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主持人: 王朝鐘　中山醫學院生化研究所

一、中文摘要

原兒茶酸(protocatechuic acid, PCA)是一簡單多酚類化合物，經由乾燥的洛神花萃取而來。CD-1 老鼠局部塗抹 5 nmol TPA，一天塗抹 2 次，連續塗抹 5 天後取上皮組織以 H.E. stain 在顯微鏡下觀察發現老鼠背部上皮有增生的現象。此外另以免疫組織化學染色法分析上皮組織發現上皮細胞層脂質過氧化及訊息傳遞蛋白增加，包括 c-Fos, Phosphotyrosine, MEK1, ERK1, ERK2 及 PKC。比起未塗抹者分別增加 2.39, 1.19, 2.34, 2.26, 2.47 及 2.45 倍。而 PCA 的預先塗抹不但抑制了 TPA 引起的增生，而且脂質過氧化現象也明顯的減少。TPA 刺激引起 c-Fos, MEK1, ERK1, ERK2 及 PKC 蛋白的表現，受到 5 μmol PCA 的抑制，抑制百分比分別是 25, 33, 16, 49 和 16%，而提高劑量至 20 μmol 則顯示更明顯的抑制，分別爲 68, 66, 64, 65 和 68%。此外以西方墨點吸漬法測定蛋白之表現，結果發現預先處理 5 μmol PCA 會引起 c-Fos, c-Jun, c-Myc, phosphotyrosine, c-Raf, MEK1, ERK1, ERK2 和 PKC 蛋白的表現受到抑制，其抑制百分比分別爲 74%, 4%, 24%, 32%, 62%, 55%, 5%, -16%, 30%。而高劑量 20 μmol PCA 則有更明顯的抑制，其抑制百分比分別爲 96%, 71%, 47%, 73%, 91%, 91%, 27%, 42%, 83%。綜合這些結果及先前的研究顯示阻斷 TPA 引起訊息傳遞蛋白的表現，是 PCA 抑制 TPA 引起腫瘤促進作用的可能機轉。

Abstract

Hibiscus protocatechuic acid (PCA), a phenolic acid isolated from Hibiscus sabdariffa L., was a chemopreventive agent. In our earlier study shown that PCA has a inhibitory effect on the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced skin tumorgenesis. Topical application of 5 nmol TPA to the dorsal surface of CD-1 mice twice daily for 5 days caused epidermal hyperplasia, lipid peroxidation and the enhancement of the protein levels of the signaling proteins, such as c-Fos, MEK, ERK1, ERK2 and PKC were 2.39-, 2.34-, 2.26-, 2.47- and 2.45-fold as compared to control on TPA-treated mice epidermis. Topical application of PCA briefly prior to the application of TPA not only inhibited the TPA-induced hyperplasia but also lipid peroxidation was markedly reduced. The TPA-induced expression of c-Fos, MEK1, ERK1, ERK1 and PKC were also significantly inhibited by PCA (5 μmol) to the extent of 25, 33, 16, 49 and 16 % respectively. Higher dose of PCA (20 μmol) showed even more marked inhibition on the TPA-induced expressions of these proteins described above, by 68, 66, 64, 65 and 68 %, respectively, in mice epidermis. In the other side, Western blotting was used to determine the inhibitory effect of

signaling protein on PCA-pretreated mice. The results also showed that PCA could inhibit the expression of TPA-induced signaling protein. These data and our previous results indicated that the TPA-induced enhancement of the expression role in suppression of TPA-induced tumor promotions.

