

# Effects of naturally occurring plant phenols on production of reactive oxygen species and DNA damage induced by tumour promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) in mouse epidermis

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## Summary

Naturally occurring phenolics: protocatechuic, chlorogenic, tannic acids and trihydroxystilbene and resveratrol were shown to inhibit multistage carcinogenesis in animal models, including mouse epidermis. Treatment of mouse skin with tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) may induce ROS production in keratinocytes and skin infiltrating leukocytes. In our study, the effect of these phenolics on intracellular ROS production and DNA damage was examined. ROS were analysed by flow cytometry, while DNA damage by comet assay. Following treatment with TPA (6.8 nmol/mouse) two subpopulations of epidermal cells were identified. Pretreatment of mice with 16 µmol of phenolics decreased ROS production in both subpopulations. The most efficient inhibitors of ROS in whole population of keratinocytes were chlorogenic acid and resveratrol. Tannic acid reduced the most DNA damage induced by TPA treatment. These results suggest that anti-promotional effects of these two plant phenols might be partially explained by the inhibition of TPA-induced inflammation.

**Key words:** *plant phenols, TPA, mouse epidermal cells, tumourigenesis*

## INTRODUCTION

Cellular pro-oxidant state plays an important role at critical steps of skin carcinogenesis including promotion stage. Tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), increase the generation of reactive oxygen species (ROS). Several molecular events linked to tumor promotion may be mediated through these oxygen-centered free radicals. ROS may be formed in the TPA-treated epidermal cells themselves and/or released by the activated phagocytic cells infiltrating the skin during inflammation [1]. *In vitro* studies provided evidence for TPA-induced ROS generation and DNA damage. Using epidermal cells derived from BALB/c mice, Hartley et al. [2] demonstrated that TPA induces a dose-dependent increase in formation of single stranded DNA breaks. TPA increased also H<sub>2</sub>O<sub>2</sub> production in mouse epidermis treated *in vivo*, and in primary cultures of newborn mouse keratinocytes treated *in vitro*. In addition to ROS production, TPA also increased formation of oxidized DNA bases as demonstrated in HeLa [3] and in HL60 cells [4]. However, the above mentioned reports demonstrated that TPA induces DNA damage in epidermal cells and led to the generation of ROS, it is not known whether these properties were mediated through an *in vivo* immune response or whether TPA can directly damage DNA through ROS production within keratinocytes themselves.

Studies on the generation of ROS in pure populations of initiated mouse epidermal keratinocytes in culture indicated that TPA did not directly induce 8-hydroxydeoxyguanosine formation in DNA of these cells, although ROS generation was confirmed by luminol-dependent chemiluminescence [5]. Our earlier studies showed that naturally occurring plant phenols protocatechuic, chlorogenic and tannic acids as well as stilbene derivative, resveratrol, significantly reduced the TPA-induced chemiluminescence in human neutrophils *in vitro*, showing antioxidant efficacy. These compounds, which are common ingredients of herbs and edible fruits, were also potent inhibitors of the formation of adducts of DNA and polycyclic hydrocarbon, 7,2-dimethylbenz[a]anthracene (DMBA), which may be important for the anticarcinogenic activity of the tested phytochemicals [6].

The aim of present study was to evaluate the ability of three structurally diversified natural phenolic acids - simple phenolic acid – protocatechuic acid, derivative of hydroxycinnamic acid – chlorogenic acid, ester of digallic acid with glucose – tannic acid, and trihydroxystilbene – resveratrol, to modulate the intracellular ROS production and oxidative damage to DNA induced by TPA in mouse keratinocytes *in vivo*.

## MATERIALS AND METHODS

### Chemicals

Tannic acid (purity >97%), protocatechuic acid (purity >97%), chlorogenic acid (purity >95%) and resveratrol (purity >99%) were purchased from Sigma Chemical (St. Louis, MO, USA).

Other reagents of the highest available grade were obtained from common chemical suppliers.

## Animals and treatment

Female Swiss mice seven to nine weeks old were shaved on the dorsal side using surgical clippers two days before the treatment. Phenolic compounds were applied topically to the shaved dorsal skin at the dose of 16  $\mu\text{mole}/\text{mouse}$  15 minutes before a single application of 6.8  $\text{nmole}/\text{mouse}$  of TPA in acetone. A control group of mice was treated with acetone alone. Three mice were used for each experimental group. Twenty four hours after TPA treatment animals were sacrificed. All experiments were approved by Regional Ethics Committee's guidelines for animal experimentation, consent No. 33/2007.

