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SELECTIVE INHIBITION OF COLLAGEN SYNTHESIS BY OKADAIC ACID IN CULTURED HUMAN FIBROBLASTS

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To determine whether protein phosphatases can affect collagen synthesis, we examined the effect of okadaic acid, a potent specific inhibitor of protein phosphatases 1 and 2A, on collagen synthesis. Okadaic acid significantly decreased the [^{3}H]proline incorporation into the collagenase-digestible protein and the percent collagen synthesis. These effects were synergistic with phorbol myristate acetate (PMA). The time course study showed that okadaic acid inhibited collagen synthesis after a 12 h treatment while PMA inhibited at 3 h. Down-regulation of protein kinase C by chronic treatment with PMA did not abrogate the okadaic acid-dependent inhibition. These results provide evidence for the involvement of protein phosphatases in the regulation of collagen synthesis.

The synthesis and accumulation of collagen, the most abundant of mammalian proteins, play a crucial role in biological situations involving tissue development, homeostasis, and repair. Abnormalities in collagen turnover may lead to pathologic fibrosis of many organs in various diseases such as liver cirrhosis (1), pulmonary fibrosis (2), scleroderma (3) or keloid (4). Fibroblasts are responsible for the production and maintenance of collagen. A large and increasingly complex group of growth factors and cytokines is currently being evaluated as modulators of collagen synthesis in fibroblasts (5). Nevertheless, essentially nothing is known about the intracellular signalling pathways responsible for their actions on collagen synthesis. Recent studies indicate that protein kinase activity is involved in the regulation of collagen synthesis during acitvation by cyclic AMP elevating agents such as prostaglandin E_2, cholera toxin, or dibutyryl cAMP (6,7), and by a protein kinase C acitivator, phorbol myristate acetate (PMA) (8,9). In addition, calcium ion (10) was also reported to be involved in the regulation of collagen synthesis. However, very little is known about the role of protein phosphatases in collagen synthesis. In this experiment, we used okadaic acid, a potent inhibitor of protein phosphatase 1 and 2A (11), to study the possible role of protein phosphatases in the regulation of collagen synthesis. We show here that okadaic acid inhibited collagen synthesis in

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human fibroblasts and other collagen-producing cells and this inhibitory effect is independent of the protein kinase C pathway.

Materials and Methods

Cell culture

Human embryonic lung fibroblast (HEL299), NIH3T3, and human skin fibroblasts were cultured in Dulbecco's modified Eagle's media (DMEM) containing 10 % fetal calf serum (FCS), and osteoblastic MC3T3-E1 cells were cultured in alpha-minimum essential medium (α-MEM) containig 10 % FCS. The culture dishes were incubated in a humidified atmosphere of 5 % CO_2 at 37 ℃.

Collagen and noncollagen protein synthesis

Cells were plated at a density of 1 x 10^5 (HEL299 and skin fibroblasts) or 5 x 10^4 (NIH3T3 and MC3T3-E1) cells/well in 24-well culture plates. Cell monolayers approaching confluency were rinsed with Hank's balanced salt solution and incubated for a desired period in the labeling medium containing 1 % bovine serum albumin and 50 μg/ml ascorbic acid with or without testing agents. Protein synthesis in cell cultures was assessed by measuring the amount of [^{3}H] proline incorporated into collagenase-digestible protein (CDP) and noncollagen protein (NCP) during the last 3 or 6 h of culture. Cells were labeled with 2 μCi of [^{3}H] proline. The medium was removed and the cells were sonicated in collagenase buffer (0.25 M Tris, 0.025 M $CaCl_2$, 0.125 M N-ethylmaleimide, pH 7.4) and then recombined with the medium. Protein was precipitated with ice-cold trichloroacetic acid and dissolved in 0.2 N NaOH. Aliquots of the cell homogenates were digested with highly purified bacterial collageanse to determine CDP and NCP labeling according to Peterkofsky and Diegelman's method (12). The percent collagen synthesis was calculated by the use of the correction factor 5.4 to correct for the relative abundance of proline in collagen compared to noncollagen proteins (13). Cellular DNA contents were measured by fluorophotometric assay using Hoechst 33258 according to Labarca and Paigen's method (14). Data was expressed as the mean ± SD. Statistical analysis was carried out by the Student's t-test.

Results and Discussion

In HEL299 cells, 24 h of treatment with 0.1 - 25 ng/ml of okadaic acid caused a concentration-dependent inhibition of [^{3}H]proline incorporation into CDP which was maximally reduced by 54 % at a concentration of 25 ng/ml okadaic acid (Figure 1). These changes were not the result of an overall decrease in protein synthesis since NCP syntheis was actually increased by the addition of okadaic acid at a concentration of 5 ng/ml or greater. Threrefore, okadaic acid over the same concentration range reduced collagen synthesis expressed as a percentage of total protein synthesis. It is very unlikely that the effect of okadaic acid on collagen and NCP synthesis in our culture system was affected by cell growth since we measured collagen and NCP synthesis in confluent density-arrested cells and we could not find any significant difference in DNA contents between okadaic acid-treated cells and control cells. This is supported by the study of Herschman et al (15) who reported that okadaic acid inhibited cell growth in sparsely plated rapidly growing 3T3 cells but neither sitmulated nor inhibited incorporation of [^{3}H]thymidine into DNA in confluent density-arrested 3T3 cells. The expression of collagen

