Chemico-Biological Interactions 179 (2009) 145–153

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Perillyl alcohol attenuates Ras-ERK signaling to inhibit murine skin inflammation and tumorigenesis

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ARTICLE INFO

Article history:
Received 17 October 2008
Received in revised form
22 December 2008
Accepted 22 December 2008
Available online 31 December 2008

Keywords:
Perillyl alcohol
Oxidative stress
Ras pathway
Apoptosis
Skin tumorigenesis

ABSTRACT

In the present study, the chemopreventive effect of topical application of perillyl alcohol (POH) on 9,10-dimethylbenz(a)anthracene (DMBA)-initiated and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-promoted skin tumorigenesis and its possible mechanisms of action in Swiss albino mice were investigated. We evaluated the effect of pretreatment of POH (6 and 12 mg/kg body weight) on TPA (2 μg/200 μl of acetone)-induced skin edema, hyperplasia, peroxidase damage and modulation in activities of catalase, glutathione reductase, glutathione peroxidase, glutathione-*S*-transferase and reduced glutathione contents. Application of POH 30 min prior to TPA treatment, showed a protective effect in almost all the investigated parameters. Additionally, pretreatment with POH showed a significant inhibition of ornithine decarboxylase (ODC) activity and [^{3}H] thymidine incorporation into epidermal DNA. In promotion phase, a significant reduction was found in tumor incidence and tumor burden in mice pretreated with POH (12 mg/kg body weight) with extension of the latency period from 4 to 8 weeks as compared to those treated with TPA alone. POH significantly suppressed the Ras/Raf/ERK pathway and induced apoptosis in Swiss albino mice skin. Our findings suggested that the chemopreventive efficacy of POH is probably due to the inhibition of oxidative stress responses, inhibition of the Ras cell proliferation pathway and induction of apoptosis in murine skin tumor promotion phase.

1. Introduction

Chemoprevention, use of naturally occurring and synthetic agents, is an important and practical strategy for the management of cancer. Perillyl alcohol (POH), a hydroxylated monoterpene, is an active dietary component found in the essential oils of citrus peel, mints, celery seeds and several other plants [1]. Multiple animal studies have found that POH is effective in cancer chemoprevention. POH has been reported to be an effective chemotherapeutic agent against pancreas [2], liver [3], prostate [4] and breast [5] tumors, and chemopreventive agent for colon tumors [6], melanomas [7] and neuroblastomas [8] in earlier studies. Phase I and phase II clinical studies demonstrated the chemotherapeutic activity of POH at various doses on different cancer patients with mild side-effects [9]. However, studies on all potential targets of action of POH to inhibit the tumor are meager.

Many *in vitro* as well as *in vivo* studies have been reported on the mechanism of action of POH to retard the tumor cell growth. It inhibits the post-translational modification (prenylation) of Ras protein by inhibiting the activity of farnesyl transferase enzyme (FTase) *in vitro* [10]. POH inhibits another prenylating enzyme geranylgeranyl transferase I and II [11]. Prenylated Ras protein (active form) gets localized to the cell membrane, which is a vital event in transduction of signals and helps in regulation of many cellular functions. Inhibition of Ras prenylation is a necessary step for interfering with Ras downstream effectors as reported in DMBA/TPA-induced melanoma in TPras transgenic mice [12]. POH has been shown to induce the Go/G1 cell cycle arrest [13,14], alter the pro-apoptotic proteins Bax, Bak and anti-apoptotic Bcl-2 proteins and thereby increase the apoptosis event [3,6,15,16]. During the process of tumorigenesis, POH has also been shown as an effective inhibitory agent after the initiation stage [6,17]. The chemopreventive activity of POH may be due to the inhibition of FTase enzyme, blocking the cell cycle, inhibition of cell survival pathways, and/or induction of apoptosis in the transformed cell, which ultimately inhibits tumor cell proliferation [12–17].

Abbreviations: POH, perillyl alcohol; DMBA, 9,10-dimethylbenz(a)anthracene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ROS, reactive oxygen species; CAT, catalase; GST, gltuatione-*S*-transferase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; LPO, lipid peroxidation; ODC, ornithine decarboxylase; ERK1/2, extracellular signal-regulated protein kinase 1/2; DAB, 3,3′-diaminobenzidine.

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doi:10.1016/j.cbi.2008.12.016

The multistage mouse skin tumorigenesis model is an ideal system to study a number of histological, biochemical and cellular function changes that take place during the different stages of chemical tumorigenesis [18,19]. Application of TPA, a phorbol type tumor promoter, has been reported to increase the reactive oxygen species (ROS) in the systems, which result in many of biochemical alterations in the cell [20]. TPA induces the enzyme ornithine decarboxylase (ODC) activity [21], which can be used as a biomarker of skin tumorigenesis. Studies have shown that skin application of TPA results in inflammatory responses such as development of edema and hyperplasia, induction of ODC activity, increased incorporation of thymidine into DNA, stimulation of Ras p21 cell proliferation pathway and alteration in the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax protein [12,18–22].

