

AD_____

copy
3/29/01

Award Number: DAMD17-97-1-7100

TITLE: Mechanisms of Altered Control of Proliferation by Cyclic
Amp/Protein Kinase A During Mammary Tumor Progression

PRINCIPAL INVESTIGATOR: Walter Imagawa, Ph.D.

CONTRACTING ORGANIZATION: University of Kansas ~~Medical Center~~
Kansas City, Kansas 66160-7810
7700

REPORT DATE: June 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010406 142

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE June 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Jun 99 - 30 Jun 00) 31 May	
4. TITLE AND SUBTITLE Mechanisms of Altered Control of Proliferation by Cyclic Amp/Protein Kinase A During Mammary Tumor Progression			5. FUNDING NUMBERS DAMD17-97-1-7100	
6. AUTHOR(S) Walter Imagawa, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Kansas Medical Center Kansas City, Kansas 66160-7810 1700			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) We hypothesize that alterations in the regulation of growth by cAMP during mammary tumor progression are related to MAP kinase (ERK, JNK, p38) signaling modules known to be affected by cAMP and pertussis toxin (PT)-sensitive G proteins. Mammary epithelial cells from normal mouse mammary glands were compared to ovarian-independent mouse mammary tumors (OIT) in serum-free, collagen gel cell culture. The role of cAMP-pathway responsive transcription factors in PT or PD98059 (a specific ERK pathway inhibitor) inhibition of cAMP mitogenesis showed that CREB activation is not targeted by these inhibitors. Thus, other PT-senssitive or ERK-dependent cAMP pathways are involved. Further examination of lysophosphatidic acid (LPA) signaling showed that it can stimulate CREB and ATF2 phosphorylation, thus interacting with the cAMP pathway. The p38 inhibitor, SB202190 (SB), was used to evaluate the importance of the PT-responsive p38 pathway in cAMP mitogenesis. Surprisingly it alone was mitogenic, activated ERK and JNK kinase activity and stimulated p38 phosphorylation. It synergized with LPA and cAMP in stimulating proliferation or normal mammary epithelium but inhibited the proliferation of OITs. These novel data implicate p38 as playing a central role in proliferation control. H-89, a cAMP-dependent protein kinase A (PKA) inhibitor also stimulated proliferation of normal mammary epithelium but not OIT and could potentiate cAMP and LPA mitogenesis suggesting that nonPKA pathways are critical for cAMP mitogenesis. Thus, while cAMP alone does not significantly stimulate MAP kinase activity it appears to participate in multiple kinase networks to affect proliferation. Proliferation of mammary epithelium is controlled by multiple pathways and it is the balance between positive and negative signals that determines the final proliferative response of the cells. These networks are disrupted at many points during mammary tumorigenesis.				
14. SUBJECT TERMS Breast Cancer, Idea Award			15. NUMBER OF PAGES 19	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

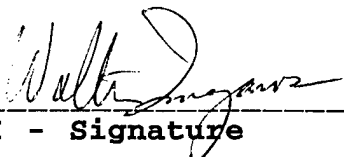
X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI - Signature

7/3/2000
Date

Table of Contents

	Page no.
Front Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Abbreviations	5
Introduction	6
Body	6
Materials and Methods	6
Results and Discussion	8
Objectives	8
Effect of cAMP and LPA on transcription factor phosphorylation and pathways of activation	8
Requirement for p38 activation for cAMP and LPA mitogenesis in normal and tumor mammary epithelium	10
Effect of the p38 inhibitor, SB202190, on MAP kinase activity in normal MEC and OIT	11
Effect of the p38 inhibitor, SB202190, on CREB and ATF-2 phosphorylation in normal MEC and OIT	12
The effect of SB on AKT phosphorylation	12
Figures	13
Research Accomplishments	17
Reportable Outcomes	17
Conclusions	17
Bibliography	19

Abbreviations

cAMP, 3'-5' cyclic adenosine monophosphate
C56, Compound 56 (EGF receptor phosphorylation inhibitor)
CK I, II, casein kinase I and II
CREB, cAMP response element binding protein
EGF, epidermal growth factor
ERK, extracellular regulated mitogen-activated protein kinase (MAP kinase)
ECL, enhanced chemiluminescence
IGF-I, insulin-like growth factor I
JNK, c-jun protein kinase
LPA, 1-oleoyl-lysophosphatidic acid
MAP Kinase, mitogen activated protein kinase
MAPKAPK2, MAP kinase activated protein kinase 2
MEC, mammary epithelial cell
MEK, MAP kinase kinase
MLCK, medium light chain kinase
OIT, ovarian-independent mammary tumor
PD, PD098059 (MEK inhibitor)
PDT, pregnancy-dependent mammary tumor
PKA, protein kinase A
PKC, protein kinase C
PKG, protein kinase G
PT, pertussis toxin

