Induction of Rat Hepatic Ornithine Decarboxylase by Tumor-promoting Phorbol Esters

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Abstract

A single i.p. administration of 0.5mg 12-0-tetradecanoylphorbol-13-acetate (TPA)/kg body weight resulted in a rapid, transient stimulation of rat liver ornithine decarboxylase (ODC) activity. The activity reached a peak (85-fold greater than control) at 4 to 5 hr after TPA treatment and returned to control level by 12 hr. The increase of ODC activity occurred in a dose dependent manner, maximal stimulation was obtained with 0.5mg TPA/kg body weight. The increase of rat liver ODC activity by TPA appeared to be under transcriptional control since administration of 2mg actinomycin D/kg body weight or 5mg cycloheximide/kg body weight 1 hr prior to application of TPA prevented the increase of ODC activity. Furthermore TPA-stimulated ODC induction was inhibited either by the pretreatment of 5mg colchicine/kg body weight or 5mg indomethacin/kg body weight.

A number of phorbol esters (17 nmol/mouse or 800 nmol/kg rat body weight) were tested for their ability to induce ODC in mouse skin and rat liver and were found to be potent in the following order: TPA> 4β-phorbol-12, 13-didecanoate (4β-PDD)> phorbol-12, 13-dibenzoate (PDB). The non-tumor promoting phorbol esters, 4α-phorbol, 4β-phorbol, 4α-phorbol-12, 13-didecanoate (4α-PDD) and phorbol-12, 13-diacetate (PDA), did not affect ODC activity.

These results indicate that the stimulation of ODC activity by the tumor promoting phorbol esters correlate well with their promoting ability on mouse skin. Therefore, it is suggested that phorbol esters-stimulated hepatic ODC induction relate to tumor promotion during hepatocarcinogenesis.

Introduction

The two stage model of chemical carcinogenesis, originally based on the experiments of BERENBLUM[1], has been studied most extensively in mouse skin[3, 28] and, during recent

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years, in liver[11, 16, 22, 23]. In the first stage called initiation, chemical carcinogens irreversibly induce damage in the cell and this damage is fixed possibly in the DNA of the target cell. For the initiation of cells usually a single application of the carcinogen is required. In the second stage called promotion, repeated administration of a tumor promoter finally leads to the development of visible tumors. When repeatedly applied after a chemical carcinogen (initiator), 12-0-tetradecanoylphorbol-13-acetate (TPA) has been shown to increase tumor incidence in mouse skin[29, 30] and liver[7, 8, 9, 27].

Ornithine decarboxylase (ODC, L-ornithine carboxy-lyase; E.C. 4.1.1.17) is the rate-limiting enzyme in the polyamine biosynthetic pathway. The activity of ODC can be rapidly and markedly induced in almost all cells and tissues which have been stimulated to grow or divide[4, 25, 26]. TPA has been shown to result in a remarkable induction of epidermal ODC activity when applied topically to mouse skin [15, 17]. Furthermore, it has been observed that the ability of TPA and a series of structurally related phorbol esters to induce ODC correlates closely with their cocarcinogenicity in mouse epidermis[17, 18]. The induction of ODC appears to be an important biochemical marker for cell growth and proliferation and may be an essential factor for the mechanism of tumor promotion.

It has been reported that TPA injected i.p. into rat resulted in a marked increase in hepatic ODC activity[2, 5, 32]. Due to the marked ability of phorbol esters to increase the activity of ODC in the skin, and to the extensive studies concerning tumor promotion during hepatocarcinogenesis, we investigated the possibility that phorbol esters might regulate ODC activity in the rat liver.

Materials and Methods

Animals: Male Wistar-Imamichi rats (200–250g) were housed in wire cages (5 per cage) in a controlled environment at 23°C with a 12 hr light/dark cycle. Animals were given food and water ab libitum until the day of experimentation. All injections were administered i.p. and rats were routinely killed between 1 and 3 p.m. to avoid variations due to circadian rhythms.

Female Charles River CD-1 mice (20–25g) were housed and given food and water as in the case of rats above. The dorsal hair of each mouse was shaved with clippers at least 2 days before use, and only those mice showing no hair regrowth were used. All chemicals were topically applied to the shaved areas and mice were routinely killed between 1 and 3 p.m..

Chemicals: Phorbol esters administered i.p. to rat were dissolved in 40% ethanol and 60% 0.15 M NaCl and delivered in a volume of 0.1 or 0.2 m*l*. Phorbol esters applied topically to mouse skin were dissolved in reagent grade acetone and delivered in a volume of 0.2 m*l*.