二、緣由與目的

原兒茶酸 (protocatechuic acid, PCA)是一簡單多酚類化合物，經由乾燥的洛神花萃取而來。洛神花茶爲夏季經常被使用的飲品，具有解熱、降血壓、利尿等功能。而近年來 PCA 的抗氧化特性已被研究證實 (1, 2)。一般而言，酚類化合物經常同時具有抗氧化性及抗癌性 (3)，因此 PCA 的抗癌特性更是科學家們所感興趣的。近來有學者指出 PCA 爲一種 chemopreventive agent 並且在動物實驗中被證實確有抑制化學致癌的能力 (4-6)，包括由 7, 12-dimethylbenz[a]-anthracene (DMBA) 所引起的 buccal pouch carcinogenesis, N-butyl-N-(4-hydrobutyl)-nitrosamine (BBN)所誘發的 bladder carcinogenesis, 4-nitroquinoline 1-oxide 引發的 oral carcinogenesis, N-methyl-N-nitrosourea 誘導的 glandular stomach carcinogenesis, Azoxymethane 導致的 colon aberrant crypt foci 及 diethylnitrosamine 引起的 hepatocarcinogenesis 均具有抑制作用 (7-10)。其主要預防機轉仍不甚清楚，可能是在抑制癌症發展階段的 initiation 與 postinitiation 之步驟中，並且可能和抗氧化及抑制細胞生長有關。

最近我們已有發表論文指出 PCA 可以抑制 TPA 所引起的小白鼠皮膚癌促進作用，並且證實 PCA 可以抑制由 TPA 所刺激引起的 inflammation, hyperplasia 及 ornithine decarboxylase (ODC) activity，至於 PCA 的抑制機轉則至今未見報告。在早先的報告中指出 PKC activity 可在 TPA 的刺激下而增強並進行下游訊息的調節，包括活化 mitogen-activated protein kinases (MAPKs)，活化後之 MAPKs 會藉由其磷酸化而調節基因表現的相關蛋白激媒，需要 TPA 刺激而表現之 transcriptional gene 包括 c-jun 及 c-fos，此基因係經由 TPA 刺激 PKC 活化及 MAPKs 之活化而被激活(12-20)。

本計劃之主要目的在於探討 PCA 抑制 TPA 引起之老鼠上皮細胞的癌促進作用的機轉，而在我們的結果中顯示 PCA 的抑制作用乃在於經由抑制 PKC 所引起的 MAPKs 活化作用，並經由此抑制作用而抑制了位於細胞核的原致癌基因，藉此使得 TPA 的促癌作用被抑制。

三、結果與討論

在 hyperplasia 實驗中利用 5 nmol TPA 引起老鼠背部上皮輕度形態改變，另以不同濃度之 PCA (5 μmol, 10 μmol, 20 μmol)做前處理，塗抹於 CD-1 mice 皮膚，每天 2 次，連續 5 天後取下其背部皮膚，用冷凍切片法取下 15 μm 厚的上皮，以 reactive aldehyde 的表現作爲脂質過氧化作用的標誌分別觀察。利用 TPA 引起脂質過氧化作用所產生的 aldehyde 和 Schiff reagent 當中的 fushin 反應的原理證實有無脂質過氧化反應，其它各組未經 TPA 處理或是預先處理不同濃度 PCA 的組別均沒有脂質過氧化反應的表現 (Table.1 及 Fig.1)。此結果顯示 PCA 具有抑制脂質過氧化作用的特性。

分別以 TPA 及不同濃度 PCA 處理

在老鼠上皮，每天2次，連續五天後取下上皮之蛋白質以Western Blot的方法偵測在 PCA 對 TPA 所引起之 MAPK pathway 活化之抑制作用，分別測定的是 c-Raf, MEK-1, ERK-1, ERK-2, TPA 在 c-Raf, MEK-1, ERK-1, ERK-2 的 induction 倍數分別爲 45, 3.8, 2 及 10.38 倍，在預先處理 PCA 的組別中，依三種不同濃度分別對 c-Raf, MEK-1, ERK-1, ERK-2 產生顯著的抑制作用，其抑制百分比(與 solvent control 比較)在 c-Raf 分別爲 62%, 75%, 91%；在 MEK-1 爲 55%, 80%, 91%；在 ERK-1 爲 5%, 20%, 27%；在 ERK-2 爲-16%, 4%, 42% (Fig.2)。

