行政院國家科學委員會專題研究計畫 成果報告

第一型蛋白酵素活化接受器在人類正常頰黏膜及口腔黏膜下纖維化症的表現(第 3 年)
研究成果報告(完整版)

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中 華 民 國 100 年 10 月 28 日

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Keywords: arecoline; buccal mucosal fibroblasts; protease-activated receptor-1; oral submucous fibrosis; regulatory mechanisms

Abstract

The major thrombin receptor protease-activated receptor-1 (PAR-1) is consistently and dramatically upregulated in a variety of fibrotic diseases. The aim of this study was to compare PAR-1 expression in normal human buccal mucosa and oral submucous fibrosis (OSF) specimens and further explore the potential mechanism that may lead to induce PAR-1 expression. Thirty OSF specimens and ten normal buccal mucosa were examined by immunohistochemistry. Primary buccal mucosal fibroblasts (BMFs) were challenged with arecoline, a major areca nut alkaloid, by using Western blot analysis. Furthermore, glutathione precursor N-acetyl-L-cysteine (NAC), phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, tyrosine kinase inhibitor herbimycin A, cyclooxygenase-2 inhibitor NS-398, and extracellular signal-regulated protein kinase (ERK) inhibitor PD98059 were added to find the possible regulatory mechanisms. PAR-1 expression was significantly higher in OSF specimens ($p<0.05$) and expressed mainly by fibroblasts and inflammatory cells. Arecoline was found to elevate PAR-1 expression in a dose- and time-dependent manner ($p<0.05$). The addition of NAC, LY294002, herbimycin

A, NS398, and PD98059 markedly inhibited the arecoline-induced PAR-1 expression ($p<0.05$). Taken together, our findings demonstrated that PAR-1 expression is significantly upregulated in areca quid chewing associated-OSF. In addition, arecoline-induced PAR-1 expression was downregulated by NAC, LY294002, herbimycin A, NS398, and PD98059.

Introduction

Oral submucous fibrosis (OSF) is regarded as a precancerous condition, [1] and is a chronic insidious disease of oral subepithelial connective tissue resulting in stiffness of the oral mucosa and inability to open the mouth. There are over 5 million OSF victims in the world.[2] Areca quid chewing has been recognized as one of the most important risk factors for OSF.[3,4] However, the precise mechanisms involved in fibrogenesis are not completely understood.

Thrombin is a multifunctional serine protease that plays a central role in hemostasis by converting soluble fibrinogen into an insoluble fibrin clot and by promoting platelet aggregation.[5] Thrombin has also a cytokine-like activity exerted viaspecific cell surface receptors called protease-activated receptors (PARs). PARs belong to the seven transmembrane domain G protein-coupled receptor superfamily. The *PAR-1* gene is located in the short arm of chromosome 5 and comprises 2 exons separated by a large intron.[6] In addition to its procoagulant effects, many studies have shown the implication of thrombin and PAR-1, its main receptor, on inflammation, fibrogenesis, and ECM remodeling. A central role for PAR-1 has been demonstrated in the inflammation and fibrosis induced in experimental lung injury,[7] and liver fibrosis.[8,9] Recently, the knockout of PAI-1 was found to reduce carbon tetrachloride-induced fibrosis in mice.[10]

The above evidences suggest that PAR-1 might also have a central role in the pathogenesis of areca quid chewing related-OSF. The present work was undertaken to identify the *in situ* localization of PAR-1 expression in normal buccal mucosa and OSF specimens. More specifically, we set out to explore whether expression of PAR-1 could be triggered in human BMFs by arecoline, the major areca nut alkaloid, *in vitro.* In addition, glutathione precursor N-acetyl-L-cysteine (NAC), phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, tyrosine kinase inhibitor herbimycin A, COX-2 inhibitor NS-398, and extracellular signal-regulated protein kinase (ERK) inhibitor PD98059 were added to find the possible mechanisms and their protective effects.