Phorbol esters, cycloheximide, actinomycin D, and indomethacin were obtained from Sigma Chemical Co. (St. Louis, MO); DL-(1-^{14}C) ornithine hydrochloride (specific activity 58 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, UK). All other chemicals were reagent grade and were purchased from commercial sources.

Assay of rat hepatic ODC activity: At appropriate times after treatment, rats were killed by cervical dislocation. The livers were rapidly removed and homogenized in 5 volumes of ice cold buffer consisting of 50 mM sodium phosphate, pH 7.2, 1 mM dithiothreitol, 0.1 mM EDTA and 0.04 mM pyridoxal phosphate. The homogenates were centrifuged at 20,000×g for 30 min at 4°C. The resulting supernatant was used as the source of ODC activity. ODC activity of the soluble liver extracts was determined as the amount of $^{14}CO_2$ released from

0.5μCi of DL-[1-^{14}C] ornithine hydrochloride. The concentration of reagents in the assays for the determination of ODC activity were the same as reported by WEINER et al[32]. Assays were routinely carried out at 0.3mM L-ornithine in a final volume of 1ml. After incubation for 30 min at 37°C, the reaction was stopped by addition of 0.5ml of 4 N sulfuric acid, incubation was continued for another 1 hr, and radioactivity in each sample was measured in 18ml of toluene scintillation fluid with 20% ethanol in a Beckman Model LS 8000 scintillation counter at 80% efficiency.

Assay of mouse skin ODC activity: At 4 hr after treatment, mice were killed by cervical dislocation, and skin from individual mice was excised. The skin from 4 mice were pooled, and homogenized in 3 volumes of ice cold buffer consisting of 50mM sodium phosphate, pH 7.2, 0.1mM pyridoxal phosphate and 0.1 mM EDTA. The homogenates were centrifuged at 20,000×g for 30 min at 4°C. The resulting supernatant was used as the source of ODC activity. ODC activity of the soluble skin extracts was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C] ornithine hydrochloride. The concentration of reagents in the assays for the determination of ODC activity were the same as reported by O'BRIEN et al[17]. Assays were routinely carried out at 0.3mM L-ornithine in a final volume of 1ml. After incubation for 1 hr at 37°C, the reaction was stopped by addition of 0.5ml of 4 N sulfuric acid, incubation was continued for another 1 hr, and radioactivity in each sample was measured in 18ml of toluene scintillation fluid with 20% ethanol in a Beckman Model LS 8000 scintillation counter at 80% efficiency.

Protein determination: The protein concentration of the mouse skin extracts was measured by the method of LOWRY et al.[13], with bovine serum albumin as standard.

Results

Increase of rat liver ODC activity after i.p. administration of TPA: As shown in Figure 1, i.p. injection of TPA (0.5mg/kg body weight) led to a rapid and marked increase in ODC activity in the rat liver. The maximal increase in the activity of ODC occurred 4–5 hr following TPA injection and reached a level 85-fold above the value obtained from animals receiving the injection vehicle alone. TPA-stimulated ODC activity had returned to control levels by 12 hr (data not shown). The injection vehicle alone had no effect upon ODC activity.

Dose-response of i.p. administration of TPA and rat liver ODC activity: In Figure 2 the effects of increasing doses TPA on rat liver ODC

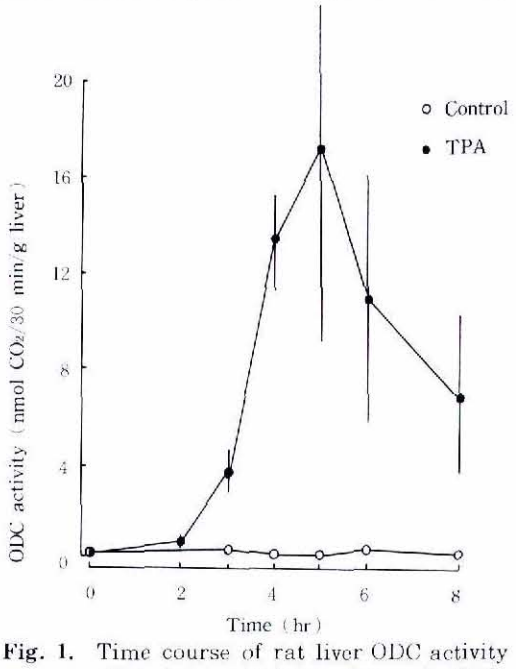

Fig. 1. Time course of rat liver ODC activity after i.p. administration of TPA. Rats received a single i.p. injection of 0.5mg TPA/kg body weight and were killed at the times indicated. Each point represents the mean±S.E.M. of 5 individual rat liver supernatants in which ODC activity was determined in duplicate.

