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Long-chain (sphingoid) bases inhibit multistage carcinogenesis in mouse C3H/10T½ cells treated with radiation and phorbol 12-myristate 13-acetate

(sphingolipids/protein kinase C/tumor promotion/transformation)

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ABSTRACT **Sphingosine and other long-chain (sphingoid) bases inhibit protein kinase C, the putative cellular receptor for the tumor promoter phorbol 12-myristate 13-acetate (PMA), and exert potent effects on diverse cell functions. We tested the ability of long-chain bases to modulate multistage carcinogenesis in mouse C3H/10T½ cells exposed to γ-rays and PMA. Sphingosine and sphinganine completely blocked the enhancement of radiation-induced transformation by PMA (promotion) and partially suppressed transformation by radiation alone. *N*-Acetylsphingosine, a ceramide analog, did not inhibit transformation. Sphingosine was rapidly taken up by the cells and metabolized; hence, the long-chain bases were added daily to achieve prolonged inhibition. Long-chain bases inhibited protein kinase C activity in C3H/10T½ cells and suppressed the down-regulation of this enzyme by PMA. Our results establish that long-chain bases are highly effective inhibitors of carcinogenesis in this model. Our results also indicate that the suppressive effects may be mediated, in part, by inhibition of protein kinase C. The data suggest that sphingosine and other long-chain bases derived from complex sphingolipids may act as cancer-preventative agents.**

Transformation of normal cells to malignancy proceeds through several discernible stages. Two early states of transformation have been characterized as initiation and promotion (1–4). Initiation is achieved by exposing cells to a single dose of a physical or chemical carcinogen. Promotion is induced by repetitive treatments with noncarcinogenic tumor promoters, such as phorbol 12-myristate 13-acetate (PMA), and leads to an augmented outgrowth of initiated cells and enhanced transformation rates (reviewed in refs. 3 and 4).

The major cellular receptor for phorbol ester tumor promoters, and potentially others, is a family of Ca^{2+}- and phospholipid-dependent protein kinases termed protein kinase C, which binds phorbol esters with high affinity (5–7). Protein kinase C is known to play a central role in signal transduction and growth control (7); however, its function in multistage carcinogenesis is poorly understood (3, 5–8). The identification of agents that have the capacity to inhibit protein kinase C and tumor promotion is of great advantage for elucidating the role of this enzyme in carcinogenesis (3, 9). In addition, such agents may act as endogenous antipromoters (9) or as dietary antirisk factors (4, 9, 10) and play an important role in cancer prevention.

Free long-chain bases (sphingosine and sphinganine) have recently been shown to be potent inhibitors of protein kinase C in cell-free systems and in intact cells (9, 11, 12). These findings led to the hypothesis that long-chain bases may also affect tumor promotion (9). If this were the case, these bases could influence carcinogenesis since they are the backbone moieties of complex sphingolipids (sphingomyelin, gangliosides, etc.) and are natural constituents of cells (13, 14) and food products such as milk (15).

Complex sphingolipids have long been associated with the regulation of cell growth, differentiation, and transformation (13, 14), but free long-chain bases have only recently been thought of as bioactive compounds (9, 11, 12). Hence, the present work was undertaken to study in cultured mouse C3H/10T½ fibroblasts (16) the effects of long-chain (sphingoid) bases on PMA-induced promotion in radiation-initiated cells (17, 18) and to correlate their effects on promotion with their modulation of protein kinase C. We report that sphingosine and sphinganine inhibit radiation-induced transformation and PMA-induced promotion. Furthermore, they alter the activity of protein kinase C and partially suppress the PMA-mediated down-regulation of this enzyme in C3H/10T½ cells.