Materials and methods

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of 10 normal buccal mucosa from non-areca quid chewers, and 30 OSF specimens from areca quid chewers, were drawn from the files of the Department of Pathology, Chung Shan Medical University Hospital. Diagnosis was based on histological examination of hematoxylin- and eosin-stained sections. Institutional Review Board permission at the Chung Shan Medical University Hospital was obtained for the use of discarded human tissues. Five ↘m sections were stained with the polyclonal anti-PAR-1 antibody (1:100 dilution) using a standard avidin-biotin-peroxidase complex method. 3-amino-9-ethylcarbazole (AEC, DAKO, Carpinteria, CA, USA) was then used as the substrate for localizing the antibody binding. Negative controls included serial sections from which either the primary or secondary antibodies were excluded. The preparations were counterstained with hematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

Cell culture

Three healthy individuals, without areca quid chewing habit, attending the Oral Medicine Center (Chung Shan Medical University Hospital, Taichung, Taiwan) were enrolled with informed consents for this study. Biopsy specimens were derived from

histologically normal oral mucosa at the time of surgical third molar extraction. Fibroblast cultures were grown and maintained by using explant method as described previously. Cell cultures between the third and eighth passages were used in this study.

PAR-1 expression analysis

BMFs were grown in DMEM medium supplemented with 10 % fetal calf serum (FCS) and antibiotics (100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of fungizone). Cells arrested in G_0 by serum deprivation (0.5 % FCS; 48 h) were used in the experiments. Nearly confluent monolayers of BMFs were washed with serum-free medium and immediately thereafter exposed at the indicated incubation times to 0, 20, 40, 80, and 160 μg/ml arecoline. Cell lysates were collected at 24 h for Western blot analysis. Cultures without FCS were used as negative control. Subsequently, various pharmacological agents without cytotoxic concentrations were added to test their regulation effects during 24 h coincubation period.

Western blot

Briefly, cells were solubilized with SDS-solubilization buffer (5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl , pH 7.5 and 0.5 % Trition X-100, 2 mM phenylmethysulfonyl fluoride, and 1 mM N-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12,000g at 4➡C and the protein concentrations

determined with Bradford reagent using bovine serum albumin (BSA) as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10 % SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3 % BSA for 2 h, rinsed, and then incubated with primary antibodies anti-PAR-1 (1:1000) in PBS containing 0.05 % Tween 20 for 2 h. After 3 washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:1000 in the same buffer, washed again as described above and treated with 1:1000 streptavidin-peroxidase solution for 30 min. After a series of washing steps, the reactions were developed using Diaminobenzidine (DAB, Zymed). The intensities of the obtained bands were determined using a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean ± SD.

Statistical analysis

Three replicates of each concentration were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test.