## Epidermal cells separation

Epidermal cells were isolated by a modified trypsinization procedure as described previously [7]. Briefly, dorsal skins of mice were removed and spread out, hair side down on Petri dish. Connective tissue was removed using surgical scalpel and dried skins cut into small pieces were transferred to another Petri dish filled with 0.25% trypsin in PBS and incubated for 30 min in 37°C and up to 1.5 hours at a room temperature. The upper layer of skin pieces was scraped into Eagle's culture medium containing 10% of FCS. Isolated cells were stirred slowly for 30 min using magnetic stirrer, filtered through nylon mesh and centrifuged at 1000 rpm for 10 min at a room temperature and resuspended in 5 mM glucose in PBS. The viability of cells was evaluated with Trypan Blue solution used also for cells counting.

## Flow cytometric analysis of intracellular ROS production

The intracellular ROS production by epidermal cells was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA) [8]. DCFH-DA is a nonpolar compound that rapidly diffuses into cells where it is hydrolyzed to non-fluorescent polar derivative, 2',7'-dichlorofluorescein (DCFH), and trapped within the cells. In the presence of intracellular hydroperoxides and peroxidases, DCFH is oxidized to highly fluorescent compound 2',7'-dichlorofluorescein (DCF). Thus, cellular fluorescence intensity is directly related to levels of intracellular ROS. Cells ( $1 \times 10^6$ ) were incubated with 15  $\mu\text{M}$  DCFH-DA for 30 min. Intracellular DCF fluorescence of epidermal cells was determined by flow cytometry (Cytoron Absolute, Orto, USA) at 488 nm excitation wavelength. The DCF fluorescence was measured with green filter (515–548 nm) in the range of signal linear amplification. The fluorescence intensity was expressed as the value of the "mean channel", calculated by ImmunoCount 2 software (Ortho).

## Comet assay

Comet assay in alkaline conditions was performed according to Hartmann et al. [9] with minor modifications: clean glass microscopic slides were dip in 1% water solution of regular agarose and left horizontally to dry in air. A portion of 40  $\mu\text{l}$  of this agarose solution was placed on the precoated slides covered with a 16x16 mm coverglass and left to harden on the ice-cold plate, then coverglasses were removed. Low melting point (LMP) agarose (1% in PBS) was warmed and kept in 37°C in water bath. Cells (approx. 40 000 in 10  $\mu\text{l}$ ) were mixed with 30  $\mu\text{l}$  of warm LMP agarose and placed on the solidified layer of the regular agarose, covered with a coverglass and left on the ice-cold plate to solidify. Then, coverglasses were carefully removed and slides were immersed in the ice-cold lysis buffer (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris-base, pH 8.0, with 10% DMSO and 1% Triton X-100 added prior to use) for 1 h in +4°C in the dark. Then the slides were carefully drained from the excess of lysis buffer and immersed in the electrophoresis buffer (0.3 M NaOH, 0.001 M EDTA, pH 13) for 40 minutes in +4°C (the DNA unwinding phase). Electrophoresis was carried out in the above mentioned buffer, (30 minutes, 300 mA, 0.7-1.0 V/cm) on ice. Then slides were removed from the tank and rinsed (3x5 minutes) in the ice-cold neutralizing buffer (0.4 M Tris, pH 7.4), placed in the ice-cold ethanol (96%) for dehydration and left on air to dry. Before the assessment in a fluorescence microscope, slides were rehydrated under coverglasses and stained with water solution of ethidium bromide (0.05 mg/ml, 10  $\mu\text{l}$  per agarose field). The “comets” were divided into 5 groups according to the degree of DNA damage and the results were expressed as a total damage score for the sample [10].

## Statistical analysis

Differences between the means of treatment were compared after analysis of variance (ANOVA) by the t-Student test, with  $p < 0.05$ .

## RESULTS

### Effect of TPA and plant phenols on DCFH oxidation in murine epidermal cells

In order to characterize the effects of TPA on mouse epidermis, single cell suspensions of epidermal cells were analysed using laser light scatter properties (fig. 1). Analysis of cell size (right scatter) and the “green fluorescence” (DCF-fluorescence) (forward scatter) revealed two subpopulations of epidermal cells isolated from acetone and TPA-treated mice. Cells were isolated 24 h following

treatment of mice with TPA (6.8 nmol/mouse) and differed in ~17% higher level of oxidized 2',7'-dichlorofluorescein than cells treated with acetone alone (fig. 2). Moreover, the ROS production was higher in subpopulation 2 of epidermal cells in comparison with control cells (~33% increase) than in subpopulation 1.