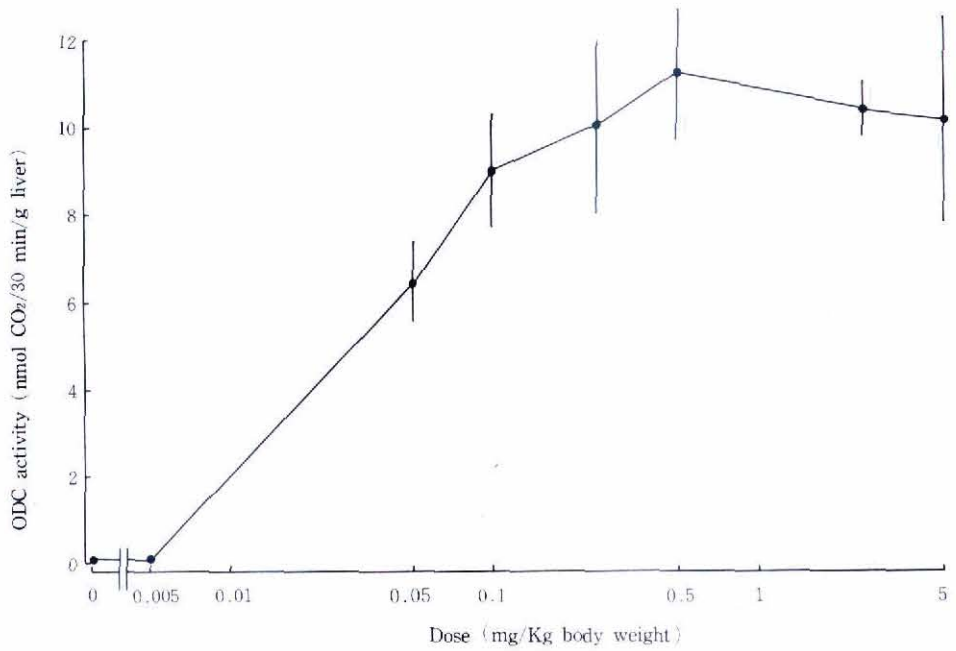

Fig. 2. Effect of increasing doses of TPA, administered by i.p. injection, on rat liver ODC activity. Rats were given TPA at the doses indicated and killed 4 hr after treatment. Each point represents the mean ± S. E. M. of 5 individual rat liver supernatants in which ODC activity was determined in duplicate.

activity are shown. Administration of 0.1~5 μgTPA/kg body weight had no effect on basal liver ODC activity when determined after 4 hr. However, a remarkable increase of ODC activity was observed after 4 hr when rats were given doses to 50μg/kg body weight. TPA-stimulated ODC activity were maximal at 0.5 mg/kg body weight. Raising the doses to 5 mg/kg body weight caused no further increase of ODC activity. This suggests that TPA saturates the system by which liver ODC activity is stimulated.

Effect of actinomycin D and cycloheximide on the increase of liver ODC activity by TPA: In Table 1 the effect of actinomycin D and cycloheximide on TPA-stimulated rat liver ODC activity is shown. 2mg actinomycin D/kg body weight or 5mg cycloheximide/kg body weight were administered i.p. 1 hr prior to the injection of 0.5mg TPA/kg body weight. The increase of liver ODC activity by i.p. administration of TPA was inhibited either by actinomycin D or cycloheximide. These data indicate that the increase of TPA-stimulated ODC activity is the induction of ODC by TPA.

Effect of colchicine on the induction of rat liver ODC by TPA: In Table 2 the effect of colchicine on the TPA dependent induction of rat liver ODC is shown. 5mg colchicine/kg body weight was administered i.p. 1.5 hr prior to the injection of 0.5mg TPA/kg body weight. The induction of liver ODC by i.p. administration of TPA was inhibited by colchicine.

Effect of indomethacin on the induction of rat liver ODC by TPA: In Table 3 the effect of indomethacin on the TPA dependent induction of rat liver ODC is shown. 5mg indomethacin/kg body weight was administered i.p. 1 hr prior to the injection of 0.1mg TPA/kg body weight. The induction of liver ODC by i.p.

Table 1. Effect of actinomycin D and cycloheximide on TPA-stimulated rat liver ODC activity.