Introduction

The purpose of this research is to identify the mechanisms underlying the change in the proliferative response to 3'-5' cyclic adenosine monophosphate (cAMP) that occurs during mammary tumor progression (1). cAMP is a potent mitogen for normal mammary gland epithelium, weakly mitogenic for hormone-dependent mammary tumors and growth inhibitory to hormone-independent mammary tumors. Pertussis toxin (PT) inhibited cAMP-stimulated proliferation in normal mammary epithelium but neither cAMP-inhibited nor basal proliferation in hormone-independent mammary tumors (2). PT is a bacterial toxin that ADP-ribosylates G α i subunits and blocks activation of receptor-coupled heterotrimeric G α β γ proteins. These findings show that through postreceptor crosstalk, pertussis toxin-sensitive G α β γ pathways modulate cAMP-mediated proliferation. These preliminary results led to the hypothesis that during mammary tumor progression, a critical alteration occurs in growth regulation related to signaling pathways affected by cAMP and pertussis toxin-sensitive G proteins. Using a serum-free, primary cell culture system, we have examined intracellular mitogen-activated kinase (MAP kinase) pathways that may be altered during the progression of normal mammary epithelium to hormone-independent mammary tumors.

Body

Materials and Methods

Reagents. Cell culture: Ham's F-12, Medium 199, and Dulbecco's Modified Eagle's medium (DMEM) were from GIBCO/BRL (Grand Island, NY); collagenase (CLS Type 2) was from Worthington Biochemical Co. (Freehold, NJ), Percoll was from Pharmacia Biotech (Piscataway, NJ). Rat tail collagen, solubilized in acetic acid, was prepared as described previously (3). Antibodies: nonspecific (i.e. total) antibodies to ERK 1 (C-16), ERK 2 (C14) and total and phosphospecific antibodies to jun kinase (JNK), CREB, and ATF-2 were from Santa Cruz Biotechnology (Santa Cruz, CA); total and phospho-specific antibodies to p38 and AKT and phosphospecific antibodies to ERKS 1, 2 were from New England Biolabs. Biochemicals: Inhibitors to MEK1 (PD 098059), p38 kinase (PD16936, SB202190), and protein kinase A (H-89) were from Calbiochem (San Diego, CA). Protein A and G agarose, dibutyryl-cyclic AMP (cAMP), pertussis toxin, 1-oleoyl lysophosphatidic acid (LPA) were from Sigma Chemical Co. (St. Louis, MO) or Avanti Biochemicals (Birmingham, AL). LPA was prepared by brief sonication of an aqueous suspension in Saline A containing fatty acid-free bovine serum albumin (0.1 mg/ml).

Cell culture and tissues. Ovarian-independent mammary tumors were raised by subcutaneous transplantation of tumor pieces in virgin DDD mice. These hormone-independent tumors grow

rapidly in virgin hosts. Normal tissues were from mature virgin Balb/cAnNCrIBR mice obtained from Charles River.

Normal and tumor tissues were dissociated with collagenase (0.1%) and purified epithelial cells obtained by Percoll gradient centrifugation as described previously (3). For growth experiments, cell organoids were cultured for 10 days within collagen gels as described (3). The basal medium used for cell growth was composed of a 1:1 (v:v) mixture of Ham's F-12 and DMEM buffered with 20 mM HEPES and 0.67 g/l sodium bicarbonate, and supplemented with 10 µg/ml insulin, 100 U/ml soybean trypsin inhibitor, 1 µg/ml α -tocopherol succinate and other additives as indicated. Cell number was determined by fluorometric DNA assay using diaminobenzoic acid (4) and standard curves using diploid tumor cells.

For kinase assays, cells were cultured on collagen I-coated 6 cm petri dishes at 3-5 million cells per plate. For some experiments, cells were cultured in collagen gels. Cells were cultured in culture medium containing porcine serum (5%), EGF (10 ng/ml), and insulin (10 µg/ml) for 3-4 days until at least 75% confluency was achieved. The cultures were then washed and cultured in serum-free medium (above) for 2 days before the addition of test factors. cAMP (100 µg/ml) was added in combination with the cAMP phosphodiesterase inhibitor RO-20-1724 (10⁻⁵ M, Calbiochem). Pertussis toxin (100 ng/ml) was added overnight (to allow activation by the cells) prior to the addition of test factors. Kinase inhibitors SB202190 (p38), PD98059 (MEK/ERK), and H-89 (PKA), were added 2 hrs prior to cAMP or lysophosphatidic acid (LPA) except where indicated. Pertussis toxin (PT) was added overnight.

