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吉澤, 滋

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Tumor-promoting Activity of Staurosporine, a Protein Kinase Inhibitor on Mouse Skin

Shigeru Yoshizawa, Hirotu Fujiki, Hiroko Sugari, Masumi Suganuma, Michie Nakayasu, Rie Matsushima, and Takashi Sugimura

National Cancer Center Research Institute; and National Cancer Center, Chuo-ku, Tokyo 104, Japan

ABSTRACT

Staurosporine, which is a potent inhibitor of protein kinases, inhibited both inductions of adhesion of mouse promyelocytic leukemia cells (90% effective dose = 0.9 nM) and 7,12-dimethylbenz(a)anthracene (DMBA) tumor-promoting activity by 50% in mouse skin. The two-stage carcinogenesis experiment revealed that the group treatment with a single application of 100 μg of 7,12-dimethylbenz(a)anthracene, followed by repeated applications of 50 μg of staurosporine, resulted in 85% of tumor-bearing mice at week 30, whereas group treatment with staurosporine alone or 7,12-dimethylbenz(a)anthracene alone gave 4.7% and 6.8%, respectively. Experiment 2 showed that group treatment with 7,12-dimethylbenz(a)anthracene followed by applications of 10 μg of staurosporine resulted in 33% of tumor-bearing mice at week 30. In addition, staurosporine treatment reduced the percentage of tumor-bearing mice treated with teleocidin from 100% to 67% in week 15. These results demonstrated that staurosporine is a weak tumor promoter of mouse skin compared with teleocidin, but staurosporine has some potency to inhibit tumor promotion by teleocidin.

INTRODUCTION

TPA-type tumor promoters, such as TPA, teleocidin, and aplysia toxin, activate calcium-activated, phospholipid-dependent protein kinase (protein kinase C) and, thus, induce biological activities and tumor promotion (1, 2). If inhibitors of protein kinase C block this phosphorylation, they should also inhibit the tumor promotion induced by TPA-type tumor promoters. In fact, naltmycin-carnitine, a protein kinase C inhibitor, has this effect (3, 4).

A potent protein kinase inhibitor, staurosporine (Fig. 1), was isolated from Streptomyces spp. by Tamaoki et al. (5). Staurosporine inhibited protein kinase C activity within a nanomolar range of concentrations, as well as the activity of cyclic-AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, and the p55src tyrosine kinase (5, 6). Although staurosporine did not selectively inhibit protein kinase C, the effects of staurosporine on various biological activities induced by phorbol esters had been reported. Staurosporine enhanced differentiation of HL-60 cells (7) and inhibited induction of EB virus EA in Raji cells (8) and NADPH-oxidase activation in human neutrophils (9). Two research groups independently found that staurosporine inhibited tumor promotion of TPA in two-stage carcinogenesis experiments on mouse skin (10, 11). Staurosporine induced morphological changes of primary mouse epidermal cells similar to phorbol 12,13-dibutyrate (9). Thus, the study with staurosporine suggested the presence of dual effects on tumor promotion.

Here, we report that staurosporine induced irritation on mouse skin and HDC activity in mouse skin and had a weak tumor-promoting activity on mouse skin initiated with DMBA, although staurosporine showed inhibition of biological activity of teleocidin in HL-60 cells and Raji cells. Staurosporine is a unique tumor promoter possessing dual effects: tumor-promoting activity and antipromoting activity on mouse skin.

MATERIALS AND METHODS

Materials

Staurosporine was kindly provided by Dr. H. Nakato, Tokyo Research Laboratories, Kyowa Hakko Kogyo, Tokyo, Japan. Teleocidin was isolated from Streptomyces mediocutus (12). DMBA was purchased from Sigma Chemical Co., St. Louis, MO, and TPA was from LC Services Corporation, Woburn, MA. Sodium o-butoxymethyl is obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. [3H].jTPA was from American, United Kingdom, and [125I]E2 was from Sigma Chemical Co., in Boston, MA. Anti-human IgG conjugated with fluorescein isothiocyanate was obtained from Dakopatts A/S, Glostrup, Denmark.

Animals

Female CD-1 mice were purchased from the Japanese Charles River Co., Ltd., Kanagawa, Japan, and kept as reported previously (13).

Assay of Inhibition of Activation of Protein Kinase C by Filter

The effect of staurosporine on protein kinase C activated by 2.3 μM TPA was examined as described previously (14). Protein kinase C was purified from mouse brain by DEAE-cellulose column chromatography (15).

Assay of Inhibition of Induction of HL-60 Cell Adhesion

Adhesion of HL-60 cells cultured in RPMI-1640 medium with 10% fetal calf serum was induced by incubation with 4.5 mM teleocidin for 48 h (16). The inhibitory effect of various concentrations of staurosporine on cell adhesion was examined in the presence of teleocidin.