Treatment	ODC activity (nmol CO_2/30 min/g liver)
0.15 M NaCl + Vehicle	0.158 ± 0.041
0.15 M NaCl + TPA	13.442 ± 0.451
Actinomycin D + TPA	1.408 ± 0.611*
Cycloheximide + TPA	1.560 ± 0.348*

Rats received an i. p. injection of 0.5mg TPA/kg body weight. One hour prior to TPA injection, 5mg cycloheximide/kg body weight or 2mg actinomycin D/kg body weight were administered i. p.. Control animals only received 0.15 M NaCl 1 hr prior to TPA administration. Four hours after administration of TPA, rats were killed for the immediate preparation of liver homogenates. ODC activity was measured in 20.000×g, 30min supernatants in duplicate. Each value represents the mean ± S. E. M. of individual determinations from 5 rats. *$p<0.001$ versus TPA
Vehicle, 40% ethanol and 60% 0.15 M NaCl

Table 2. Effect of colchicine on TPA-induced rat liver ODC activity.

Treatment	ODC activity (nmol CO_2/30 min/g liver)
0.15 M NaCl + Vehicle	0.47 ± 0.06
0.15 M NaCl + TPA	15.76 ± 2.57
Colchicine + TPA	3.81 ± 0.81*

Rats received an i. p. injection of 0.5mg TPA/kg body weight. 1.5hr prior to TPA injection, 5mg colchicine/kg body weight was administered i. p.. Control animals only received 0.15 M NaCl 1.5 hr prior to TPA administration. Four hours after administration of TPA, rats were killed for the immediate preparation of liver homogenates. ODC activity was measured in 20.000×g, 30min supernatants in duplicate. Each value represents the mean ± S. E. M. of individual determinations from 5 rats.
*$p<0.005$ versus TPA; Vehicle, 40% ethanol and 60% 0.15 M NaCl

Table 3. Effect of indomethacin on TPA-induced rat liver ODC activity.

Treatment	ODC activity (nmol CO_2/30 min/g liver)
Vehicle + Vehicle	0.18 ± 0.09
Vehicle + TPA	14.11 ± 2.11
Indomethacin + TPA	5.44 ± 1.52*

Rats received an i. p. injection of 0.5mg TPA/kg body weight. One hour prior to TPA injection, 5mg indomethacin/kg body weight was administered i. p.. Four hours after administration of TPA, rats were killed for the immediate preparation of liver homogenates. ODC activity was measured in 20.000×g, 30min supernatants in duplicate. Each value represents the mean ± S. E. M. of individual determinations from 5 rats.
*$p<0.01$ versus TPA; Vehicle, 40% ethanol and 60% 0.15 M NaCl

Table 4. Effect of phorbol esters on ODC activity in mouse skin and rat liver.

Treatment	Skin promoting ability[a)]	Skin ODC activity[b)] (nmol CO_2/hr/mg protein)	Liver ODC activity[c)] (nmol CO_2/30 min/g liver)
Vehicle	−	0.003	0.353 ± 0.055
4α-phorbol	−	0.003	0.395 ± 0.066
4β-phorbol	−	0.002	0.435 ± 0.165
4α-PDD	−	0.003	0.487 ± 0.257
PDA	−	0.004	0.914 ± 0.571
PDB	+	0.114	4.614 ± 1.135*
4β-PDD	++	0.212	10.756 ± 1.039*
TPA	++++	0.234	14.677 ± 0.422*

a) Promoting ability are taken from Ref. 3

b) The effect of a single topical application of 17 nmol of each of phorbol esters on the activities of mouse skin ODC. Groups of 5 mice were treated with either 0.2 m*l* acetone or 0.2 m*l* of one of phorbol esters tested and were killed 4 hr after. ODC activity from soluble skin extracts was determined as described in "Materials and Methods". Each value represents the mean of triplicate determinations of ODC activity from soluble skin extracts prepared from 5 mice.

c) The effect of i. p. administration of 800 nmol of each of phorbol esters on the activities of rat liver ODC. Groups of 5 rats were treated with either 40% ethanol and 60% 0.15 M NaCl or phorbol esters and were killed 4 hr after. ODC activity from soluble liver extracts was determined as described in "Materials and Methods". Each value represents the mean ± S. E. M. of individual determination from 5 rats.

*$p < 0.001$ versus Vehicle; 4α-PDD, 4α-phorbol-12, 13-didecanoate; PDA, phorbol-12, 13-diacetate; PDB, phorbol-12,13-dibenzoate; 4β-PDD,4β-phorbol-12, 13-didecanoate; TPA, 12-0-tetradecanoyl-phorbol-13-acetate.

administration of TPA was inhibited by indomethacin.

Effect of phorbol esters on ODC activity in mouse skin and rat liver: A number of phorbol esters were tested for their ability to induce mouse skin and rat liver ODC. As shown in Table 4, the tumor-promoting phorbol esters, PDB, 4β-PDD and TPA increased ODC activity in mouse skin and rat liver in the following order: TPA>4β-PDD> PDB. The non-tumor promoting phorbol esters, 4α-phorbol, 4β-phorbol, 4α-PDD and PDA, caused no detectable increase in mouse skin and rat liver ODC activity.