Preparation of Cell Extracts. After incubation for various times, collagen gel cultures were terminated by aspiration of the culture medium followed by blotting of the gels on filter paper and transfer of the dehydrated gels to 0.6 ml of lysis buffer containing 20 mM Tris (pH 7.4), 0.3 M NaCl, 50 mM NaF, 2 mM EDTA, 1% (v/v) Triton X-100, 25 mM β -glycerophosphate, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10% glycerol, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml leupeptin, 2 µM benzamidine, 5 µg/ml aprotinin. The lysates were mixed by vortexing and left on ice for 60 min. before centrifugation (15 min, 13,000 x g, 4°C). Cells cultured on collagen-coated plates were terminated by quick freezing on dry ice followed by direct lysis on ice with 0.35 ml of lysis buffer. The cells were scraped and transferred, 1.5 ml microcentrifuge tubes and incubated on ice for 30 minutes. Extracts were obtained by centrifugation at 13,000 x g for 15 minutes. Supernatants were used for immunoprecipitations and western blot analysis. Protein concentration was determined using the BCA assay by Bradford.

Western Immunoblotting. Sample lysates containing 20-40 µg of total protein were electrophoresed on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with TBST buffer containing 5% (w/v) dry milk and 0.1% Tween and incubated with the manufacturer's recommended dilution of antibody (dilutions

were adjusted as necessary). For all immunoblotting, enhanced chemiluminescence (ECL, Amersham Corp.) was used to detect binding of horseradish peroxidase-conjugated secondary antibody to bound primary antibody. Membranes were stripped for reprobing with another antiserum by shaking at 65° C for 30 min. in Tris (63 mM, pH 6.7), 2% SDS, and 100 mM 2-mercaptoethanol then washed in TBST and reblocked. For data analysis, ECL-detected bands were quantified using a Molecular Dynamics Personal Densitometer using ImageQuant software.

Results and Discussion

Objectives:

The objectives of the present studies were to 1) Examine the effect of cAMP and lysophosphatidic acid (LPA) on transcription factor phosphorylation in normal and ovarian-independent mammary tumors (OIT), 2) Assess the importance of the p38 MAP kinase pathway in cAMP and lysophosphatidic acid mitogenesis in normal and OIT cells. These objectives are related to Task 1 of the Statement of Work but are an extension using new inhibitors to delineate MAP kinase pathways involved in cAMP mitogenesis. We have de-emphasized work on the role of IGF-I in cAMP mitogenesis (Task 2) since previous work indicated that cAMP activation of growth or MAP kinases is not dependent upon insulin/IGF-I. New work has focused on, a) the regulation of transcription factor phosphorylation, and b) the roles of the stress activated MAP kinase p38 and protein kinase A (PKA) in cAMP and LPA mitogenesis.

1. Effect of cAMP and LPA on transcription factor phosphorylation and pathways of activation.

Effect of cAMP on CREB and ATF phosphorylation:

We showed previously that cAMP stimulates the proliferation of normal mammary epithelium without stimulating the activity of MAP kinases (ERK, p38, JNK). However, pertussis toxin (PT) which inhibits G β i-coupled pathways and PD98059 that is a specific inhibitor of the MEK/ERK pathway both inhibit cAMP mitogenesis. Both factors inhibited basal ERK activity suggesting that although cAMP did not activate MAP kinases, cAMP mitogenesis required a permissive level of ERK activity. We sought to determine if the effects of PT and PD98059 could also be directed at the level of transcription, especially at transcription factors activated by cAMP-dependent protein kinase A (PKA). One of these factors is the cAMP response element binding protein (CREB).

CREB phosphorylation was examined in normal mammary epithelial cells (MEC) cultured in the presence of cAMP with or without inhibitors. After working through the conditions needed to observe CREB phosphorylation we determined that immunoprecipitation of CREB was unnecessary and that direct immunoblotting of lysates provided the most reproducible results. We observed that the PKA inhibitor (H-89) but not PT or PD98050 inhibited cAMP-stimulated CREB phosphorylation (**Fig. 1**). The elevation in CREB phosphorylation occurring in the

cAMP/inhibitor combinations was not reproducible. This antiserum also detects phosphorylated ATF-1 which was phosphorylated in parallel to CREB (not shown). Preliminary analysis of ATF-2 phosphorylation, another transcription factor substrate of PKA, in cAMP-treated cells is now underway.

Currently, we cannot assess the effect of CREB phosphorylation on transcriptional activation although our working assumption is that phosphorylation is indicative of activation. Suitable transfection assays for primary cultures of these cells will be pursued in the future. These data show that PT or PD98050 do not inhibit cAMP-induced growth by inhibiting downstream PKA-dependent transcription factor phosphorylation through cross-talk between their respective signaling pathways. Thus, cAMP stimulates proliferation by pathways not dependent upon the induction of CREB phosphorylation by PKA.