In vivo effects of POH on TPA-induced histological, biochemical and cellular alterations in murine skin model system have not been reported so far and the molecular mechanisms involved in chemopreventive activity of POH is not fully understood. In an effort to understand the *in vivo* mechanism of action of POH during the skin tumor promotion phase, in the present study we investigated the effects of POH on TPA-induced inflammatory response (edema and hyperplasia), oxidative stress, ODC enzyme activity, [^{3}H] thymidine incorporation, Ras/Raf/ERK1/2 signaling pathway and induction of apoptosis during the promotion phase in DMBA/TPA-mediated mouse skin tumorigenesis.

2. Materials and methods

2.1. Chemicals

POH, DMBA, TPA, aprotinin, leupeptin, pepstatin, 3,3′-diaminobenzidine (DAB), 5′-dithio (bis) 2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), glutathione reductase (GR), oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1-chloro 2,4-dinitrobenzene (CDNB), ethylenediamine tetra acetic acid (EDTA), pyridoxal phosphate, phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, dithiothreitol, Tween-80 and Brij-35 were purchased from Sigma Chemical Co., MO, USA. [^{14}C] ornithine and [^{3}H] thymidine were purchased from Amersham (Little Chalfont, UK). Primary monoclonal antibodies anti-Ras, Raf, phospho-extracellular signal-regulated protein kinase (p-ERK1/2), β-actin were purchased from BD Biosciences Pharmingen (San Jose, USA) and Bax and Bcl-2 were purchased (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG) were purchased from Genei (Bangalore, India). All other chemicals and solvents were obtained commercially at highest purity available.

2.2. Animals

Six- to eight-week-old female Swiss albino mice (20–25 g) were obtained from Central Animal House Facility of Jamia Hamdard, New Delhi, India. The mice were housed in a well-ventilated room at 25 ± 2 °C under 12 h light:12 h dark cycle and provided with pellet diet (Pranav Agro Industries Ltd., Delhi, India) and drinking water ad libitum. Research protocols used on the experimental animals were approved by Indian Animal Ethical Committee, CPCSEA and followed the guidelines. The dorsal skin of the mice was shaven with an electric clipper (Oster A2, USA) followed by the application of hair removing cream (Anne French, Geoffery Manners, Bombay, India) at least two days before treatment.

2.3. Experimental protocol for biochemical estimations

Effect of pretreatment with POH on TPA-mediated cutaneous oxidative stress was studied by random allocation of mice to five groups of six mice each. Each mouse of group 1 received topical application of 200 μl vehicle (acetone alone). The mice of group 3 received topical applications of POH (6 mg/kg b. wt. in 200 μl acetone/mice), and those on groups 4 and 5 received POH (12 mg/kg b. wt. in 200 μl acetone/mice). Thirty minutes after these treatments, the animals of groups 2–4 received topical applications of TPA (2 μg/200 μl acetone/animal). Mice of group 2 were treated with TPA alone served as a positive control. The treatments were carried out for three days. Mice were sacrificed by cervical dislocation after 12 h the last TPA treatment. Skin of each mouse was excised and processed for evaluation of edema, histopathology and biochemical studies.

To study the effect of pretreatment of POH on TPA-induced cutaneous ODC activity, 30 mice were randomly allocated to five groups of 6 mice each. Treatment schedule of the animals in this study also, was exactly the same as in case of oxidative stress study except that animals of all the groups were sacrificed after 6 h of TPA treatment as opposed to 12 h and were processed for cytosolic preparation. For cutaneous [^{3}H] thymidine incorporation study also, the treatment protocol was exactly the same as described above. The only difference being those 18 h after the treatment with TPA/acetone, the animals of all the groups were injected intraperitoneally with [^{3}H] thymidine (20 μCi/animal/200 μl saline) and were sacrificed by cervical dislocation 2 h after [^{3}H] thymidine treatment.

2.4. Evaluation of edema and hyperplasia

The evaluation of skin edema was performed using method of Afaq et al. [19]. Briefly, the skin punch of 1 cm diameter was collected and dried for 24 h at 50 °C. The skin punch was reweighed and loss of water content was determined. The degree of intracellular edema was determined by using the difference in water gain between control and TPA-treated and POH/TPA-treated.

For hyperplasia study, skin was removed, fixed in 10% buffered formalin and embedded in paraffin. Five micrometer sections were cut and stained with H&E and observed under the microscope (Motic Digital Microscope). The thickness of the epidermis was measured at least in six different regions.

2.5. Biochemical assays

GSH was determined by the method of Jollow et al. [23]. GR and GPX activities were measured by the method of Mohandas et al. [24]. GST activity was estimated by the method of Habig et al. [25]. CAT activity was measured by the method of Claiborne [26].

2.6. Lipid peroxidation (LPO)

The assay of LPO was done following the method of Wright et al. [27]. The amount of malonaldehyde (MDA) formed in each of the samples was assessed by measuring the absorbance at 535 nm against a reagent blank. The results were expressed as nmol MDA formed/h per g tissue at 37 °C using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.7. Ornithine decarboxylase activity

ODC activity was assayed by the method of Verma et al. [28]. In brief, the reaction mixture contained 400 μl of the enzyme solution to be assayed and 95 μl of co-factor mixture containing 0.32 mM pyridoxal phosphate, 0.4 mM EDTA, 4.0 mM dithiothreitol, 0.4 mM ornithine, 0.02% Brij-35 and 0.05 μCi DL [^{14}C] ornithine in