在 protooncogenes 表現上，則同樣以 Western Blot 來觀察蛋白表現的量，包括 c-Fos, c-Jun 及 c-Myc 的表現，TPA 在 c-Fos, c-Jun 及 c-Myc 的 induction 倍數(與 solvent control 比較)分別爲 5.56, 1.15 及 3.78 倍，在預先處理 PCA 的組別中，依三種不同濃度分別對 c-Fos, c-Jun 及 c-Myc 產生抑制作用，其抑制百分比(與 solvent control 比較) 在 c-Fos 分別爲 74%, 94%, 96%；在 c-Jun 爲 4%, 23%, 71%；在 c-Myc 爲 24%, 38%, 47% (Fig.3)。

由於在 TPA 處理過的皮膚上會有 PKC 被誘發而表現的現象，因此我們仍以上述之方法來測定 PKC 的表現，另外在磷酸化酪氨酸蛋白質 (phosphotyrosine protein, Py) 的測定，則可以藉此觀察 PCA 對 TPA 所引起的蛋白質磷酸化的抑制情形，在 TPA 處理的組別中對 PKC-α 及 Py 的 induction 倍數分別爲 1.4 及 1.3 倍，而在預先處理 PCA 的組別中，依三種不同濃度分別對 PKC-α 及 Py 產生抑制作用，其抑制倍數(與 solvent control 比較) 在 PKC-α 分別爲 30%, 72%, 83%；在 Py 爲 32%, 35%, 73% (Fig.4)。

由此結果可以得知在老鼠上皮給予 TPA 後會使得過氧化物急速增加，此種增加則誘使蛋白質進行磷酸化而啓動 MAP kinases cascade 進行訊息之傳遞，最後活化細胞核內之 protooncogenes 而引發癌化的進行。PCA 在此過程中應是因爲抑制了過氧化物之產生進而使下游訊息傳遞受到抑制而達成保護細胞免於癌化的危機。

四、計劃成果自評

本計劃之目標爲找出 PCA 抑癌作用之機轉，研究成果中顯示 PCA 可能係經由抑制了過氧化物引發之訊息傳遞，使得下游之 protooncogenes 免於被誘發，藉此保護個體不會進行癌化的過程。

在本計劃中所探討之途徑僅爲 PCA 抑癌之可能途徑之一，應有其它相關途徑也可能包含於其中，值得進一步探討，另外在 PCA 與 DNA 之直接作用關係上亦爲一相當有趣的課題，值得再做相關的研究。

五、參考文獻

1. Tseng, T.H., Wang, C.J., Kao, E.S. and Chu, C.Y. (1996) Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoside in rat primary hepatocytes. Chem.-Biol. Interact., 101, 137-148.
2. Ohkawa, Y., Tanizawa, H., Takino, Y., Miyase, T., Ueno, A., Kageyama, T., Shikanl, E. (1991) Studies on the antioxidative activity in citrus fruit II, in proceedings of 111th Annual Meeting of Japanese

Pharmacological Society (Abstract), P.174.