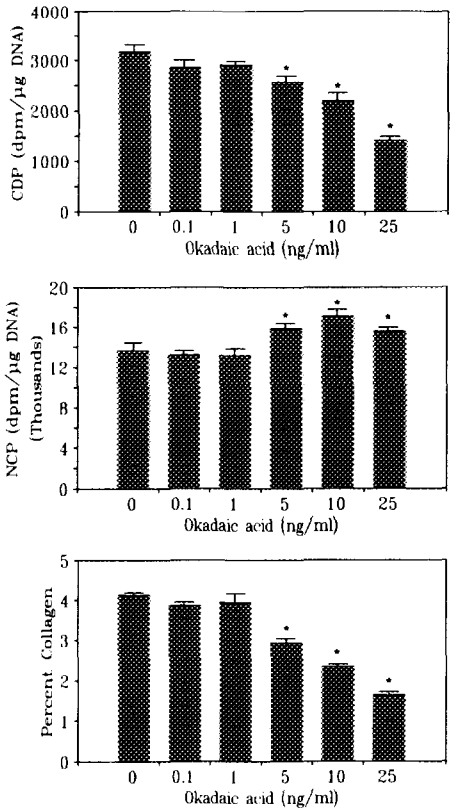
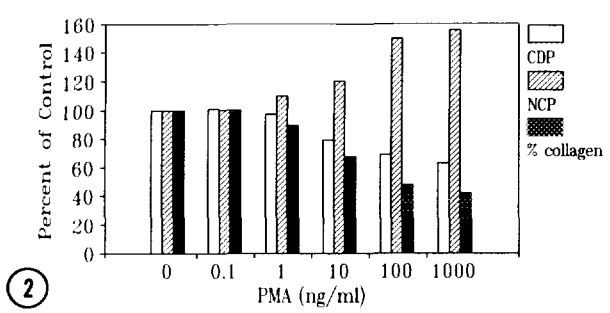


Figure 1. Dose response experiment for the effect of okadaic acid on CDP, NCP and percent collagen synthesized in HEL299 cells. Confluent cultures were treated with okadaic acid (0.1 - 25 ng/ml) for 24 h and labeled with 2 μCi/ml of [^{3}H] proline for the last 6 h. Each value represents the mean ± SD of six measurements. *$p<0.05$ by the Student's t-test as compared with untreated cultures.

synthesis as a relative rate represents a selective effect on collagen synthesis and eliminates consideration of changes in amino acid transport or other factors that would modify the specific activity of the precursor proline pool (10). We prefer to use percent collagen as an indicator of collagen synthesis in this paper. The effect of okadaic acid on collagen synthesis mimicked that of PMA which also inhibited collagen synthesis in the same cells (Figure 2). In a more detailed second experiment we measured the time course of collagen synthesis following treatment of cells with okadaic acid or PMA. Okadaic acid gave a different time course of collagen synthesis compared to PMA (Figure 3). The inhibition of collagen synthesis by okadaic acid started 12 h after treatment while the inhibition by PMA was noted at 3 h, suggesting that the mechanisms underlying okadaic acid-dependent inhibition in collagen synthesis are qualitatively different from that of PMA.

When okadaic acid was combined with 2 ng/ml PMA, a concentration of PMA which alone has little effect on collagen synthesis, the inhibitory effect was more potent than okadaic acid alone at concentrations ranging from 5 to 25 ng/ml (data not shown). This synergistic effect suggests a functional interaction between an



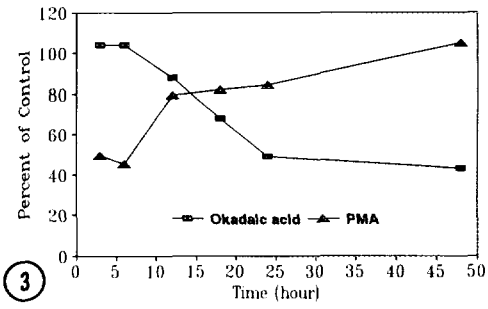


Figure 2. Dose response experiment for the effect of PMA on CDP, NCP and percent collagen synthesized in HEL299 cells. Confluent cultures were treated with PMA (0.1 - 1,000 ng/ml) and labeled with 2 μCi/ml of [^{3}H] proline for 6 h. Data are expressed as the percentage of control. Each value was obtained from six measurements.

Figure 3. Time response experiment for the effect of okadaic acid and PMA on percent collagen synthesis in HEL299 cells. Confluent cultures were treated with okadaic acid (10 ng/ml) or PMA (100 ng/ml) for the indicated time and labeled with [^{3}H] proline for the last 3 h. Data are expressed as the percentage of control. Each value was obtained from six measurements.

okadaic acid-sensitive protein phosphatase and a PKC-dependent signalling pathway in the regulation of collagen synthesis.

