then their sections were subjected to Mayer's hematoxylin–eosin staining (Wako Pure Chemicals). After terminating the staining, tissue secretions were washed with ethanol and xylene, and then viewed with a light microscope equipped with a digital camera. 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 Science.

Quantitative analysis of intracellular lipids The lipid composition of extracts of intracellular lipids was analyzed by an automatic TLC, Iatroscan (Iatron Laboratories, Tokyo, Japan) using authentic tripalmitin, palmitic acid, cholesterol, cholesterol palmitate (Doosan Serdary Research Laboratories, Englewood Cliffs, New Jersey), and palmityl palmitate (wax ester) (Nu-Chek-Prep, Elysian, Minnesota) as described previously (Sato *et al*, 2001a). The amounts of lipid components were also calculated using an internal control concomitantly performed using authentic cholesterol acetate (2 μg) (Doosan Serdary Research Laboratories). Otherwise, the sonicated-cell lysates were used to measure the level of intracellular 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 (60 μg per mL). The intracellular DNA content was measured using authentic salmon sperm DNA (6.25–100 μg per mL) and 3,5-diaminobenzoic acid dihydrochloride (Sigma) as described previously (Sato *et al*, 2001a).

DGAT activity Hamster sebocytes were homogenized in 20 mM Tris-HCl (pH 7.6), 0.25 M sucrose, 2 mM $MgCl_2$, and 50 mM KCl, and then centrifuged at $12,000 \times g$ for 15 min at 4°C. The harvested supernatants were further centrifuged at $105,000 \times g$ for 60 min at 4°C. The precipitates were resuspended in 20 mM Tris-HCl (pH 7.6), 0.15 M KCl, and 1 mM EDTA, and then centrifuged under the same conditions. The resultant precipitates were resolved in distilled H_2O and then subjected to the measurement of DGAT activity (Coleman and Bell, 1976). Briefly, aliquots (3 μg) of the samples were incubated with 40 nM 1,2-dioleoyl glycerol (Cayman Chemical), and 5 nM [^{14}C]palmitoyl-CoA (Amersham Biosciences, Tokyo, Japan) in 175 mM Tris-HCl (pH 7.6), 8 mM $MgCl_2$, and 1% (wt/vol) fatty-acid-free bovine serum albumin for 10 min at 37°C. The reaction was terminated by adding 2-propanol:heptane:distilled H_2O (40:10:1; vol/vol/vol) solution. After adding heptane:dis-

tilled H_2O (2:1; vol/vol) solution, the radioactivity of synthesized ^{14}C-labeled TG in the extracted heptane layer was measured by a liquid-scintillation counter. We note that the main component of the heptane extraction on silica-gel 60 F_{254}-coated TLC (Merck, Darmstadt, Germany) by iodic visualization had the same relative flow as that of authentic TG (2 mg per mL) (data not shown). In addition, we confirmed that the radioactivity of synthesized ^{14}C-labeled TG decreased in the presence of 1 mM EDTA or 0.1% (wt/vol) Tween 20 (data not shown) as described previously (Coleman and Bell, 1976), suggesting that this assay is reliable for the measurement of DGAT activity *in vitro*.

Enzyme immunoassay for PGE_2, $PGF_{2\alpha}$, PGD_2, and 15d-PGJ_2 Amounts of PGE_2, $PGF_{2\alpha}$, PGD_2, and 15d-PGJ_2 in harvested culture media were measured using enzyme immunoassay kits for PGE_2, $PGF_{2\alpha}$, and PGD_2 (Cayman Chemical), and for 15d-PGJ_2 (Assay Designs, Ann Arbor, Michigan) according to the manufacturers' instructions.

Western blot analysis for COX Cells and sebaceous glands were homogenized in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (wt/vol) NP-40, 0.1% (wt/vol) SDS, 1 mM EDTA, 10 μg per mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and then subjected to sonication as described previously (Noguchi *et al*, 2003). After centrifugation at 8000 $\times$ *g* for 15 min at 4°C, proteins (80 μg) in the resulting supernatant were subjected to SDS-polyacrylamide gel electrophoresis using 10% (wt/vol) acrylamide gel, and then electrotransferred onto a nitrocellulose membrane as described previously (Sato *et al*, 2001b). The membranes were reacted with rabbit anti-(human COX-1) or anti-(human COX-2) antibody (Cayman Chemical), which was then complexed with horseradish peroxidase-conjugated goat anti-(rabbit IgG)IgG (Sigma). Immunoreactive COXs were visualized with enhanced chemiluminescence–Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's instructions.

Statistical analysis Data are presented as the mean $\pm$ standard deviation, 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.

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Supplementary Material

The following material is available online for this article.

Figure S1
Expression of COX-1 and COX-2 in indomethacin- and NS-398-treated hamster sebocytes.

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