3. Huang, M.T. and Ferraro, T. (1992) Phenolic compounds in food and cancer prevention. in: Huang, M.T., Ho, C.T. and Lee, C.Y. (Eds), Phenolic compounds in Food and Health II: antioxidants and Cancer Prevention, American Chemical Society, Washington, DC., pp. 8-34.
4. Tanaka, T., Kojima, T., Kawamori, T., Yoshimi, N. and Mori, H. (1993) Chemoprevention of diethylnitrosamine-induced hepatocarcinogenesis by a simple phenolic acid, protocatechuic acid in rats. Cancer Res., 53, 2775-2779.
5. Tanaka, T., Kawamori, T., Ohnishi, M., Okamoto, K. and Mori, H. and Hara, A. (1994) Chemoprevention of 4-nitroquinoline-1-oxide-induced oral carcinogenesis by dietary protocatechuic acid during initiation and post-initiation phase. Cancer Res., 54, 2359-2365.
6. Kawanori, T., Tanaka, T., Kojima, T., Suzui, M., Ohnishi, M. and Mori, H. (1994) Suppression of azoxymethane-induced rat xolon aberrant crypt foci by dietary protocatechuic acid. JPN. J. Cancer Res., 54, 2359-2365.
7. Tanaka, T., Kojima, T., Kawanori, T. and Mori, H. (1995) Chemoprevention of digestive organs carcinogenesis by natural product protocatecuic acid. Cancer, 75 (Suppl.), 1433-1439.
8. Hirose, Y., Tanaka, T., Kawamori, T., Ohnishi, M., Makita, H., Mori, H., Satoh, K. and Hara, A. (1995) Chemoprevention of urinary bladder carcinogenesis by the natural phenolic compound protocatechuic acid in rats, Carcinogenesis, 16, 2337-2342.
9. Tseng, T.H., Hsu, J.D., Lo, M.H., Chu, C.Y., Chou, F.P., Huang, C.L. and Wang, C.J. (1998) Inhibitory effect of Hibiscus protocatechuic acid on tumor promotion in mouse skin. Cancer Lett., 126, 199-207.
10. Verma, A.K., Pong, R.C. and Erickson, D. (1986) Involvement of protien kinase C in ornithine decarboxylase gene expression in primary culture of newborn mouse epidermal cells and in skin tumor promotion by TPA. Cancer Res., 46, 6149-6155.
11. Tseng, C.P., Kim, Y.J., Kumar, R. and Verma, A.K. (1994) Involvement of protein kinase C in the transcriptional regulation of TPA-inducible genes modulated by AP-1 or non AP-1 transacting factors. Carcinogenesis, 15, 707-711.
12. Cooper, G.M.: Oncogenes in mitogenic signal transduction pathway. In Oncogenes (Cooper G.M. ed). Boston, Jones and Bartjott publishers, 1990, pp 255-276.
13. Filvaroff, E., Stern, D.F. and Dotto, G.P. (1990) Tyrosine phosphorylation is an early and specific event involved in primary keratinocyte differentiation. Mol. Cell Biol., 10, 1164-1173.
14. Thomas, S.M., Demarco, M., Da'Arcangelo, G., Halegoua, S. and Brugge, J.S. (1992) Ras is essential for nerve growth factor and TPA-induced tyrosine phosphorylation of MAP kinases. Cell, 48, 525-534.

15. Boulton, T.G., Nye, S.H., Robbins, D.J., IP, N.Y., Radziejewska, E. and Yancopoulos, S.D. (1991) ERKs: a family of protein serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell, 65, 663-675.
16. Davis, R.J. (1994) MAPKs: new JNK expands the group. Trend Biochem, Sci., 19, 470-473.
17. Bernstein, I.R. and Colburn, N.H. (1989) AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. Science, 244, 566-569.
18. Dong, Z., Birret, M.J., Watts, R.G., Matrisian, L.M. and Colburn, N.H. (1994) Blocking of tumor promoter induced AP-1 activity inhibits induced transformaiton in JB6 mouse epidermal cells. Proc. Natl. Acad. Sci., 91, 609-613.
19. Sakai, M., Swart, G. and Monaci, P. (1989) Structure and expression of the rat c-jun messenger RNA: tissue distribution and increase during chemical hepatocarcinogenesis. Cancer Res., 49, 5633-5637.
20. Frain, M., Swart, G. and Monaci, P. (1989) The liver-specific transcription factor LF-B1 contains a highly diverged homobox DNA binding domain. Cell, 59, 145-147.

Table 1 Inhibitory effect of topical application of PCA on TPA-induced hyperplasia and inflammation

Treatment[a]	No. of layers	Epidermal thickness (μm)[b]	Leukocyte infiltration[c]
Acetone	1-2	12.5 ± 0	+
TPA	5-8	53.6 ± 3.9	++++
PCA	1-2	12.3 ± 0	+
TPA *plus*			
PCA (5μmol)	5-6	50.1 ± 2.3	+++
PCA (10μmol)	3-4	41.1 ± 2.3*	+
PCA (20μmol)	2-3	32.1 ± 2.3**	+

[a] Female CD-1 mice were treated with 200 μl acetone, TPA (5 nmol) in 200 μl acetone or TPA together with PCA in 200 μl acetone twice a day for 5 days. The aminals were killed 18 h after the test dose and skins were processed for histological tests.