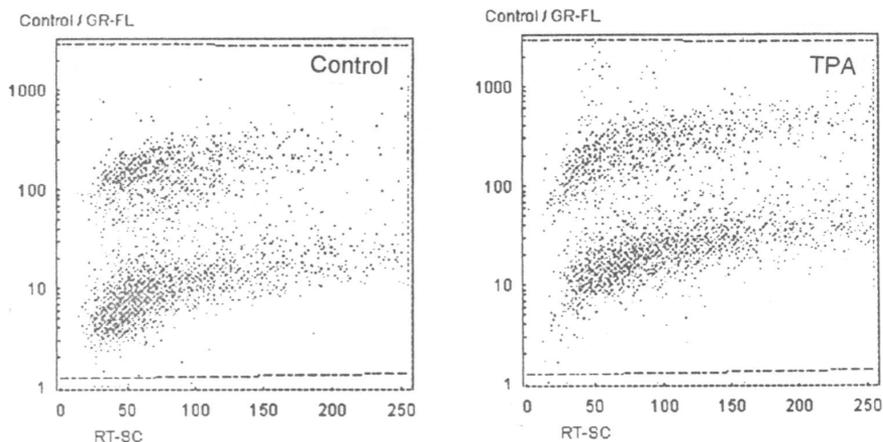


Figure 1.

Flow cytometric graphs presenting the “green fluorescence” (DCF-fluorescence) of mouse epidermal keratinocytes following treatment with acetone or 6.8 nmol TPA. Two subpopulations of keratinocytes were present both in acetone-treated and TPA-treated epidermis

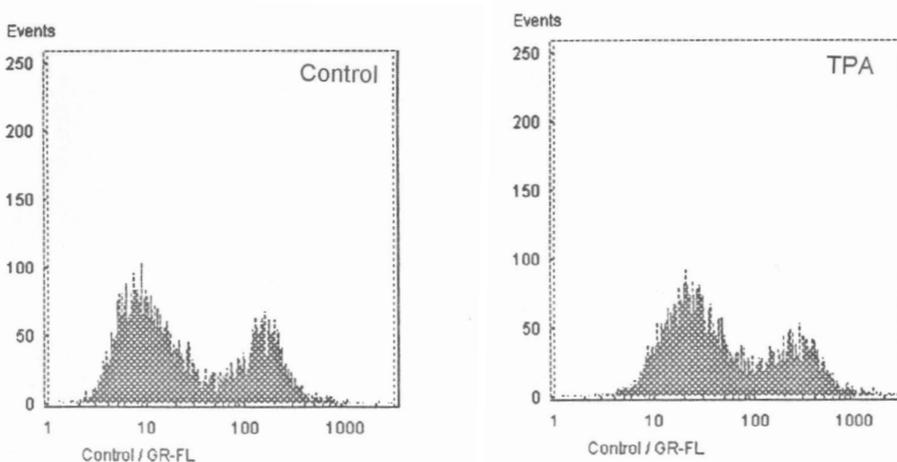


Figure 2.

Flow cytometric histograms presenting the fluorescence of untreated and TPA-treated mouse epidermal keratinocytes

Pretreatment of mice with 16  $\mu\text{mol}$  of phenolics decreased the intracellular ROS production in whole epidermal cells (tab. 1), although, the observed differences were more pronounced in subpopulation 2 of keratinocytes. The most efficient inhibitors of ROS in this subpopulation were chlorogenic acid, which decreased the ROS level by 36% in comparison with TPA treated cells and protocatechuic acid (~22% decrease). However, the statistical significance of these data ranged only from  $p < 0.10$  to  $p < 0.20$ .