Assay of Inhibition of Induction of EB Virus EA

Raji cells (3 × 10⁶ per ml) were incubated in 1 ml of RPMI-1640 medium containing 10% fetal calf serum with 2.3 mM staurosporine, 4 mM sodium o-butoxymethyl, and 2.0 mM staurosporine. After 48-h incubation, EA-positive cells stained with the indirect immunofluorescence method were counted (17). Cell numbers were counted after staining with trypan blue.
Irritant Test on Mouse Ear

Various amounts of staurosporine in 10 μl of acetone were applied to the ears of 8-wk-old CD-1 mice. The extent of irritation was expressed as the minimum dose of the compound causing irritation, as described previously (18).

Induction of HDC in Mouse Skin

Staurosporine in 0.1 ml of acetone was applied to the skin of the backs of CD-1 mice, and a crude enzyme extract was prepared from the skin 18 h later as described previously (19). HDC activity was expressed as pmol of CO₂ released per mg of protein per h of incubation.

Induction of ODC in Mouse Skin

Staurosporine in 0.2 ml of acetone was applied to the skin of the backs of CD-1 mice. After 4 h, a crude enzyme extract was prepared from the epidermis, and its ODC activity was measured as described previously (16). Enzyme activity was expressed as nmol of CO₂ per mg of protein per 30 min of incubation.

Two-Stage Carcinogenesis Experiments

Experiment 1. Initiation was achieved by a single application of 100 μg of DMBA dissolved in 100 μl of acetone to the skin of the backs of 8-wk-old female CD-1 mice (13). From 1 wk after initiation, 50 μg (107 nmol) of staurosporine dissolved in 100 μl of acetone were applied to the initiated skin parts of the mice, twice a wk, until wk 30. Control groups were treated with DMBA alone or staurosporine alone. The percentages of tumor-bearing mice and the average numbers of tumors per mouse were determined weekly as described previously (20).

Experiment 2. The experiment was carried out by the same procedure. From 1 wk after initiation with 100 μg of DMBA, 10 μg (21 nmol) of staurosporine were applied, twice a wk. In addition, this experiment included a group treated with DMBA and staurosporine plus 2.5 μg (5.7 nmol) of teledocin, which was applied 15 min after each application of staurosporine. Control groups treated with DMBA and teledocin or DMBA alone were also observed. In both Experiments 1 and 2, each group consisted of 15 mice, because of the limited availability of staurosporine.

RESULTS

Inhibitory Effects of Staurosporine on Biological Activities Induced by Teledocin. Based on our evidence that staurosporine inhibited the activation of protein kinase C by 2.3 μM teledocin, and that the effective dose for 50% inhibition was 2.6 μM, comparable to the results of Tamaoki et al. (4), we then studied whether it inhibited the other biological activities induced by teledocin. Treatment with 4.5 μM teledocin for 48 h induced adhesion of HL-60 cells, and this induction was inhibited by staurosporine dose dependently at concentrations of 6 to 30 nm (Fig. 2). The ED₅₀ value was 9.0 nm. In this experiment, the treatment with teledocin and staurosporine did not show any toxicity to HL-60 cells at concentrations of staurosporine up to 30 nm, determined by trypan blue staining. Incubation of Raji cells with 2.3 μM teledocin for 48 h induced expression of EB virus EA in 55% of the cells, whereas in the absence of teledocin, less than 1% of the cells expressed EB virus EA. As Fig. 3 shows, incubation of the cells with staurosporine at concentrations of 0.21 nm to 2.1 μM together with 2.3 μM teledocin and 4 mM sodium N-butyrate resulted in a dose-dependent decrease in the percentage of EA-positive cells, the ED₅₀ value of staurosporine being 3.4 nm. The viability of Raji cells was more than 80% at concentrations of up to 1 μM staurosporine in the presence of 2.3 μM teledocin and 4 mM sodium N-butyrate. These two experiments showed that staurosporine strongly inhibited two of the biological activities induced by teledocin. However, a single application of 10 nmol of staurosporine clearly enhanced the effects of teledocin on ODC activity (Table 1).

Biological Activities of Staurosporine. Staurosporine induced irritation of mouse ear, the dose causing redness of the ear 24 h after its application being 200 ng (0.43 nmol). Since that of teledocin was 0.088 nmol (12), the potency of staurosporine for irritation on mouse ear was about 50 times weaker than that of teledocin. In addition, applications of 170 nmol and 340 nmol of staurosporine induced HDC activity at levels of 196 and 173 pmol of CO₂/mg of protein per 60 min, respectively, which was about 10 times weaker than that of teledocin (Table 2).

Staurosporine, at concentrations of up to 500 nmol, did not induce ODC activity in mouse epididymis 4 h after application, whereas teledocin induced it as usual (Table 1). Furthermore, staurosporine did not induce HL-60 cell adhesion at a concentration of 50 nm. In Raji cells, staurosporine itself, at concentrations of up to 2.1 μM, did not induce EB virus EA in the presence of 4 mM sodium N-butyrate. Table 3 summarizes the effects of staurosporine.