To further determine whether the okadaic acid-dependent inhibition was mediated by PKC, cells were pretreated for 36 h with 1 μg/ml of PMA to down-regulate the PKC (16) and then treated with okadaic acid. Under these conditions, okadaic acid still inhibited collagen synthesis indicating that okadaic acid-dependent inhibition of collagen synthesis dose not require PKC (data not shown).

We found that okadaic acid also caused a concentration-dependent inhibition of collagen synthesis in other collagen-producing cells such as MC3T3-E1 osteoblast-like cells, NIH3T3 cells and human skin fibroblasts (Table 1).

In order to examine the effect of okadaic acid on stimulated collagen synthesis, we used TGF-β to activate collagen synthesis in HEL299 cells or used 5 keloid-derived skin fibroblasts which showed much higher collagen synthetic activities than normal skin fibroblasts. The increased collagen synthesis induced by TGF-β (Figure 4) and in all keloid-derived fibroblasts (data not shown) was dramatically inhibited by okadaic acid. This result may imply that serine/threonine phosphatases are involved in TGF-β-induced collagen synthesis. However, since okadaic acid itself can suppress collagen synthesis and there is no evidence whether serine/threonine phosphatases are involved in the induction of collagen synthesis by TGF-β, it is very hard to say at this moment that protein phosphatases are directly responsible for the TGF-β-induced collagen synthesis. Nevertheless, there are some reports (17,18) which revealed the involvement of protein phosphatases in the effects of TGF-β in different cellular systems. Therfore it is necessory to investigate what role protein phosphatases play in the regulation of collagen synthesis by TGF-β.

Table 1. Effect of okadaic acid on CDP, NCP and percent collagen synthesized in MC3T3-E1, NIH3T3 and human skin fibroblasts

Cell	Concentration (ng/ml)	CDP	NCP	%collagen
		(percent of control)		
MC3T3-E1	0	100.0	100.0	100.0
	0.1	83.3	91.0	92.4
	1	83.0	87.1	95.5
	10	75.8	86.3	89.4
	50	51.5	90.0	59.1
NIH3T3	0	100.0	100.0	100.0
	0.1	80.6	99.3	81.9
	1	78.0	99.0	79.8
	10	60.1	91.5	66.0
HSF30	0	100.0	100.0	100.0
	1	100.8	105.1	97.3
	10	65.4	144.7	51.7
	25	14.8	134.4	27.4
	50	23.5	183.3	15.0

Confluent cultures were treated with okadaic acid for 24 h and labeled with 2 μCi [^{3}H]proline for the last 6 h. Data are expressed as the percentage of control. HSF30, human skin fibroblast.

Although inatracellular signalling pathways in the regulation of collagen synthesis has not yet been extensively studied, calcium ion (10) and PKC (8,9) is known to be involved in the inhibition of collagen synthesis and type I collagen gene expression in human fibroblasts. The participation of PKC in the regulation of collagen synthesis evokes the possibility that protein phosphatases could potentially play a role in regulating collagen synthesis. Although the okadaic acid-induced pattern of protein phosphorylation is distinct from that observed in cells treated with PMA (19), okadaic acid mimics multiple changes in gene expression induced by PMA and acts synergistically with PMA (20,21). This led us to examine the effect of okadaic acid, a protein phosphatase inhibitor, on collagen synthesis in fibroblasts. As far as we know this is the first report which provides evidence that okadaic acid

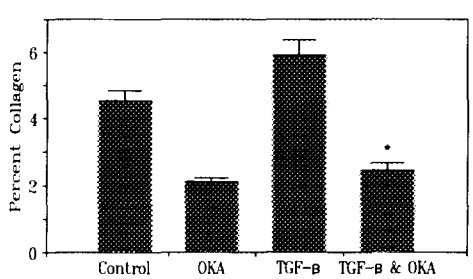


Figure 4. Effect of okadaic acid on TGF-β-stimulated collagen synthesis by HEL299 cells. Confluent cultures were treated with okadaic acid (10 ng/ml) or TGF-β (5 ng/ml) or both for 24 h and labeled with 2 μCi/ml of [^{3}H] proline for the last 6 h. Each value represents the mean ± SD of six measurements. *p<0.05 by the Student's t-test as compared with cultures treated with TGF-β alone. OKA, okadaic acid.

inhibits collagen synthesis in human fibroblasts and other collagen-producing cells. Furthermore the inhibitory effect of okadaic acid is synergistic with PMA and independent of the PKC pathway. Now we are studying the effect of okadaic acid on type I collagen gene expression and already have some data which shows that okadaic acid apparently decreased α1(I)procollagen mRNA levels in fibroblasts and MC3T3-E1 cells and also decreased α2(1) promoter activity in cells stimulated with TGF-β. These experiments are currently in progress. Our study clearly supports the view that the regulation of collagen synthesis by protein phosphorylation is achieved not only by modulating phophorylating activity but also by modulating phophatase activity. Identification of the protein phosphatase, kinase, and transcription factors involved in the okadaic acid-dependent regulation of collagen synthesis is needed to understand the signalling pathways controlling collagen synthesis and eventually provide useful information about the mechanisms underlying the pathogenesis of fibrotic diseases.

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