Results

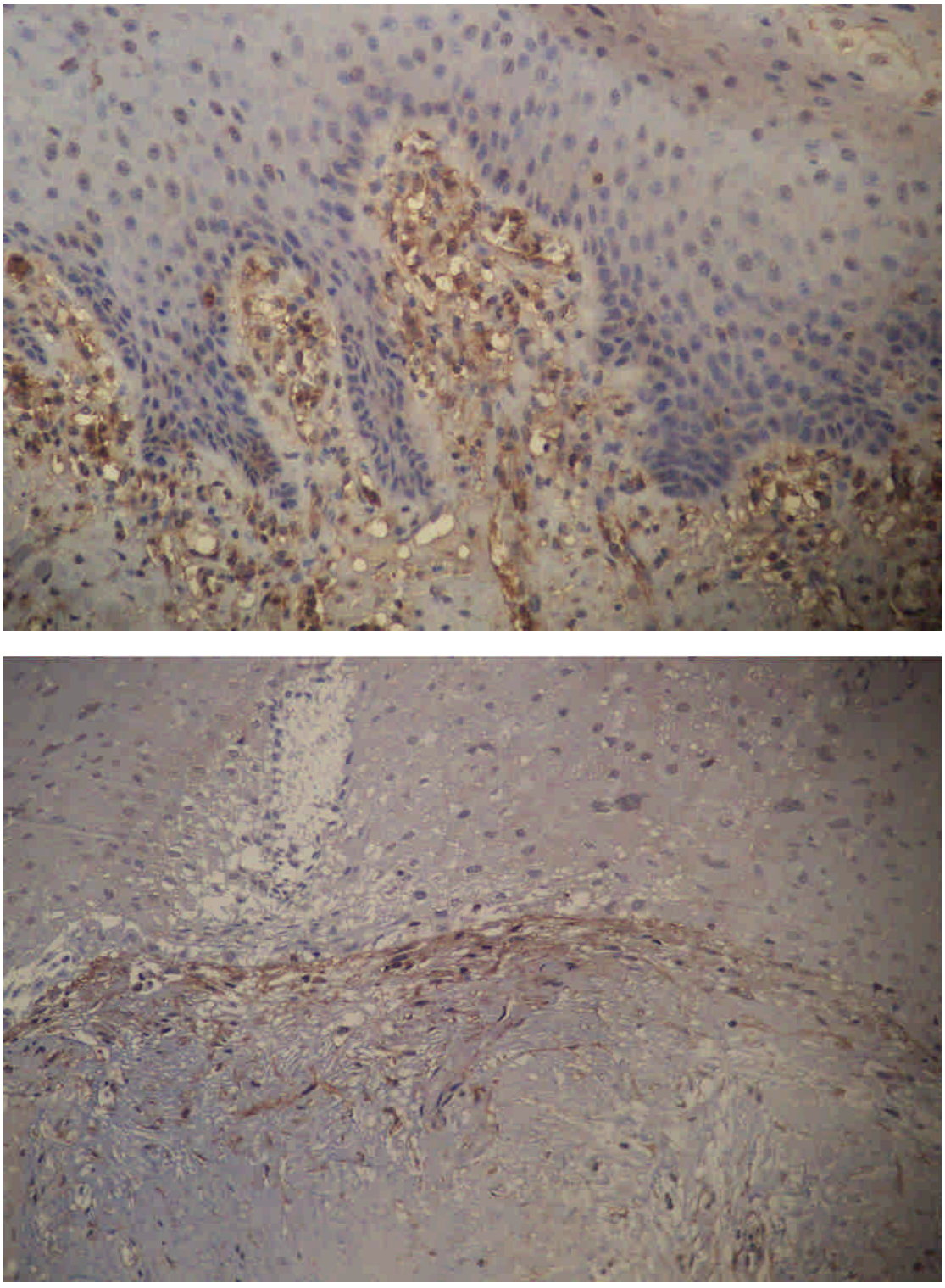

Fig. 1 (a) Very faint immunoreactivity of PAR-1 was observed in normal human buccal mucosa and almost totally limited to the lamina propria. (200x). (b)

PAR-1 was noted subepithelially and expressed in the cytoplasm of fibroblasts, and inflammatory cells. (200x)

