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一、中文摘要

檳榔嚼塊已經被發現具有基因毒性、致突變性及動物毒性，雖然在流病調查上顯示與口腔癌之角色密不可分，但對於直接致癌之報告則較少，因此它在腫瘤促進作用之重要性應被研究，本研究探討檳榔嚼塊 (BQ) 及它的添加料荖花及紅灰 (LPA)再腫瘤促進作用之角色。

研究顯示，LPA 及 BQ 造成有意義的小鼠上皮組織增生及發炎，但只 LPA 組能夠促進 ornithine decarboxylase 有意義增加，上皮組織經 LPA (25，50 及 75 mg/ml)處理後，過氧化氫分別增加為 2.41，3.90 及 3.76 倍，而 myeloperoxidase 增加 1.43，2.70 及 2.29 倍，另以 50，100 及 150 mg/ml 之 LPA 或 BQ 處理小白鼠上皮組織增加 PKC-α及 NF-κB 表現，而此種作用顯示添加料 LPA 較 BQ 強，因此在 BQ 致使上皮組織增生之過程中，添加料 LPA 具有促進作用，由於腫瘤促進作用之前期可能發生增生及發炎，因此臆測 BQ 具有腫瘤促進作用，且其作用主要是因為 LPA 促進上皮組織 PKC-α及 NF-κB 表現。

關鍵詞：檳榔嚼塊；紅灰-荖花添加物；癌促進作用；水腫作用；發炎作用；蛋白激脢 C; NF-κB

Abstract

Components of betel quid (BQ) have been investigated for genotoxicity, mutagenicity, and animal toxicity. However, little information exists regarding their carcinogenic characteristics. Considerable attention has already been focused on tumor promoters that occur environmentally for human uptake. In this study, the promoting effects of BQ and lime-piper additives (LPA) in BQ on epidermal hyperplasia in CD-1 mouse skin are investigated.

In the present study, we found that BQ and LPA at concentrations of 25, 50, 75 mg/ml caused significant induction of hyperplasia, but only LPA caused an increase of epidermal ornithine decarboxylase (ODC). Treatment of mouse skin with LPA caused remarkable increases in the production of H_2O_2 by 2.41-, 3.90-, and 3.76-fold (for the above-indicated concentrations respectively); as well as marked increases of myeloperoxidase (MPO) by 1.43-, 2.70-, and 2.29-fold. Application of LPA or BQ (50, 100, 150 mg/ml) also caused inductions of protein kinase C-α (PKC-α) and NF-κB. In these inductions, LPA exhibited more significant effect than BQ. Thus, LPA might make a major contribution to the BQ-induced expressions of PKC and NF-κB. These results indicated that BQ has the potentiality as promoting agents, and that LPA should play a major role in increasing the effects of BQ-caused skin hyperplasia and inflammation. The promoting effects of BQ and LPA on mouse skin were associated with the induction of the expressions of PKC and NF-κB.

二、緣由及目的

Betel, known as *Areca catechu*, is a masticatory substance common to many oriental countries since a warm and humid climate is ideal for growing the *Areca* nut. In Taiwan, betel quid (BQ) is a natural masticatory. The most popular BQ is 'red lime betel nut' consisting of fresh nut of *Areca catechu*, *Piper betle* flower and slacked lime paste which stains red from the addition of an extract of Chinese herb *Acacia catechu*. The other popular is 'white lime betel nut' composed of *Areca* nut, *Piper betle* leaf and slacked lime [1]. Fresh areca nut contains lipids, fiber, alkaloids, polysaccharide and complex polyphenolic compounds, mainly hydroxychavicol and safrole [2]. The alkaloids in areca nut are arecoline (7.5 mg/g weight), arecaidine (1.5 mg/g weight), guvacoline (2.0 mg/g weight) and isoguvacine (2.9 mg/g

weight) [3]. Several phenolic compounds are found in *Piper betle* flower including safrole, hydroxychavicol, eugenol, methyl eugenol, isoeugenol, flavone and quercetin [4]. In *piper betle* leaf, a large amount of carotenes (80.5 mg/g weight) is found, as well as smaller amounts of phenolic compounds (21.9 mg/g weight) and ascorbic acid (1.9 mg/g weight) [2]. *Acacia catechu* contains apparently (+)-catechin and (-)-epicatechin [2]. In previous studies, different components existed in BQ were found to possess mutagenicity or genotoxicity [5-7], to induce chromosomal aberrations [8], and to increase the frequency of sister chromatid exchange of DNA in Chinese hamster ovary cells [9]. Some experiments on whether BQ or its components induced tumors or caused oral diseases showed positive results [10-14], whereas some were not, especially those used betel leaf aqueous extract [15-17]. The different effects on tumorigenicity of BQ observed in previous study seemed to depend on the applied components, such as areca nut, arecoline, arecaidine or betel leaf. These controversial results led us to suspect that the mutagenic properties of BQ might be mainly caused by its additives. Actually, epidemiological study has showed that a high frequency of oral cancers in human can be linked to chewing BQ (4). Moreover, many investigations have demonstrated that chewing and smoking habits act synergistically, and that persons with mixed habits form a substantial fraction of the high-risk population. However, up to the present, there is no direct evidence to show that BQ is a carcinogen, even though BQ should play an important role in cancer progression. We found that most investigations on BQ were focused on its carcinogenic properties, but not on its tumor promoting properties. Furthermore, little research had clarified that it was BQ or its additives possessing the main ability to cause cancer. Therefore, a series of experiments were conducted in this study to evaluate the possible promoting properties of BQ and its additives. The kind of BQ we chose to evaluate the effect on carcinogenesis was 'red lime betel nut' consisting of the fresh nut of *Areca catechu*, *Piper betle* flower and slacked lime paste. LPA means lime-piper additives consisted of *Piper betle* flower and slacked lime paste. To investigate the promoting effect of BQ or LPA in CD-1 female mice, some short-term markers for promoting agent were observed, including morphological changes in mouse skin that represent skin inflammation and hyperplasia, induction of myeloperoxidase (MPO) activity and H_2O_2 formation, and induction of ornithine decarboxylase (ODC) activity.