**Table 1.**

Effect of plant phenolics on ROS production in mouse keratinocytes following treatment with TPA

	Gate A (DCF fluorescence of all cells)	Gate B (upper – Subpopulation 1)	Gate C (low – Subpopulation 2)
Control	108.0 $\pm$ 5.1	148.3 $\pm$ 0.9	71.8 $\pm$ 7.2
TPA	126.6 $\pm$ 5.5 <sup>a</sup> (100%)	167.1 $\pm$ 9.1 (100%)	95.2 $\pm$ 9.8 (100%)
+ Protocatechuic acid	111.8 $\pm$ 2.9 <sup>b</sup> (88.3%)	150.0 $\pm$ 27.3 (89.8%)	75.0 $\pm$ 15.5 (78.8%)
+ Chlorogenic acid	110.7 $\pm$ 5.2 <sup>b</sup> (87.4%)	155.3 $\pm$ 24.5 (92.9%)	61.7 $\pm$ 14.9 (64.8%)
+ Tannic acid	113.7 $\pm$ 11.6 (89.8%)	141.0 $\pm$ 22.6 (84.4%)	87.3 $\pm$ 2.7 (91.7%)
+ Resveratrol	109.3 $\pm$ 5.0 <sup>b</sup> (86.3%)	143.3 $\pm$ 14.2 (85.7%)	88.3 $\pm$ 13.9 (92.7%)

Data are expressed as mean channel number of linear intensity of green fluorescence DCF.

Values are means  $\pm$ SEM of 9 independent experiments. The percentage of respective control is shown in parentheses. Significantly different from solvent treated control: <sup>a</sup>  $p < 0.05$ . Significantly different from TPA-treated control: <sup>b</sup>  $p < 0.05$ , paired t-test.

## Comet assay

The results of comet assay are presented in figure 3. The TPA treatment significantly increased DNA damage in comparison to the vehicle treated control cells (by 77%). Tested plant phenols displayed diverse protective potential: tannic acid was the most powerful and resveratrol the least powerful DNA damage preventive agent (50% and 71%, respectively, in comparison to values received in TPA-only treated mice).

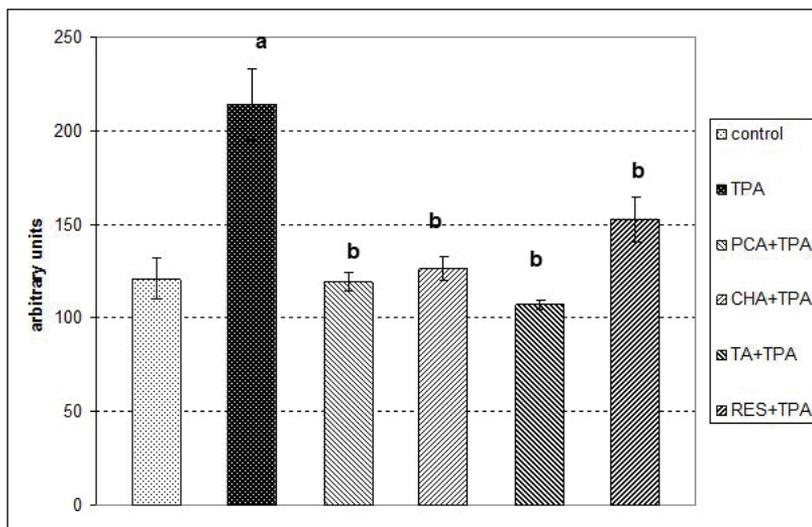


Figure 3.

DNA damage in keratinocytes isolated from epidermis of mice treated with TPA only or plant phenolics and TPA. Results are means  $\pm$  SEM from at least three independent measurements.

PCA – protocatechuic acid, CHA – chlorogenic acid, TA – tannic acid, RES – resveratrol

<sup>a</sup> significantly different from control,  $p < 0.05$ ; <sup>b</sup> significantly different from TPA-treated animals,  $p < 0.05$

## DISCUSSION

Reactive oxygen species (ROS) are continuously generated from the mitochondrial respiratory chain and other reactions of normal metabolism and are of powerful oxidative potential. ROS are capable to attack lipids, nuclear acids and proteins, resulting in oxidative damage to cells [11]. The keratinocytes ability to produce ROS may be one of their normal function as the principal cell involved in barrier functions of the host. Since the skin is continually challenged with infectious agents, the production of ROS by keratinocytes may play a role in host defense mechanisms. Keratinocytes have been shown to be phagocytic, and the observed oxidative metabolism may be related to this functional activity, as it is observed in leukocytes. Keratinocytes contain enzymes both for the generation and the detoxification of ROS. A number of investigators has reported that the normal balance between the production and detoxification of ROS is dramatically altered in epidermal cells following TPA treatment [12].