MATERIALS AND METHODS

Materials. Sphingosine, sphinganine, PMA, fatty acid depleted-bovine serum albumin, chemicals used in the protein kinase C assays, and dimethyl sulfoxide (DMSO) were purchased from Sigma. The *N*-acetylsphingosine was prepared as described (19). [γ-^{32}P]ATP was obtained from Amersham. [^{3}H]Sphingosine was prepared for us by New England Nuclear using catalytic hydrogenation and was purified immediately before use by thin-layer chromatography to remove radiochemical decomposition products. Dulbecco modified Eagle's medium (DMEM) and trypsin/EDTA were purchased from GIBCO, fetal bovine serum was purchased from HyClone; Giemsa stain and buffered formalin were obtained from Fisher.

Cell Culture Methods. Mouse embryo fibroblasts C3H/10T½ clone 8, at passage 8, were seeded into 100-mm (diameter) plastic Petri dishes (Corning) at 5×10^3 cells per plate in 10 ml of DMEM supplemented with 10% heat-inactivated (65°C for 2 hr) fetal bovine serum and incubated at 37°C in 5% CO_2 in air (4, 16). Experimental cells were irradiated with 4 Gy 24 hr after seeding using a ^{137}Cs source, Gammacell-40 (Atomic Energy of Canada, Ottawa) operating at a dose rate of 1.36 Gy (1 Gy = 100 rad). Control unirradiated cells were maintained under the same conditions. One set of irradiated cells and appropriate controls were treated daily with either PMA (0.16 μM in DMSO, final concentra-

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide.

tion of DMSO being 1 μl per plate) (16) or the long-chain base [sphingosine, sphinganine, or *N*-acetylsphingosine at 3 μM delivered as the 1:1 (mol:mol) complex with fatty acid-free bovine serum albumin] (10, 20). Both compounds were added at various times according to the scheme illustrated in Fig. 1. Cells treated with bovine serum albumin or DMSO served as controls. Medium was exchanged daily in control and experimental plates with fresh medium that contained the appropriate compounds. Cells were fixed with 10% buffered formalin and stained with buffered Giemsa 6 weeks after the beginning of the experiments. Transformed foci types II and III were scored as described (16). Parallel sets of treated and untreated cells were fixed and stained 3 weeks after the beginning of the experiments to establish the plating efficiency and cell survival (4, 16).

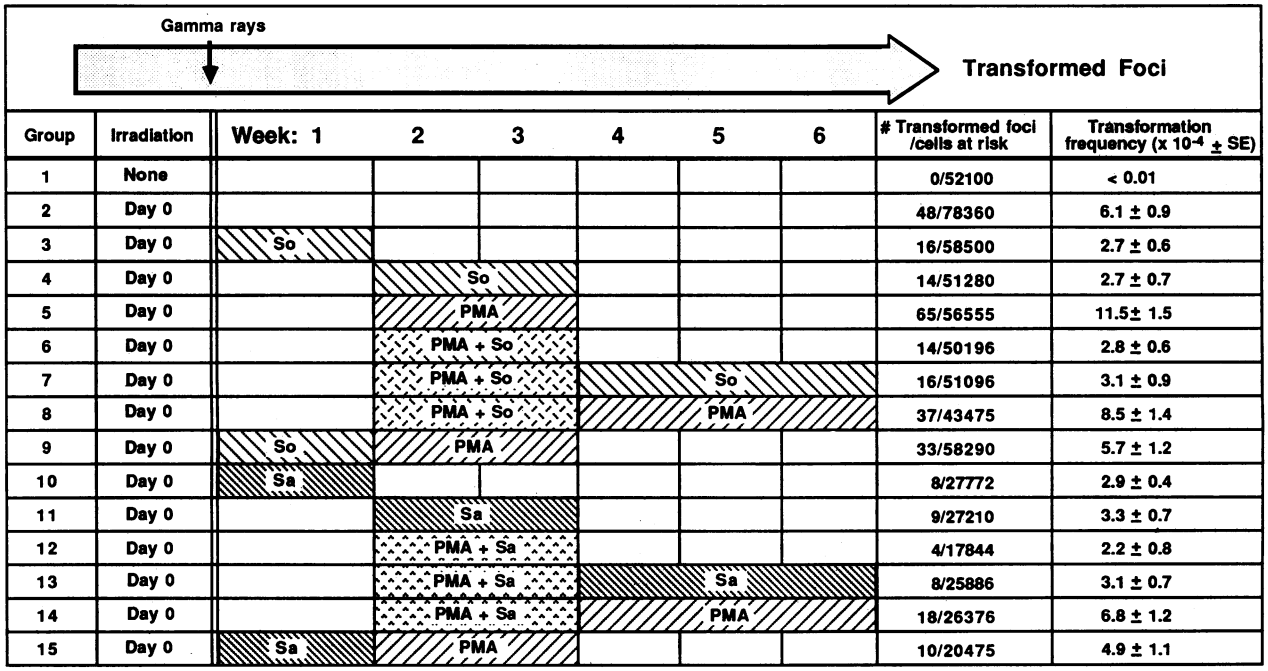
Protein Kinase C Measurements. Protein kinase C was measured in irradiated and unirradiated C3H/10T½ cells exposed to either PMA, long-chain base, or both as well as in control cells exposed to BSA and DMSO or both. The soluble and particulate activities of protein kinase C (defined as the calcium-dependent phosphorylation of histone IIIs) were measured in cells scraped from the flask using the basic method of Huang *et al.* (21).