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent serine/threonine protein kinase with fundamental importance in cellular growth control. PKC is activated by a wide variety of growth factors, hormones, and neurotransmitters; and it has been shown to be a high affinity receptor for the phorbol ester tumor promoters, as well as other agents possessing tumor promotion activity [18-20]. Since the alterations in PKC have been linked to the increased cell proliferation in response to tumor promotion, the effect of BQ and LPA on PKC expression in mouse skin was also investigated in this study. Additionally, an inappropriate regulation of NF-κB-mediated transcription has also been associated with cancer and inflammatory responses [21-23]. Therefore, we also determined the expression of NF-κB in the skin treated with BQ and LPA. The results of present study could clarify the roles of BQ and LPA on carcinogenesis.

三、結果與討論

BQ and LPA induced mouse hyperplasia and inflammation

The effects of topical application of BQ- or LPA-induced alteration of cutaneous morphology were examined (Fig.1 and Table 1). Topical application of BQ or LPA (50, 100 and 150 mg/ml) twice a day for 4 days to the dorsal surface of CD-1 mice resulted in increases of 1.5- to 2.5-fold in the epidermal thickness. Inflammatory cell infiltration of the dermis was also observed. Topical application of BQ or LPA caused moderate or severe increase, respectively, in leukocyte infiltration and intercellular edema in the dermis (Fig.1 and Table 1). LPA treatment showed apparent induction of leukocyte and intercellular edema in the dermis. Results showed that LPA had potential effect on the BQ-caused mouse hyperplasia and inflammation.

BQ and LPA induced H_2O_2 production and MPO activity

The effect of topical application of BQ or LPA on the H_2O_2 production was examined. As shown in Table 2, BQ (25, 50 and 75 mg/ml) increased the formation of H_2O_2 by 1.02-, 1.10-, and 3.38-fold and LPA increased by 2.41-,

3.90-, and 3.76-fold in comparison with that of the control (Table 2). In addition, the activity of MPO was induced to 1.65-, 1.95- and 1.82-fold by BQ, and 1.43-, 2.70- and 2.29-fold by LPA (Table 2). These results indicated that topical administration of BQ or LPA promoted skin hyperplasia and inflammation in mouse skin that were correlated with the production of peroxide. Moreover, administration of LPA showed more severe skin hyperplasia and inflammation.

BQ and LPA induced ODC in mouse epidermis

BQ or LPA was topically applied to the mouse skin to test their effect on ODC activity. As demonstrated in Table 3, topical application of LPA at the concentrations of 25, 50 and 75 mg/ml to the backs of CD-1 mice caused the epidermal ODC activity to increase by 1.49-, 1.66-, and 1.58-fold as compared to the control. However, there was no significant increase in the activity of ODC in the BQ-treated mouse epidermis.

Increased expressions of PKC and NF-κB by BQ and LPBQ

The results determined by Western blot and densitometric quantiation showed there were significant alterations in the protein levels of PKC and NF-kB (Fig. 2). After BQ (150 mg/ml) treatment, the protein levels of PKC and NF-kB were significantly increased, with induction folds of 4.2 and 1.7, respectively. Higher inductions in the PKC and NF-kB proteins expressions (5- and 3-fold) were found in the mouse epidermis after LPA treatment at the same dose.

四、計畫成果自評

1. 本計畫結果發現檳榔嚼塊具有腫瘤促進作用，可能與口腔癌有密切關係。
2. 檳榔嚼塊枝腫瘤促進作用可能係因其添加料紅灰及苤花 (LPA)之促進作用。
3. 其作用機轉可能係 LPA 促進 PKC-α及 NF-κB 之表現。
4. 本研究成果已被 Chemico-Biol. Interact. 接受刊登。

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六、圖與表 (見下頁)

Table 1
Morphological examination of BQ- or LPA-treated mouse skin

Treatment[a]	No. of epidermal layer[c]	Epidermal thickness (μm)	Leukocyte infiltration[d]
Control	1–2	10.83 ± 2.04	--
BQ			
50 mg/ml	2–3	16.43 ± 4.76*,[b]	+
100 mg/ml	2–3	23.57 ± 4.76**	+
150 mg/ml	2–3	20.00 ± 2.67**	+
LPA			
50 mg/ml	2–3	20.00 ± 5.98**	++
100 mg/ml	2–3	24.38 ± 4.95**	++
150 mg/ml	3–4	25.43 ± 4.77**	++