Our observations demonstrate that epidermal cells produce ROS and the level of their production is enhanced following TPA exposure. These observations suggest that keratinocytes may function as effector cells that produce ROS as well as

act as target cells for ROS-induced damage during the process of tumor promotion. Moreover, application of the fluorescent dyes in conjunction with flow cytometry allowed us to isolate two cellular subpopulations. Recently, Carr et al. [13], using a similar approach isolated cellular subpopulation called side population with epidermal stem cell-like characteristics (EpSPs) characterized by lower ROS and antioxidant protein levels. Moreover, these cells were more resistant to UV radiation than their progeny. Thus, we can speculate that subpopulation 2 in our study contain EpSPs, which, in contrast to UV, is more sensitive to TPA action resulting in increase of ROS production. Interestingly, in our early studies using Percoll density gradient isolation we showed that basal keratinocytes (stem cells alike) were also more sensitive to initiating dose of carcinogens, DMBA and benzo(a)pyrene (B[a]P) [7]. This may suggest that these cells are also prone to TPA promoter action. In this context the reduced level of ROS in subpopulation 2 by protocatechuic and chlorogenic acids might contribute to their anti-promotional and anti-inflammatory activities. The pro-oxidant and inflammatory conditions induce the expression of protooncogenes *c-fos* and *c-jun*. In this regard our earlier studies have shown that both protocatechuic acid and chlorogenic acid as well as tannic acid and resveratrol reduced the level and binding to specific DNA sequence of c-Jun and c-Fos protein [14, 15]. In the case of tannic acid and resveratrol this effect was most pronounced for c-Jun. Thus it is possible that inhibitory action of these compounds on ROS production is related to inhibition of protooncogenes expression or activity. Such a mechanism was also proposed for the known anti-inflammatory phytochemical, curcumin [16]. The inhibition of ROS production in whole epidermal cells population correlated with reduced DNA damage assessed with Single Cell Electrophoresis Assay (Comet assay). Tannic acid was the most efficient inhibitor of "comets" formation, while resveratrol the least potent. The lack of correlation with the inhibition of ROS in cell subpopulation 2 may indicate that more specific assay like formation of 5-hydroxymethyl-2'-deoxyuridine or 8OHdG induced by TPA should be applied in order to evaluate oxidative damage. On the other hand these results correlate with the effect of these compounds on B[a]P-DNA adducts profile observed in our earlier studies [6, 17, 18].

## CONCLUSIONS

These results suggest that anti-promotional and anti-inflammatory effects of structurally diverse plant phenols tested in this study might be partly explained by inhibition of TPA induced ROS production and DNA damage. The further studies are required in order to explain the detailed mechanism of this activity particularly in specific epidermal cell subpopulations.

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## WPŁYW FENOLI ROŚLINNYCH NA PRODUKCJĘ REAKTYWNYCH FORM TLENU I OKSYDACYJNYCH USZKODZEŃ DNA INDUKOWANYCH ESTREM FORBOLU (TPA) W NASKÓRKU MYSZY.

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### Streszczenie

Kwasy fenolowe: protokatechowy, chlorogenowy i taninowy, a także pochodna stilbenu, rezweratrol, to występujące powszechnie składniki wielu ziół i jadalnych owoców. Wykazano, że związki te mogą hamować proces nowotworzenia w modelach doświadczalnych, w tym naskórku myszy. Nakładanie na skórę promotera, estru forbolu, TPA, prowadzi do produkcji reaktywnych form tlenu (RFT) w keratynocytach, a także infiltrujących naskórek leukocytach. Celem badań była ocena oddziaływania fenoli roślinnych na produkcję RFT i uszkodzenia DNA w komórkach naskórka myszy poddanych działaniu jednorazowej dawki TPA (6,8 nmola) *in vivo*. Do oceny RFT wykorzystano technikę fluorymetrii przepływowej, a poziom uszkodzeń DNA oceniano metodą elektroforezy kometkowej.

Wszystkie badane związki obniżały poziom wewnątrzkomórkowych RFT w porównaniu z grupą traktowaną TPA. Najbardziej skutecznymi inhibitorami RFT w całkowitej populacji keratynocytów były kwas chlorogenowy i rezweratrol. Kwas taninowy w największym stopniu zapobiegał uszkodzeniom DNA. Dane te wskazują, że inhibicja RFT indukowanych TPA może przyczyniać się do antypromocyjnego działania badanych związków.

**Słowa kluczowe:** fenole roślinne, TPA, komórki naskórka myszy, tumorogeneza