Cellular Uptake and Metabolism of Sphingosine. Cells were exposed to 3 μM sphingosine (30 nmol/10 ml of culture medium) for up to 24 hr; then the medium was removed and the amount of sphingosine was assayed by high-performance liquid chromatography (22). To assess the disappearance of sphingosine over time, the medium was removed from one group after 6 hr, new (sphingosine-free) medium was added, and the amount of sphingosine was assayed at 24 hr. To identify some of the metabolic products of added sphingosine, ≈1 μCi (1 Ci = 37 GBq) of [^{3}H]sphingosine was added to each dish, and 4 hr later the lipids were extracted and chromatographed on silica high-performance TLC plates developed with chloroform/methanol/NH_4OH, 40:10:1 (vol/vol), to separate the long-chain bases from more complex sphingolipids (23). The radioactive regions of the chromatoplate were detected with a BioScan system 200 imaging scanner.

RESULTS

Determination of Conditions for Adding Long-Chain Bases. Since these compounds can be cytotoxic and growth inhibitory (24), experiments were conducted to determine the levels of long-chain bases that were nontoxic to C3H/10T½ cells. Daily addition of 3 μM sphingosine or sphinganine under conditions comparable to those outlined in Fig. 1 did not decrease the cell number or viability nor did the combination of PMA plus long-chain base (or PMA alone) have a cytotoxic effect (data not shown). The total cell numbers in the different groups were not noticeably different when the cells were counted to calculate the transformation frequency, which confirms that this concentration was not toxic or growth inhibitory.

Effect of Sphingosine and Sphinganine on Radiation-Induced Transformation. C3H/10T½ cells were exposed to γ-rays and treated with various combinations of PMA and/or long-chain bases as shown in Fig. 1. The cultures were fixed and stained, and transformation was scored as the number of types II and III foci expressed as the frequency of transformation per surviving cells (16, 17). Nonirradiated cells or cells treated with PMA showed no detectable transformed foci (Fig. 1); irradiation alone significantly increased the number of foci, and irradiation followed by treatment of the



Group	Irradiation	# Transformed foci /cells at risk	Transformation frequency (x 10^{-4} ± SE)
1	None	0/52100	< 0.01
2	Day 0	48/78360	6.1 ± 0.9
3	Day 0	16/58500	2.7 ± 0.6
4	Day 0	14/51280	2.7 ± 0.7
5	Day 0	65/56555	11.5± 1.5
6	Day 0	14/50196	2.8 ± 0.6
7	Day 0	16/51096	3.1 ± 0.9
8	Day 0	37/43475	8.5 ± 1.4
9	Day 0	33/58290	5.7 ± 1.2
10	Day 0	8/27772	2.9 ± 0.4
11	Day 0	9/27210	3.3 ± 0.7
12	Day 0	4/17844	2.2 ± 0.8
13	Day 0	8/25886	3.1 ± 0.7
14	Day 0	18/26376	6.8 ± 1.2
15	Day 0	10/20475	4.9 ± 1.1