[a] The mice were treated topically with BQ or LPA twice a day for 4 days. The animals were killed 18 h after the last dose and skins were processed for histological tests. Data are expressed as the mean ± S.D. from six mice/group.
[b] Statistically different from control group.
* $P < 0.01$.
** $P < 0.001$.
[c] 'No. of epidermal layer' means the number of nucleated cell layers in the epidermis.
[d] Leukocyte infiltration that was moderate (+) or severe (++) was characterized by diffuse infiltration of mononuclear infiltratory cells in the dermis when compared with the control.

Table 2
The formation of H_2O_2 and induction of MPO activity in mouse skin by topical treatment

Treatment[a]	H_2O_2 (nmol/cm^2)	Fold	MPO (units/cm^2)	Fold
Control	12.64 ± 0.45	1	1.30 ± 0.23	1
BQ				
25 mg/ml	12.98 ± 1.15	1.02	2.15 ± 0.27*	1.65
50 mg/ml	13.97 ± 10.22	1.10	2.54 ± 0.60*	1.95
75 mg/ml	42.68 ± 11.66*,[b]	3.38	2.37 ± 0.93	1.82
LPA				
25 mg/ml	30.45 ± 11.67	2.41	1.86 ± 0.23	1.43
50 mg/ml	49.32 ± 31.03*	3.90	3.52 ± 0.69***	2.70
75 mg/ml	47.58 ± 30.48*	3.76	2.98 ± 1.29**	2.29

[a] The mice were treated topically with BQ or LPA. A total of 20 h later, the animals were treated again. The mice were killed after the second treatment 1 h and the skins were removed. The formation of H_2O_2 and MPO activity were determined. The data were presented as the mean ± S.D. from six mice/group.
[b] Statistically different from control group.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.0001$.

Table 3
BQ- or LPA-induced epidermal ODC in mouse skin

Treatment[a]	ODC (pmol CO_2/mg protein/h)	Fold
Control	264.20 ± 19.70	1
BQ		
25 mg/ml	259.16 ± 88.64	0.98
50 mg/ml	229.37 ± 89.31	0.87
75 mg/ml	270.21 ± 90.25	1.02
LPA		
25 mg/ml	394.13 ± 83.17*,[b]	1.49
50 mg/ml	439.37 ± 43.40**	1.66
75 mg/ml	418.64 ± 44.31**	1.58

[a] Mice were treated with BQ or LPA. A total of 5 h later, the animals were killed and the epidermis was isolated. ODC activity was determined. The data presented the mean ± S.D. from six mice/group.
[b] Statistically different from control group.
* $P < 0.05$.
** $P < 0.01$.

Fig. 1. The morphological change of mouse epidermis was observed by topical treatment with: (A) water/absolute ethanol, 5:3, v/v; (B) BQ; and (C) LPA. Three doses (50, 100 and 150 mg/ml) of BQ and LPA were used, and a representative dose (150 mg/ml) is shown. BQ- and LPA-treated epidermis showed a markedly thickened epidermal layer (↔), with the upper dermis was infiltrated by prominent leukocytes (↑). H&E, 100 ×.

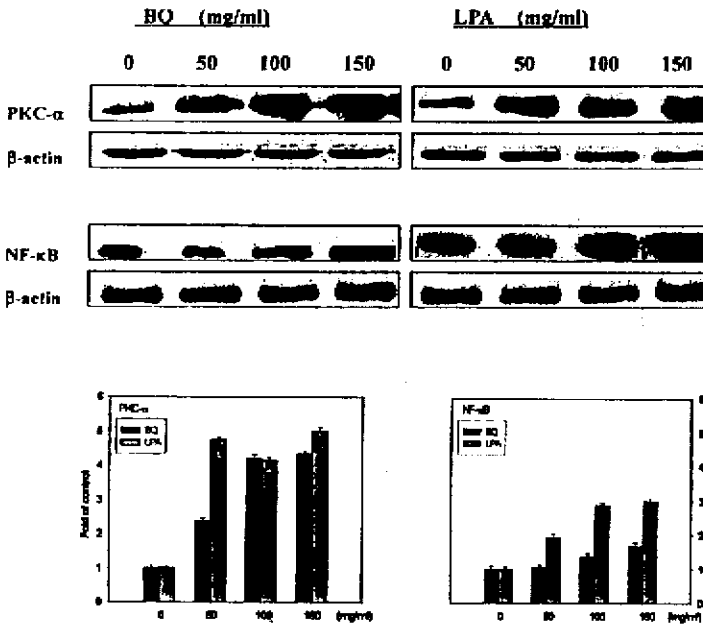

Fig. 2. Western blot of the epidermis showed expression of PKC and NF-κB in BQ- and LPA-treate mice. Six mice per group were examined; a representative is shown. The results were repeated three tim and represented as mean ± S.D.