[b] Mean ± SD, n = 6; * $P < 0.01$, ** $P < 0.005$; compared with the TPA-treated group.

[c] Leukocyte infiltration that was slight (+), mild (++), moderate (+++) or severe (++++) was characterized by diffuse infiltration of mononuclear infiltratory cells in the dermis when compared with the acetone controls.

Table 2 Inhibitory effect of PCA on the formation of H_2O_2 and activity of MPO in mouse skin treated with TPA

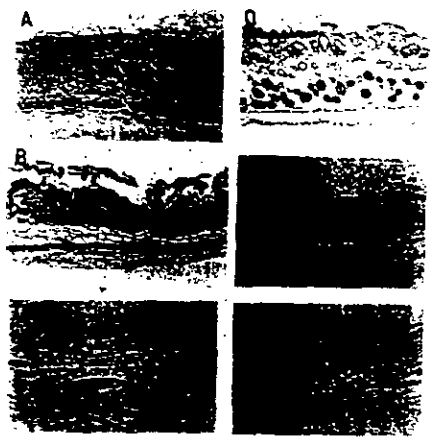
Treatment[a]	H_2O_2/nmol/cm^2[b]	MPO/unit/cm^2	Lipid peroxidation[c]
Acetone	3.48 ± 1.52	14.11 ± 0.15	--
TPA	18.72 ± 2.41	144.71 ± 9.42	+++
PCA	3.84 ± 1.81	15.20 ± 1.15	--
TPA *plus*			
PCA (5μmol)	9.14 ± 1.74*	115.45 ± 8.16*	-
PCA (10μmol)	6.11 ± 2.11**	87.64 ± 7.39**	-
PCA (20μmol)	5.81 ± 2.15**	53.76 ± 5.88***	--

[a] Female CD-1 mice were treated with 200 ml acetone, TPA (0.5 nmol) in 200 ml acetone or TPA together with PCA in 200 ml acetone twice with an interval 20 h. The mice sacrificed 1 h after the second TPA application and the skin was removed.

[b] Mean ± SD, n = 6; * $P < 0.01$, ** $P < 0.005$, *** $P < 0.001$; compared with the TPA-treated group.

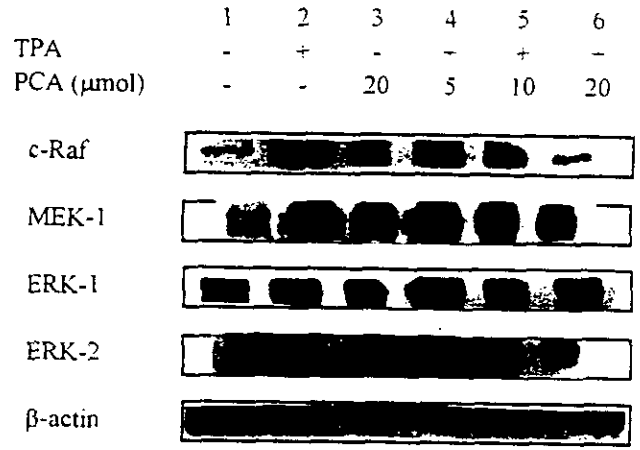
[c] Frozen section was determined in three successive sections using an histochemical assay (aldehyde detect system).

Fig.1 Inhibitory effect of PCA on TPA-induced hyperplasia in mice epidermis



Two hundred microliters of acetone, 5 nmol TPA in 200 μl acetone, or TPA together with 5 μmol, 10 μmol, 20 μmol PCA in 200 μl acetone were applied topically to the backs of CD-1 mice twice a day for 5 days. The mice (six per group) well killed 24 h later and the skins were fixed in 10% phosphate-buffered formalin solution for 18-24 h. Tissue section morphology was determined in three successive sections using an histochemical assay (Hematoxylin-Eosin stain), the manification was 20 X. The groups studied are indicated below. (a) Acetone alone twice a day for 5 days. (b) 5 nmol TPA in acetone twice a day for 5 days. (c) 5 μmol PCA in acetone twice a day for 5 days. (d) 5 μmol PCA in acetone pretreated TPA twice a day for 5 days. (e) 10 μmol PCA in acetone pretreated TPA twice a day for 5 days. (f) 20 μmol PCA in acetone pretreated TPA twice a day for 5 days.