Effect of LPA on CREB and ATF phosphorylation:

Lysophosphatidic acid (LPA) is a phospholipid mitogen that selectively affects normal and tumor MEC growth in the same manner as cAMP (2). Its mitogenic effect is similarly PT sensitive. We have attempted to gain further insight into growth-stimulatory and growth-inhibitory pathways by comparing the effect of cAMP with that of LPA on kinase pathways. Previously we showed that LPA stimulates the activity of ERK, JNK, p38 while cAMP is inhibitory to ERK in normal MEC and JNK in OIT. We have begun to look for possible cross-talk between cAMP and LPA signaling pathways by examining the effect of LPA on CREB and ATF-2 phosphorylation.

LPA (also EGF) stimulated CREB phosphorylation in normal MEC that was inhibited by pretreatment of the cells with PT and PD98059 (Fig. 2). LPA activation of ERK but not p38 or JNK is PT-sensitive suggesting that CREB phosphorylation occurs via activation of the ERK pathway. This is in contrast to cAMP which, as shown above, activates PKA not MAP kinases leading to CREB phosphorylation. Compound 56 (Calbiochem) is an EGF receptor kinase inhibitor and its inhibition of LPA-stimulated CREB phosphorylation (Fig. 2) may indicate that LPA transactivates the EGF receptor as observed in other systems (5-7). However, this inhibitory effect of Compound 56 may be a nonspecific effect. We found no evidence that LPA treatment of cells induces EGF receptor phosphorylation as assayed by immunoprecipitation of the receptor and phosphotyrosine blotting.

We have begun to examine the effect of LPA on ATF-2 phosphorylation using phospho-specific antiserum. In one experiment with normal MEC, LPA stimulated ATF-2 phosphorylation approximately 80% over basal as assayed by western immunoblotting with phospho-specific ATF-2 antiserum (not shown).

Effect of the PKA inhibitor H-89 on cAMP mitogenesis:

Since we observed that H-89 could inhibit cAMP induction of CREB phosphorylation we tested the effect of H-89 on cAMP mitogenesis. Unexpectedly, H-89 alone stimulated the

proliferation of cells cultured on the surface of collagen I-coated culture plates (**Fig. 3**) as well as within collagen gels (not shown). Inhibition of cAMP-stimulated proliferation by H-89 was not a consistent finding, both factors together could stimulate growth in an additive manner. These findings do indicate that blocking cAMP signaling through PKA (as determined by examining CREB phosphorylation, Fig. 1) does not consistently block cAMP mitogenesis. We conclude that cAMP can stimulate proliferation via PKA-independent pathways.

Preliminary data show that the effect of H-89 on proliferation does not involve stimulation of ERK or JNK phosphorylation. Surprisingly it potently stimulates Akt phosphorylation which would activate an anti-apoptotic pathway. H-89 has a K_i of 0.048 μM toward PKA which is at least 10-100X lower than the K_i 's for PKC, CK I and II, MLCK, and PKG suggesting that inhibition of these kinases is not involved in the stimulatory effect on growth. This effect may lead us to PKA pathways that are inhibitory to proliferation as observed in OIT and illustrates that the net effect of cAMP on proliferation may be the result of a balance between positive and negative pathways.

2. Requirement for p38 activation for cAMP and LPA mitogenesis in normal and tumor mammary epithelium.

Earlier we reported a higher level of active or phosphorylated p38 in OIT cells cultured within collagen gels compared to normal MEC and pregnancy-dependent mammary tumors. The total level of p38 (phosphorylated and nonphosphorylated) assessed by immunoblotting with p38 antisera showed that it was similar in normal and tumor cells. Thus, an elevation in p38 activity may be significant for the OIT phenotype and suggests that the regulation of this enzyme is altered in late stage breast tumors. cAMP, however, did not stimulate or inhibit p38 phosphorylation in normal MEC or OIT.