FIG. 1. Effect of sphingosine (So) and sphinganine (Sa) in suppressing radiogenic transformation and its enhancement by the tumor promoter PMA: two-stage transformation of C3H/10T½ cells by γ-irradiation and PMA. C3H/10T½ cells were exposed to γ-rays and treated with various combinations of 0.16 μM PMA and/or 3 μM sphingosine or sphinganine, which were added daily as the 1:1 complex with bovine serum albumin. The frequency of transformation is defined as number of transformed foci types II and III per 10^4 cells at risk. No transformation was observed in control (unirradiated) C3H/10T½ cells treated with PMA, the long-chain bases alone, or the carriers bovine serum albumin and DMSO under the same conditions. The data are given as the mean values of at least three experiments ± standard error (SE). The transformation frequencies were subjected to analysis by *t* test. The transformation frequencies in cultures treated with x-rays and So or Sa (groups 3, 4, 10, and 11) are different from those given x-rays alone (group 2) at the significance level of $P < 0.05$. Transformation frequencies in cultures treated with So or Sa and PMA (groups 6–9 and 12–15) were different from those given PMA alone (group 5) at the significance level of $P < 0.01$, except for group 8, which was not significantly different from PMA alone, indicating that continued treatment with PMA after So was removed reversed the suppression of transformation.

cells with PMA during weeks 2 and 3 postirradiation (a crucial period of promotion in this system) caused a highly reproducible doubling in transformation frequency.

Sphingosine and sphinganine reduced the transformation frequency to about half of that observed in cells exposed to radiation alone (compare groups 4 and 11 versus group 2). Moreover, they completely blocked the PMA-induced increase in transformed foci when added during weeks 2 and 3 (compare groups 6 and 12 versus group 5). The reduction in transformation frequency was not due to cytotoxicity or inhibition of cell growth (as has been observed in some systems) (24) for the reasons noted above.

The inhibition of transformation was partially reversed by removing the long-chain base and treating the cells with PMA during weeks 4 through 6 (groups 8 and 14), whereas the transformation frequency remained low when only sphingosine or sphinganine was present during the later period (groups 7 and 13). The inhibition of transformation by long-chain bases and the reversal of the inhibition by PMA in the later period support the hypothesis that long-chain bases and phorbol esters act competitively at a single site (9).

Addition of *N*-acetylsphingosine, a ceramide analog, did not affect transformation. In this experiment, the frequency with γ-irradiation alone was 6.8×10^{-4}, with PMA 13×10^{-4}, and with PMA plus *N*-acetylsphingosine 12×10^{-4} versus γ-irradiation with *N*-acetylsphingosine 5.9×10^{-4} (for comparison, PMA plus sphingosine was 5.2×10^{-4} in this experiment). Hence, it appears that the free amino group is required for inhibition of transformation.

Sphingosine and sphinganine inhibited transformation when added during the first week after irradiation (Fig. 1, groups 3 and 10). This indicates that long-chain bases have the ability to suppress early events in radiogenic transformation. This, too, was partially reversed when PMA was added during weeks 2 and 3, but not to the level observed in cells exposed to PMA alone (compare groups 9 and 15 versus group 5).

Effect of Long-Chain Bases and PMA on Protein Kinase C. One possible mechanism for the inhibitory action of long-chain bases on transformation may be by inhibiting the activation of protein kinase C by PMA (and possibly by endogenous activators). Protein kinase C activities were measured in irradiated and unirradiated C3H/10T½ cells exposed to single or combined treatments with PMA and sphinganine as well as in untreated controls. Cells were treated using approximately the same protocols as those described in Fig. 1 (i.e., they were irradiated and treated with PMA and/or sphinganine and assayed at the end of 1 week).