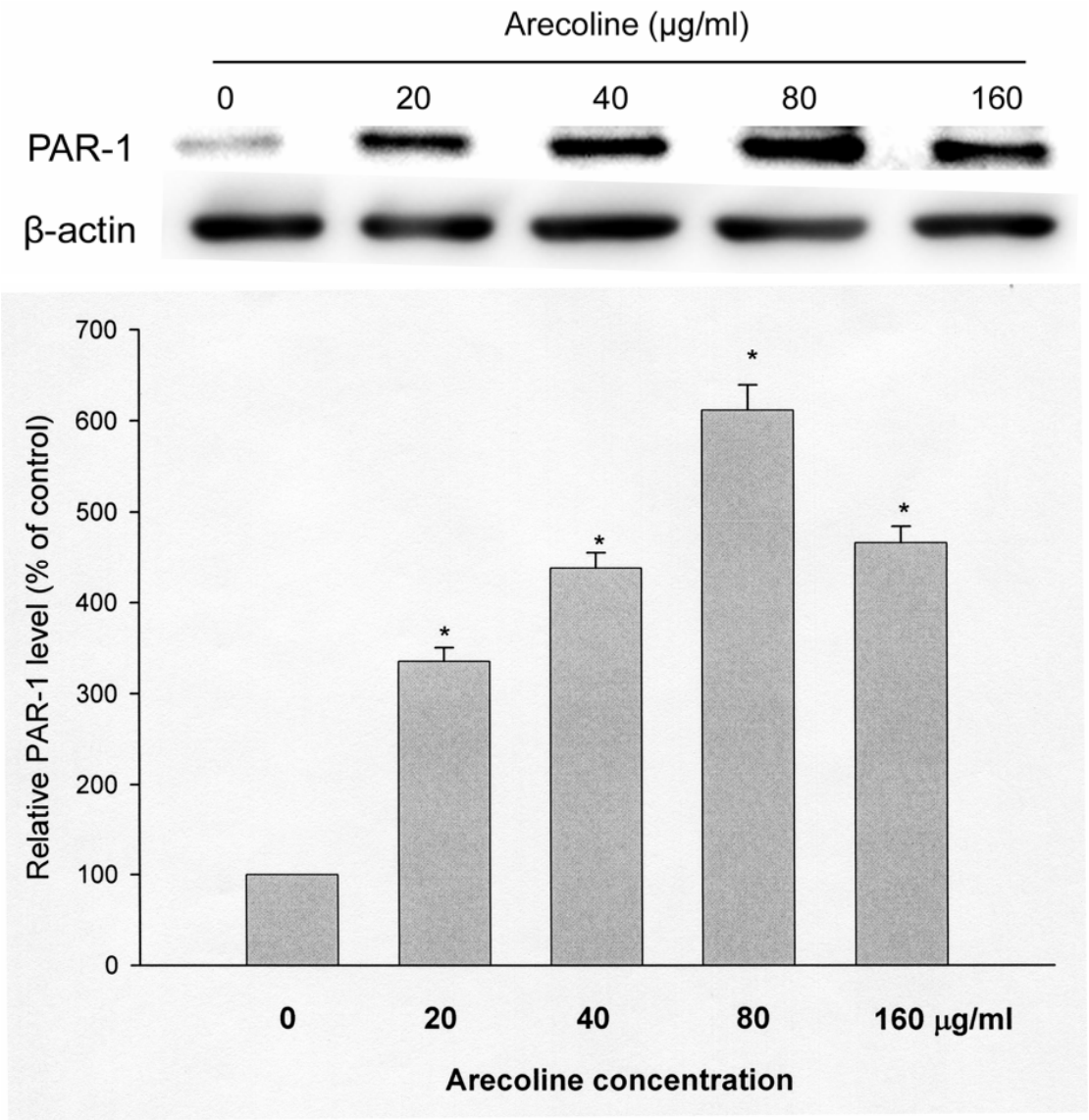

Figure 2 (a) Expression of PAR-1 in arecoline-treated BMFs by Western blot. Cells were exposed for 24h in medium containing various arecoline concentrations as indicated. β-actin was performed in order to monitor equal protein loading. (b) Levels of PAR-1 protein treatment with arecoline were measured by

densitometer. The relative level of PAR-1 protein expression was normalized against β-actin signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p < 0.05$.