We needed a means to assess the effect of inhibition of the p38 pathway on cAMP and LPA mitogenesis. Our approach was to use the specific pyridinyl imidazole inhibitors of p38, SB202190 (SB) and PD16936. Surprisingly, like H-89, these inhibitors stimulated the growth of normal MEC (**Fig. 4A,B**) but inhibited the growth of OIT (**Fig. 5**). They markedly synergize with cAMP and LPA in stimulating the proliferation of normal MEC. PD16936 differs from SB in not significantly stimulating proliferation alone but is a more potent synergist showing this effect at a concentration of 0.1 μM (Fig. 4A) while SB requires higher concentrations, >1 μM (Fig. 4B shows SB at 25 μM). The stimulatory effect of these inhibitors on proliferation raises the possibility that the p38 pathway is constitutively inhibitory to growth and contributes to the inherent growth-limited phenotype of the cells. That is, p38 activity in basal serum-free medium is not being driven by an exogenous factor that is a requirement for the maintenance of cell cycle quiescence. This interpretation is tempered by the necessity for the effect of SB202190 on p38 activity, rather than phosphorylation, to be unequivocally determined (see discussion below). Another caveat is that the inhibitor may affect the activity of kinases other than p38. A related inhibitor, SB203580, at concentrations greater than 1 μM has been reported to inhibit the

AKT/PKB (Protein Kinase B) apoptotic pathway by inhibiting phosphoinositide-dependent protein kinase 1 which phosphorylates and activates PKB (8).

3. Effect of the p38 inhibitor, SB202190, on MAP kinase activity in normal MEC and OIT.

The stimulatory effect of SB on proliferation of normal MEC and synergism with cAMP and LPA prompted us to examine the effect of this inhibitor on MAP kinase signaling. We found that SB differentially affects the phosphorylation of MAP kinases. We first observed, using phospho-specific antiserum in western immunoblots of cell lysates, that SB was a potent inducer of p38 and pJNK phosphorylation but a relatively weak inducer of ERK phosphorylation (**Fig. 6**). Time course experiments (5, 30, 120 minutes) showed that the effect of SB reached maximum at 5 minutes of treatment for ERK and 30 minutes p38 and JNK.

The effect of SB on ERK phosphorylation was altered when SB was combined with LPA or cAMP (**Fig 7**). SB now inhibited ERK activation but stimulated JNK activity and p38 phosphorylation as expected. Thus the synergistic effect on proliferation was negatively associated with ERK activation and positively associated with JNK activation and p38 phosphorylation.

The effect of SB202190 on p38 kinase activity is of great interest to us in view of the effect of this compound on p38 phosphorylation. Phosphorylation may not indicate activation since the inhibitor will compete with ATP for its binding site on the phosphorylated enzyme and inhibit its activity. However, p38 isoforms differ in their susceptibility to inhibition by SB. SB inhibits the activity of the α and β isoforms which are more ubiquitously expressed in various tissues. The γ and δ isoforms are less susceptible to inhibition by SB (8). Since the antiserum detects all isoforms of the enzyme the interpretation of this phosphorylation event in terms of kinase activation awaits the characterization of the p38 isoforms expressed in mammary epithelium. We have attempted to assess p38 activity indirectly by measuring the activity of one of its substrates, MAPKAPK 2 that is activated upon phosphorylation by p38. No results are available yet but we do note that the level of phosphorylation of another substrate of p38, ATF-2, is increased in the presence of SB. This transcription factor is the substrate for other kinases, such as PKA, and experiments examining the effect of kinase inhibitors on SB-induced ATF-2 phosphorylation may aid in interpreting this response. In any event, if SB inhibits p38 activity *in vivo*, the stimulation of p38 phosphorylation by SB suggests that p38 regulates its activity through a substrate-dependent negative feedback mechanism.

Similar examination of OIT (**Fig 8**) showed the same general responses to SB but with differences in the magnitude of the activation of individual kinases: p38 phosphorylation increased >30-fold, JNK phosphorylation increased about 3-fold but remained at a relatively low level, and ERK phosphorylation increased about 2-fold. Thus, in OIT p38 phosphorylation was the predominant response. This occurred on a background of growth inhibition (**Fig. 5**). As indicated in our 1999 annual report, cAMP does not affect p38 phosphorylation in OIT (reconfirmed in new experiments) but, like SB, inhibits proliferation. Thus, a common effect on p38 phosphorylation is not characteristic of growth inhibition by these two factors.

4. Effect of the p38 inhibitor, SB202190, on CREB and ATF-2 phosphorylation in normal MEC and OIT.

SB stimulated ATF-2 phosphorylation but inhibited CREB phosphorylation in normal MEC (Fig 9A, B). The effect of SB on CREB phosphorylation was not examined in OIT. These effects were rapid occurring at 5 minutes the shortest time period examined in time course studies. SB at 0.5 μM slightly inhibited CREB phosphorylation (not shown) but, significant inhibition was observed only at 25 μM , the highest concentration tested. The effect of SB on CREB phosphorylation is interesting since SB synergizes with cAMP to stimulate proliferation of normal MEC. This result supports the conclusion that cAMP stimulation of mitogenesis is not dependent upon CREB activation but rather involves other pathways.