The phorbol ester caused a major loss of protein kinase C (Fig. 2), as typically occurs in cells exposed to phorbol esters over extended periods of time (7). This down-regulation is thought to be mediated by PMA-induced activation of a protease that destroys protein kinase C (7). The remaining activity was mainly in the particulate fraction, which is consistent with a translocation of protein kinase C to membranes in response to PMA. Irradiation of the cells also caused some loss of protein kinase C. The loss was exacerbated when γ-ray treatment was combined with a subsequent exposure of the cells to PMA (Fig. 2); however, the residual activities are too low for reliable measurements.

Sphinganine proved to be an inhibitor of protein kinase C in these *in vitro* assays (Fig. 2), although the activity was not completely suppressed. The potency of sphinganine may be underestimated under the conditions used here since other additional lipids constitute part of the *in vitro* assay cocktail, and the inhibitory effects of long-chain bases are subject to surface dilution kinetics (9). The extent of inhibition by sphinganine alone (≈50%) was similar for control and irradiated cells; however, in the latter instance, the activities were already somewhat lower due to irradiation alone.

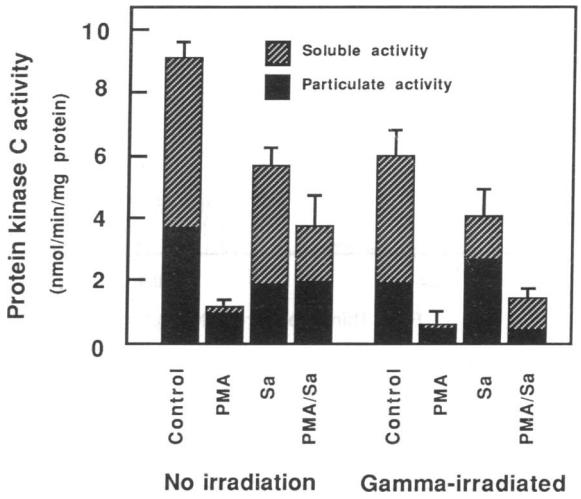


FIG. 2. Protein kinase C activities in soluble and particulate fractions from C3H/10T½ cells. The cells were exposed to γ-rays and treated with PMA and/or sphinganine (Sa) for 48 hr in essentially the same manner as for Fig. 1. The cells were scraped from the dishes, disrupted, centrifuged to separate soluble and particulate fractions, and assayed for protein kinase C activity with histone IIIs as the phosphate acceptor. The data are given as the mean values of at least three experiments ± standard error (SE).

The most striking effect of sphinganine, on protein kinase C activities, was seen in groups of cells that received PMA and sphinganine. In control cells, the total and particulate activities were much higher in cells exposed to PMA and sphinganine than in those treated with PMA alone. This suggests that inhibition of protein kinase C by sphinganine may reduce the PMA-induced down-regulation of the enzyme, as reported in other systems (25, 26). A similar effect was observed in cells exposed to radiation and subsequently treated with PMA and sphingosine (data not shown).

Cellular Uptake and Metabolism of Sphingosine. Cellular uptake of the long-chain bases was confirmed by measuring the amount of cell-associated sphingosine. Within about 6 hr, the cells contained 3–5 nmol of sphingosine per 10^6 cells—a 300-fold increase over the amount found in cells that were not sphingosine treated (12 ± 3 pmol) (Fig. 3). Upon replacement