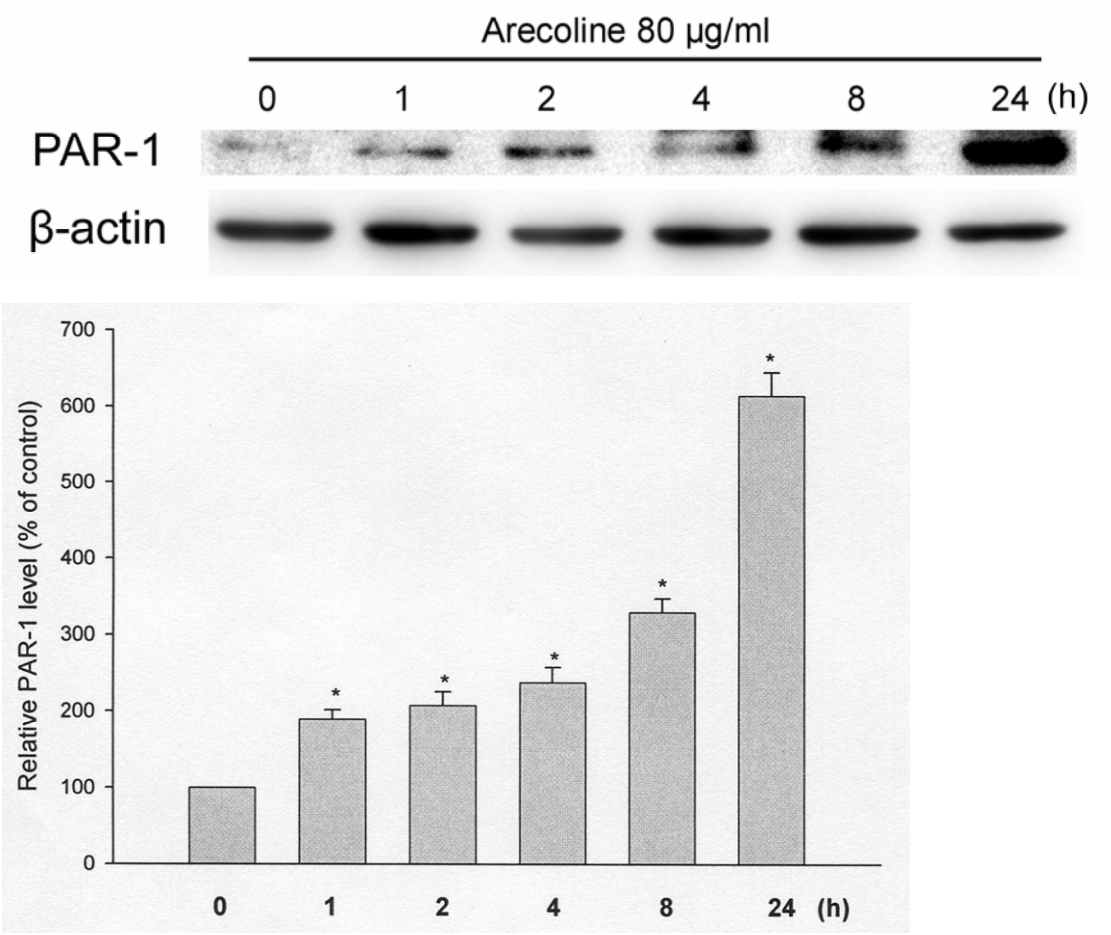

Fig. 3 (a) Kinetics of PAR-1 expression in BMFs exposed to 80 μg/ml arecoline for 0, 1, 2, 4, 8, and 24 h, respectively. β-actin was performed in order to monitor equal protein loading. (b) Levels of PAR-1 protein treated with arecoline were measured by densitometer. The relative level of PAR-1 protein expression was normalized against β-actin signal and the control was set as 1.0. Optical

density values represent the mean ± SD. * represents significant difference from control values with $p < 0.05$.