5. The effect of SB on AKT phosphorylation.

As discussed above (pt.2), a related p38 inhibitor, SB203580, can at concentrations above 1 μM can inhibit phosphoinositide-dependent protein kinase 1 leading to reduced activation of the kinase, Akt. This kinase regulates pathways leading to the inhibition of apoptosis. We asked if SB202190 might also be inhibiting Akt activation as an index of a lack of specificity toward p38. For both normal MEC and OIT we found that SB treatment inhibited of Akt phosphorylation (Fig 10). SB inhibited Akt phosphorylation in OIT at a concentration of 10 μM or higher while inhibition in normal MEC was observed only at the highest concentration of 25 μM . These results show that SB at concentrations above 5 μM can affect Akt phosphorylation although this response would be expected to attenuate a proliferative response through the stimulation of apoptosis via this pathway. SB can affect apoptosis through other pathways as well. For example, SB202190 can induce apoptosis via a caspase-dependent pathway by inhibiting p38 β that appeared to be antiapoptotic while p38 δ was proapoptotic (9). It possible that MEC express predominantly p38 δ and that SB would be anti-apoptotic by inhibiting p38 δ activity. Future work is necessary to examine the role of apoptosis in the proliferative response to SB.

The effect of SB on Akt phosphorylation in combination with cAMP or LPA reinforces the conclusion that the inhibition of Akt phosphorylation is not inhibitory to proliferation. LPA stimulates Akt phosphorylation in normal MEC which is not surprising since LPA can stimulate phosphoinositide turnover leading to the activation of the Akt pathways (Fig. 11). cAMP has no effect on Akt phosphorylation (Fig. 11). LPA and cAMP synergize with SB to stimulate growth but in the presence of SB, Akt phosphorylation is inhibited (Fig. 11). Thus, Akt regulation is not a primary response related to proliferation control.

Fig. 1 Effect of cAMP and inhibitors on CREB phosphorylation.

Normal mammary epithelial cells (MEC) were cultured in collagen gels and after 3 days inhibitor addition was initiated. PT (50 ng/ml) was added overnight, and PD98059 (50 μ M), H-89 (10 μ M) were added 2 hrs prior to the addition of cAMP. After 30 min. cAMP treatment, the gels were blotted on filter paper and transferred to lysis buffer on ice. Western immunoblot analysis of lysate proteins with phospho-specific CREB antiserum was followed, after stripping the membrane, by blotting with total CREB antiserum. Densitometry of bands was done and the results expressed as the ratio of phosphorylated CREB to total CREB (pCREB/tCREB) in arbitrary units. Representative of 3 experiments.

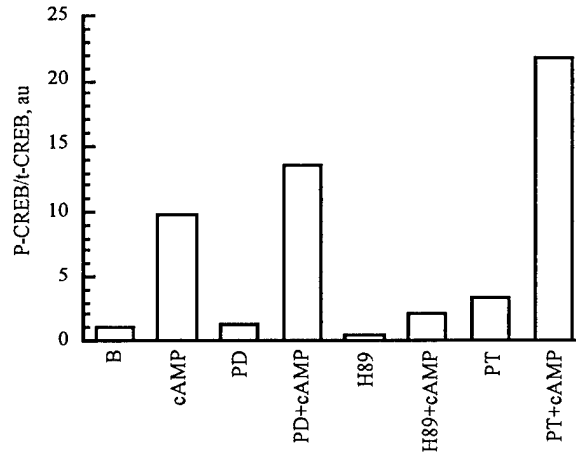


Fig. 2 Effect of H-89 on proliferation.

Normal mammary epithelial cells were cultured as monolayers in collagen-coated multiwells for 4 days in serum-free medium containing H-89 (0.1-10 μ M) or cAMP in the absence and presence of H-89. Basal is no additive control, OT is starting cell number. Mean \pm SD of triplicate cultures.

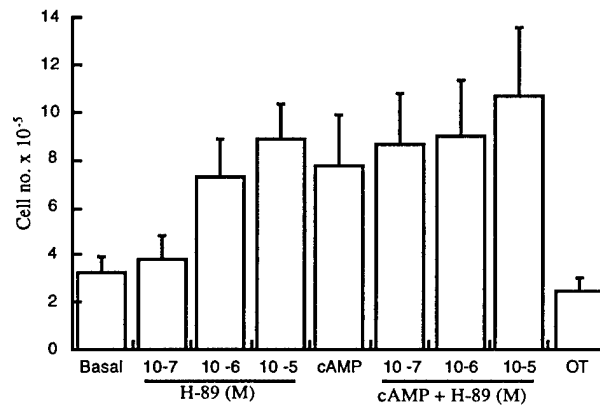
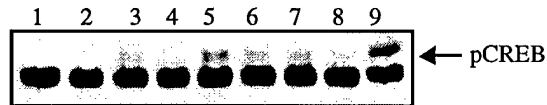


Fig. 3 Effect of lysophosphatidic acid (LPA) on CREB phosphorylation. Normal MEC were cultured as described in Fig. 2. Pertussis toxin (PT, 50 ng/ml) was added overnight. PD98059 and Compound 56 (C56) were added 2 hrs prior to the addition of LPA (50 μ M). Cells were stimulated by EGF (10 ng/ml) and LPA for 5 min. prior to cell lysis. The lower band is a nonspecific band.