Discussion

We observed that a single administration of tumor promoter, TPA, to the abdominal cavity of rats resulted in a increase in liver ODC activity. The increase of ODC activity by TPA occurred in a dose dependent manner and was temporal. This transient effect of TPA on ODC activity has already been observed in mouse skin[17], in mouse epidermal cells[20, 33], in rat kidney cells[21] and rat liver, lung and brain[32]. It has been suggested that the decrease of ODC activity is due to the action of proteolytic enzyme[24], the synthesis of a specific inhibitory protein (ODC antizyme)[10], and the conversion of the high affinity form (90.000 d protein) into the low affinity form (180.000 d protein)[14].

This ODC regulating mechanism was investigated further. The increase of stimulated rat liver ODC activity appear to be dependent on RNA and protein synthesis, since pretreatment of rats with actinomycin D or cycloheximide completely abolished the increase of TPA-stimulated ODC activity. Therefore, the increase of ODC activity by TPA is not the

activation of ODC, but the induction.

Colchicine, microtubule-disrupting agents, administered 1.5 hr prior to TPA inhibited significantly the induction of rat liver ODC by TPA. Since microtubule-containing structures may constitute a general mechanism for the transmission of signals generated at the plasma membrane into the interior of the cell where effects on gene expression is clarified[19], it is suggested that the first acting site of TPA is the membrane of rat liver. Recent experiments with TPA, the mouse skin tumor promoter par excellence, indicate that at least in mouse epidermal cells, there are specific membrane receptors[6, 12] which, it is suggested, mediate in the cellular effects of TPA. Further studies will be necessary to investigate this possible mode of action for TPA in rat liver.

Indomethacin, inhibitor of prostaglandin synthesis, administered 1 hr prior to TPA inhibited the induction of rat liver ODC by TPA significantly. VERMA et al[31], suggest that prostaglandins play a role in the mouse skin ODC induction as well as mouse skin tumor promotion, since indomethacin treatment before application of TPA to mouse skin inhibits the accumulation of prostaglandins, the induction of ODC, and the formation of skin papillomas. Our data also suggest that prostaglandins may play a crucial role in TPA-dependent liver ODC induction.

Within a series of phorbol esters, skin tumor promoting ability correlates well with the magnitude of ODC activity in mouse skin and rat liver (Table 4). As the tumor promotion process is also known to occur in mouse liver[7, 8, 9, 27], it is suggested that there is a correlation between promoting ability and the magnitude of ODC activity in rat liver. Furthermore, it is suggested that phorbol esters-stimulated hepatic ODC induction relate to tumor promotion during hepatocarcinogenesis.

Further studies concerning the cellular sites of action of phorbol esters in rat liver are currently being carried out.

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発癌プロモーターであるフォルボールエステルによるラット肝臓のオルニチン脱炭酸酵素の誘導

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摘　　要

ラットに0.5mg 12-0-tetradecanoylphorbol-13-acetate (TPA)/kg body weightを腹腔内投与すると，肝臓のオルニチン脱炭酸酵素 (ODC) 活性は著しく増加した。ODC活性の増加は一過性で，かつ濃度依存性であった。この活性の増加は2mg actinomycin

D/kg body weight および 5mg cycloheximide/kg body weight 前投与により抑制されたことから，酵素誘導によるものと思われた。この誘導は 5mg colchicine/kg body weight および 5mg indomethacin/kg body weight の前投与により抑制された。

マウス皮膚およびラット肝臓の ODC 活性に対する一連のフォルボールエステル (17nmol/mouse or 800nmol/kg rat body weight) の影響を検討した。マウス皮膚癌のプロモーター作用を有する TPA, 4β-phorbol-12, 13-didecanoate (4β-PDD) および phorbol-12, 13-dibenzoate (PDB) は皮膚と肝臓の ODC を誘導した (TPA＞4β-PDD＞PDB) が，プロモーター作用の無い 4α-phorbol, 4β-phorbol, 4α-phorbol-12, 13-didecanoate (4α-PDD) および phorbol-12, 13-diacetate (PDA) は効果が無かった。

以上の成績から，フォルボールエステルによる肝臓の ODC 誘導能とマウス皮膚におけるプロモーター作用とがよく相関することが認められた。したがって，フォルボールエステルによる肝臓の ODC 誘導は肝癌のプロモーション過程に，少なくとも何らかの影響をおよぼしているものと思われる。