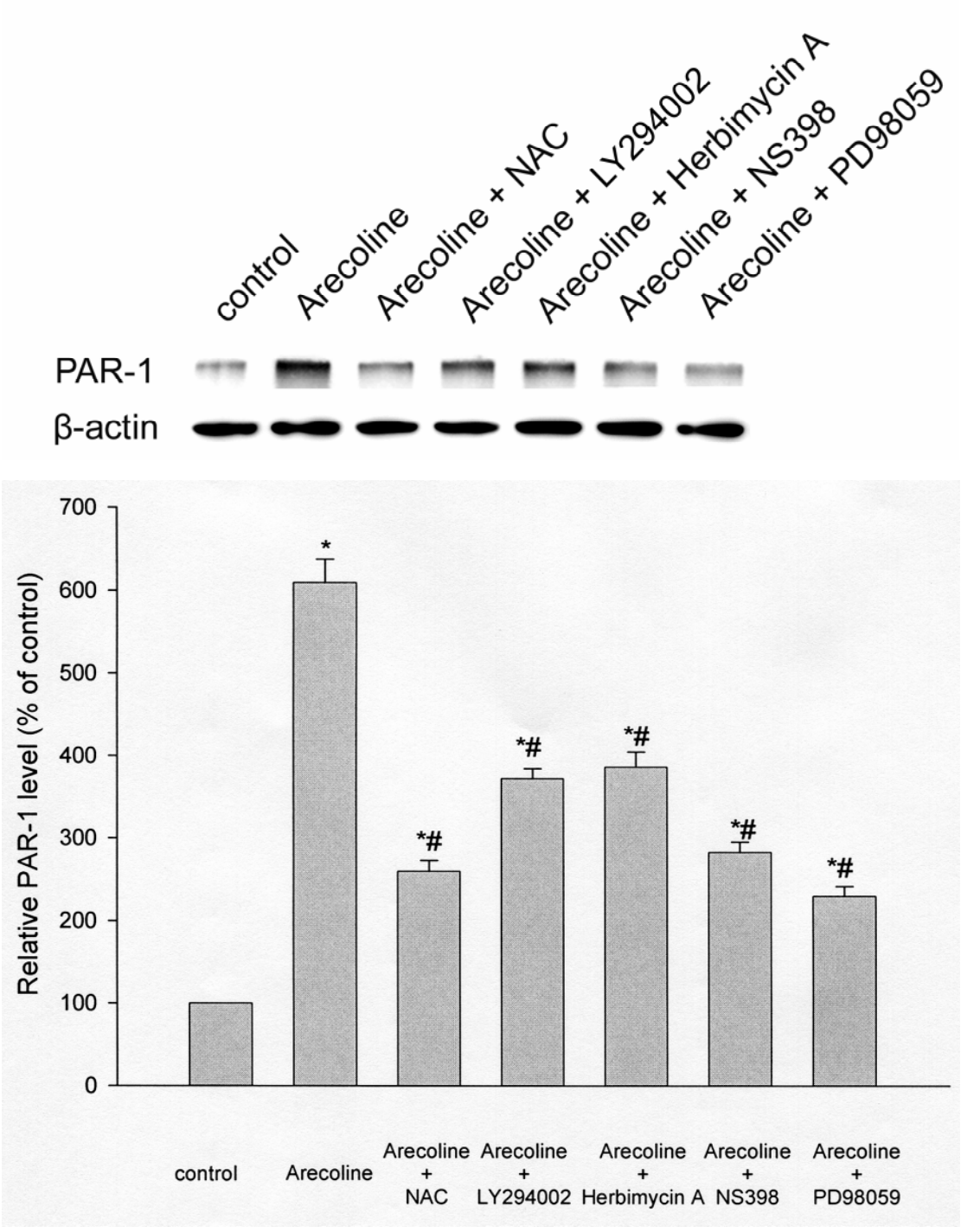

Fig. 4 (a) The regulatory effects of NAC, LY294002, herbimycin A, NS398, and PD98059 on arecoline-induced PAR-1 expression in BMFs. Cells were

coincubation with pharmacological agents in the presence of 80 μg/ml arecoline. β-actin was performed in order to monitor equal protein loading. (b) Quantization was achieved by densitometer as described in Figure 2. * represents significant difference from control values with $p < 0.05$. # represents statistically significant between arecoline alone and arecoline with pharmacological agents; $p < 0.05$.

Conclusion

This study represents that PAR-1 is elevated in OSF specimens from areca quid chewers. Arecoline was capable of stimulating PAR-1 expression in BMFs. This suggests that areca quid chewing may contribute the pathogenesis of OSF *via* PAR-1 expression. PAR-1 inhibited by NAC, PD98059, LY294002, NS398 and herbimycin A suggest that ERK, PI3K, COX2 and tyrosine kinase transduction pathways may be involved in the arecoline-stimulated PAR-1 expression. Therefore, studying the signal transduction pathway involved in PAR-1 expression may prove versatile. These results may advance our understanding of the role of PAR-1 expression in the pathogenesis of areca quid-chewing-associated OSF.

References

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國科會補助計畫衍生研發成果推廣資料表

日期:2011/10/25

國科會補助計畫	計畫名稱: 第一型蛋白酵素活化接受器在人類正常頰黏膜及口腔黏膜下纖維化症的表現
	計畫主持人: 蔡崇弘
	計畫編號: 97-2314-B-040-019-MY3　　學門領域: 牙醫學

無研發成果推廣資料

97 年度專題研究計畫研究成果彙整表

計畫主持人：蔡崇弘	計畫編號：97-2314-B-040-019-MY3
計畫名稱：第一型蛋白酵素活化接受器在人類正常頰黏膜及口腔黏膜下纖維化症的表現	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	1	100%		
		研討會論文	0	0	0%		
		專書	0	0	0%		
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（本國籍）	碩士生	0	3	100%	人次	
		博士生	0	0	0%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	0%		
		研討會論文	0	0	0%		
		專書	0	0	0%	章/本	
	專利	申請中件數	0	0	0%	件	
		已獲得件數	0	0	0%		
	技術移轉	件數	0	0	0%	件	
		權利金	0	0	0%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	0%	人次	
		博士生	0	0	0%		
		博士後研究員	0	0	0%		
		專任助理	0	0	0%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

□達成目標

■未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

■其他原因

說明：

本計劃病理切片, 組織培養, RT-PCR, 西方點墨法, 統計學部份在本實驗室長久以來建立的操作標準作業下均可穩定達成目標, 對於口腔粘膜下纖維化致病機轉達進一步的了解, 對於基因多形性探討, 含蓋抽血標本的取得相對困難, 及操作技術穩定性有待努力, 有待增強血液標本的收集, 及基因多形性實驗操作的訓練.

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

本計劃已寫成論文投稿, 針對編者的意見, 進一步補足內容或加強.

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

口腔黏膜下纖維化症在台灣是口腔常見疾病之一, 尤其與檳榔的關係, 是本土疾病特有的特性, 本研究團隊多年來致力於致病機轉的探討, 本次計劃以 PAR-1 為主題, 經過體內體外的實驗數據收集, 得到口腔粘膜下纖維化症 PAR-1 較有表現, 且用檳榔鹼的調控, 證明 PAR-1, 檳榔鹼與口腔黏膜下纖維症之間的相關, 對於提供病因及預防的參考.