Additive	Density (au)
1. Basal	285
2. PT	367
3. PD	741
4. C56	209
5. LPA	1270
6. LPA+PT	625
7. LPA+PD	767
8. LPA+C56	717
9. EGF	2909

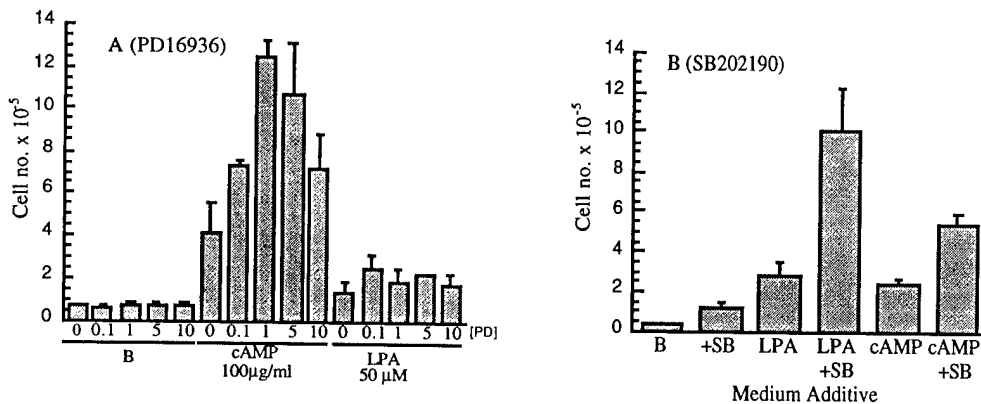


Fig. 4 Effect of inhibitors of p38 kinase on the proliferation of normal mammary epithelial cells. (A) Cells were cultured in collagen gels in basal medium (B) without or with the addition of cAMP, or LPA in the absence or presence of PD16936 (0.1-10 µM). (B) Cells were cultured in basal medium containing SB202190 (25 µM) alone or in combination with cAMP and LPA. Cells were cultured in triplicate for 10 days then terminated for DNA assay. Mean and SD of triplicates is plotted.

Fig. 5 Effect of the p38 kinase inhibitor, SB202190, on the proliferation of OIT cells. Cells from ovarian-independent tumors were cultured within collagen gels in basal serum-free medium. SB202190 (5, 10, 25 µM) was added at the initiation of the cultures (OT is starting cell number) and the cultures were terminated for DNA assay after 10 days. The results of 2 separate experiments is plotted, mean and SD of triplicates.

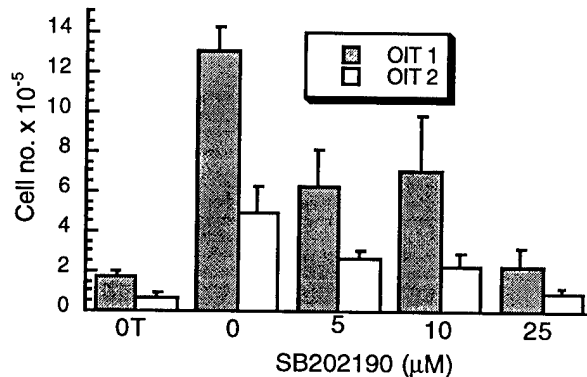


Fig. 6 Dose/response effect of SB202190 on ERK2, p38, and JNK1 phosphorylation. Normal MEC were cultured in the absence or presence of SB202190 (5-25 µM) for 120 min. Cell lysates were subjected to western immunoblotting with phospho-specific antisera and bands detected by ECL. Plotted is band density after scanning densitometry. Representative of 2 experiments.