Fig.2 Western blotting examination of the effect of PCA on TPA-induced the expression of MAP cascade kinases



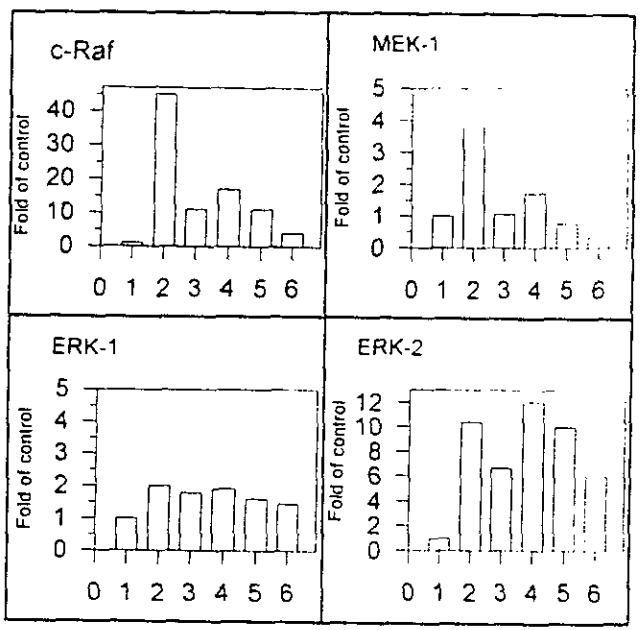
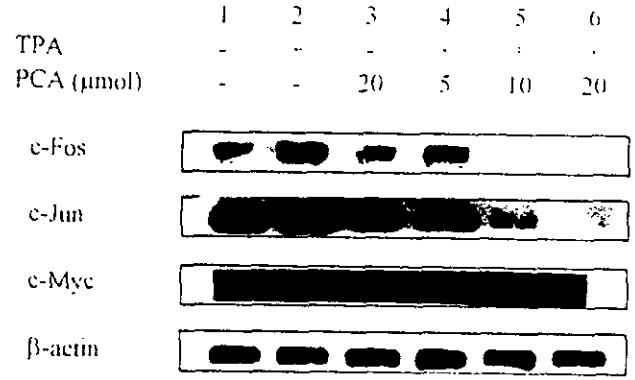


Fig.3 Western blotting examination of the effect of PCA on TPA-induced nuclear-protocogenes protein



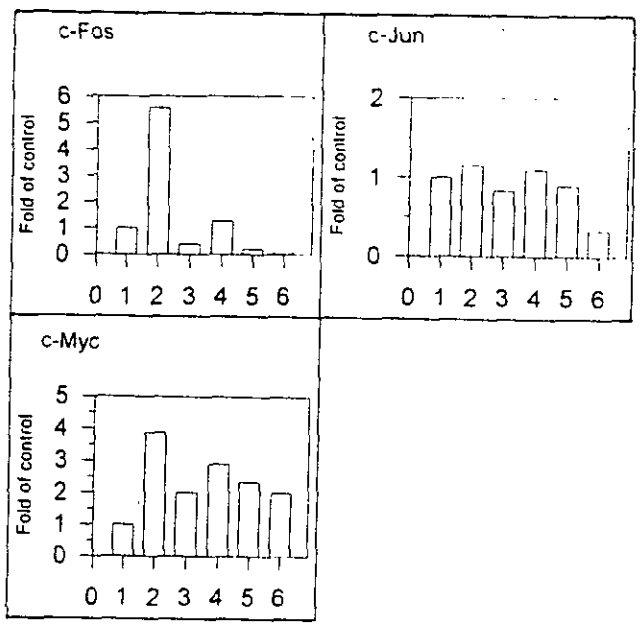


Fig.4 Western blotting examination of the effect of PCA on TPA-induced the expression of PKC-α and phosphotyrosine protein