Tumor-Promoting Activity of Staurosporine. Since staurospor-
The average numbers of tumors per mouse treated with OMBA alone did not produce any tumors, while sodium butyrate did not induce EB virus EA in Raji cells. In these two groups were 1.9 and 0.05, respectively. The average numbers of tumors per mouse treated with DMBA and teledocin plus staurosporine resulted in 67% tumor-bearing mice by Wk 15 and 80% by Wk 30, whereas the group treated with DMBA and teledocin induced tumors in 100% of the mice by Wk 15 and remained constant up to Wk 30. The staurosporine treatment reduced the average numbers of tumors per mouse from 6.1 to 3.9 in Wk 30. In the group treated with DMBA and teledocin plus staurosporine, mice did not show any significant body weight loss throughout the experiment, and the skin of the backs of these mice were not irritated, compared with those in the group treated with DMBA and teledocin. The inhibitory effect of staurosporine was statistically significant at around Wk 10 of tumor promotion. Although statistically significant inhibition was not obtained with 10 µg of staurosporine in later weeks of the experiment, a decreasing tendency in the percentage of tumor-bearing mice and in average numbers of tumors per mouse was apparent (Fig. 5). Thus, staurosporine slightly inhibited tumor promotion of teledocin, even at the dose at which staurosporine itself induced tumors.

DISCUSSION

Staurosporine is a potent inhibitor of protein kinases, such as protein kinase C, cyclic AMP-dependent protein kinase, and cyclic GMP-dependent protein kinase (26). Since staurosporine inhibited biological activities of teledocin in cell lines such as HL-60 cells and Raji cells, it was expected to inhibit tumor-promoting activity of non-TPA-type tumor promoters. In fact, staurosporine slightly inhibited the tumor-promoting activity of teledocin in a two-stage carcinogenesis experiment on mouse skin. During the preparation of this paper, two groups reported independently that staurosporine inhibited tumor-promoting activity of teledocin in a two-stage carcinogenesis experiment on mouse skin. Although our results with staurosporine were not strongly inhibitory, due to limited amounts of the compound, for comparison, we carried out our experiment. In addition to inhibition of tumor promotion, staurosporine itself had a tumor-promoting activity on mouse skin and was classified as a non-TPA-type tumor promoter, which does not tend to phosphoryl esters (1, 2). Staurosporine was phosphorylated by casein kinases and tyrosine kinases in mouse skin like the TPA-type tumor promoters. However, it did not significantly induce ODC activity in mouse skin. Recently, in collaboration with our previous studies, staurosporine stimulated protein kinase C production in rat macrophages, and with L. Levine, we demonstrated stimulation of protein kinase C activity in non-TPA-type tumor promoters. These results are well in agreement with our previous data that activation of arachidonic acid metabolism is the common effect induced by TPA-type and non-TPA-type tumor promoters.

As to the ODC induction by staurosporine, it was reported that staurosporine did not induce ODC activity in primary mouse epidermal cells (9) and that staurosporine did not affect ODC activity in T24 cells. These results were in agreement with our data. In one report, Yamamoto et al. (11) found that 100 mM of staurosporine induced ODC activity in mouse skin 5 h after application. Moreover, two test groups (10, 11) reported that staurosporine itself did not show any tumor-promoting activity in a two-stage carcinogenesis experiment. This discrepancy between our results might be due to the doses of DMBA used and of staurosporine that were used. The results of our experiment indicated that the doses of staurosporine which other groups used were not sufficient to induce any tumor-promoting activity in two-stage carcinogenesis experiments.

Staurosporine has been accumulated in the inhibition of protein kinase C. It does not directly inhibit induction of tumor promotion by the TPA-type tumor promoters. Sako et al. (9) reported that staurosporine did not effectively inhibit the effect of phorbol esters on epidermal growth factor binding or induction of ODC and stimulation of epidermal cell proliferation. Among mouse primary epidermal cells, and that staurosporine itself induced morphological changes in keratinocytes, resembling those induced by the phorbol esters. Since staurosporine is a microbial aldehyde produced by Sphingomonas spp., (5), staurosporine was, structurally, from a new family of tumor promoters than those already reported, such as phorbol esters, teledocin, alpylasitoxin, palmitoylethanolamine, and okadaic acid. K-252a is a structurally related inhibitor of protein kinases. Cyclic AMP-dependent protein kinase, and cyclic GMP-dependent protein kinase (22). The irritant activity of K-252a on mouse ear was 40 times weaker than that of staurosporine, the dose causing 50% edema of the ear was 8 µg (17 nmol). These results suggested that K-252a might be a weaker tumor promoter than staurosporine.
TUMOR-PROMOTING ACTIVITY OF STAUROSPORINE ON MOUSE SKIN