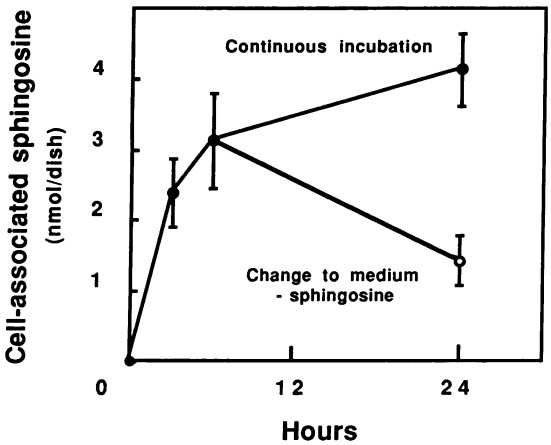


FIG. 3. Uptake and metabolism of sphingosine by C3H/10T½ cells. Cells were exposed to 3 μM sphingosine for up to 24 hr; then the medium was removed and the amount of sphingosine was assayed by high-performance liquid chromatography. To assess the disappearance of sphingosine over time, the medium was removed from one group after 6 hr, new (sphingosine-free) medium was added, and the amount of sphingosine was assayed at 24 hr (open circle). The data are given as the mean values of three experiments ± standard error (SE).

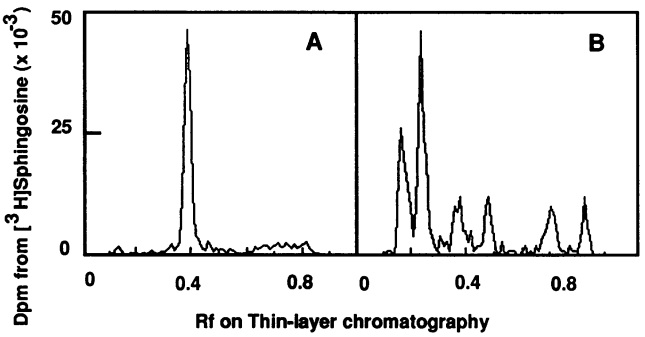


FIG. 4. Metabolism of [^{3}H]sphingosine by C3H/10T½ cells. (*A*) Scan of radiolabeled sphingosine before addition to the cells. (*B*) Approximately 1 μCi of [^{3}H]sphingosine was added to each dish of cells, 4 hr later the lipids were extracted and chromatographed on silica high-performance TLC plates developed with chloroform/methanol/NH_4OH, 40:10:1 (vol/vol), and the radioactivity was detected with a BioScan system 200 imaging scanner.

of the medium with new medium lacking sphingosine, the amount of sphingosine declined significantly by 24 hr. Such rapid uptake and metabolism of long-chain bases has been noted with other cell types (24, 27) and was the basis for our adding sphingosine daily to replace the amounts removed by metabolism. Sphingosine can be metabolized to ceramides and more complex sphingolipids or degraded by phosphorylation and lytic cleavage to ethanolamine phosphate and *trans*-2-hexadecenal (28).

As shown in Fig. 4, a large number of metabolites are formed from the radiolabeled sphingosine. Although these were not conclusively identified, the highest R_f compounds (*ca.* 0.8) comigrated with standards for ceramides, and the low R_f compounds (0.18–0.25) migrated with sphingomyelin. It is common for sphingolipids to migrate as doublets due to heterogeneity in the ceramide backbone. These observations raise the possibility that some of the effects of sphingoid bases may be due to their conversion to other bioactive sphingolipids. However, it is doubtful that ceramides are the active species in inhibiting transformation since we observed no suppression by *N*-acetylsphingosine, a ceramide analog.

DISCUSSION

The results presented here indicate that sphingosine and sphinganine inhibit multistage carcinogenesis. They support the hypothesis that long-chain bases, as potent inhibitors of protein kinase C, can block tumor promotion by phorbol esters (9). Though it should be born in mind that long-chain bases may have additional targets in cells (11, 12), these findings with C3H/10T½ cells provide evidence for an involvement of protein kinase C in transformation (3, 5–8).

Long-chain bases also partially protected protein kinase C from down-regulation by phorbol esters, as has been seen before (25). This raises the question whether the determining event in promotion is the activation of protein kinase C by phorbol esters, or the reduction in activity that occurs during the prolonged exposure to PMA, required for tumor promotion (3, 8, 18). Partial down-regulation of protein kinase C appears to play a role in the multistep process of transformation by chemical carcinogens (8), by radiation (as our data indicate), and by dominant transforming genes (29). Hence, the ability of long-chain bases to inhibit transformation might be related to the protection of a portion of the protein kinase C from down-regulation rather than a reduction in the activity of the enzyme *per se*.

The suppression of radiation-induced transformation by long-chain bases in groups that were not treated with PMA may represent the effects of these compounds on a variety of cellular and molecular processes. It may reflect an inhibition of protein kinase C activation by endogenous factors (4, 18) such as diacylglycerols and unsaturated fatty acids (7). Long-chain bases inhibit protein kinase C activation by these compounds (11, 12, 27, 30). Other agents such as hormones and various naturally occurring compounds may act as endogenous tumor promoters under the appropriate conditions (31). Transformation of C3H/10T½ cells by radiation has been associated with the activation of cellular oncogenes (17), and one mechanism by which oncogenes transform cells has been postulated to be by way of constitutive activation of protein kinase C (32).

Our findings using C3H/10T½ cells as a model for multistage carcinogenesis may be relevant to tumor promotion *in vivo*. Though there is no direct information available concerning the effects of long-chain bases on tumorigenesis in animals, sphingosine inhibits the induction of ornithine decarboxylase by PMA in mouse skin, which may reflect an effect on an early marker of carcinogenesis (33, 34).

Although various agents have been reported to act as anticarcinogens (18, 35–37) or to inhibit protein kinase C (38), long-chain (sphingoid) bases are particularly interesting in their combined action as inhibitors of transformation and modulators of protein kinase C (Fig. 5). Sphingolipids are natural constituents of cells and tissues (13, 14). Sphingolipid content and composition often differ between normal and neoplastic cells (14, 39, 40) and can be altered by treating cells with activators of protein kinase C (41, 42). Cells also contain small amounts of free sphingosine (12, 20, 22, 43–46); thus, the turnover of endogenous sphingolipids to long-chain bases may be one of the factors that influence cell susceptibility to neoplastic conversion.

Sphingolipids are constituents of foods (15) and their hydrolysis during digestion is reported to release sphingosine (47, 48), which may act as a protective factor for the small and large intestine (49). A number of foods contain significant

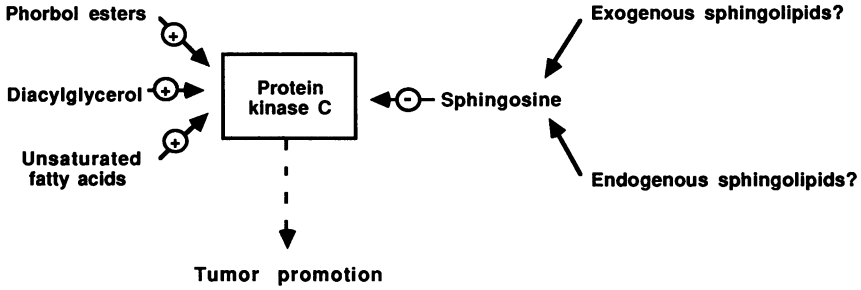


FIG. 5. Model for the modulation of C3H/10T½ cell transformation by long-chain bases. In this scheme (modified from ref. 9), phorbol esters and endogenous diacylglycerols, unsaturated fatty acids, and potentially other factors modulate neoplastic transformation by way of activation of protein kinase C, whereas long-chain bases (sphingosine or sphinganine) are inhibitory. In these experiments, the long-chain bases were derived from an exogenous source; however, *in vivo*, complex sphingolipids may also serve as precursors to free long-chain bases.

levels of sphingolipids. For example, milk contains ≈124 nmol of sphingomyelin per ml (15) and liver contains about 1 μmol of complex sphingolipids per g (44). Thus, sphingolipids may act as putative antirisk factors and play an important role in cancer prevention.

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