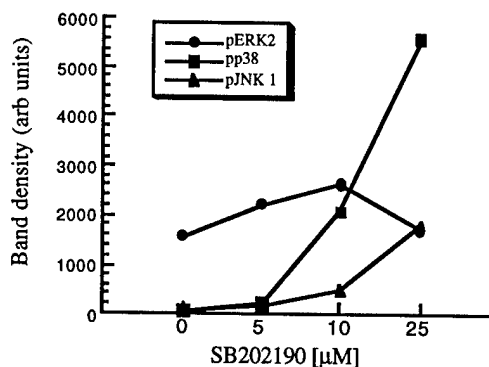


Fig. 7 Effect of SB202190 on MAP kinase phosphorylation in the presence of LPA and cAMP. Normal MEC were cultured in the absence or presence of SB for 2 hrs. prior to the addition of LPA for 5 min. and cAMP for 30 min. At the end of these incubation times, cell lysates were prepared and subjected to western immunoblot analysis with phospho-specific antibodies to ERK1, JNK1, and p38. After ECL detection, bands were quantitated using scanning densitometry. The results of a single representative experiment is plotted.

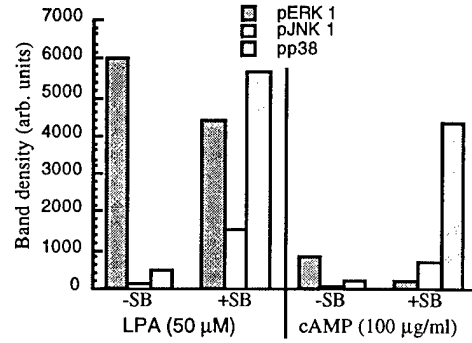


Fig. 8 Dose/response effect of SB202190 on ERK2, p38, and JNK1 phosphorylation on OIT. Ovarian-independent tumors were cultured in the absence or presence of SB202190 (1-20 μ M) for 120 minutes. Cell lysates were subjected to western immunoblotting with phospho-specific antisera to MAP kinases. Plotted is band density after scanning densitometry. Results of 2 separate experiments(1&2) are shown (open and closed symbols). Immunoblotting using antiserum to total kinase showed equivalent band intensities in all lanes.

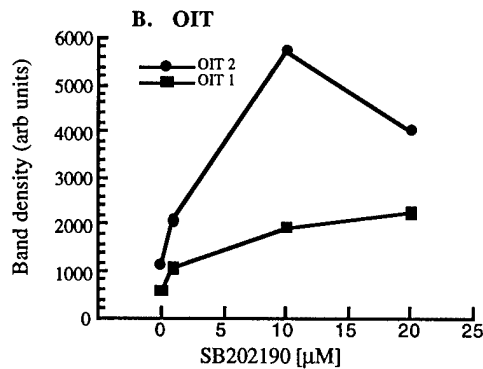
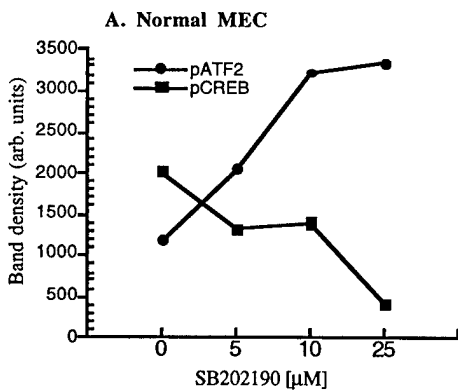
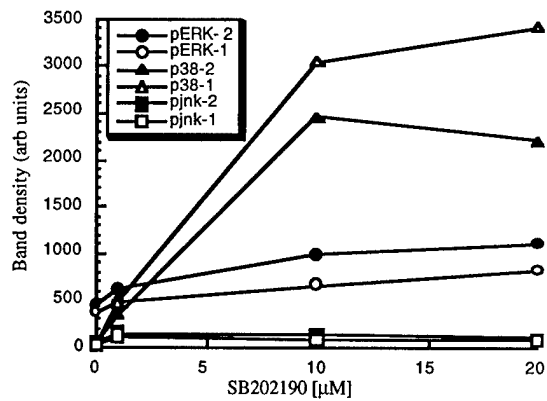


Fig. 9A, B Effect of SB202190 on ATF2 and CREB phosphorylation. See Fig. 6, 7 legend for details. Panel A shows the effect of SB on CREB and ATF2 in normal mammary epithelium. Panel B show the effect of SB on ATF2 phosphorylation two separate cultures of ovarian-independent (OIT) tumors. CREB phosphorylation was not examined in tumors. Immunoblotting using antiserum to total kinase showed equivalent band intensities in all lanes.

Fig. 10 Effect of SB202190 on Akt phosphorylation in ovarian-independent tumors and normal mammary epithelial cells. Cells from OIT or normal mammary epithelium (MEC) were stimulated by different doses of SB202190 for 2 hrs prior to lysis and western immunoblotting with phospho-specific antiserum to Akt protein kinase. Results of 2 experiments are plotted for OIT (OIT 1, OIT 2) and one for MEC. Immunoblotting using antiserum to total Akt showed equivalent band intensities in all lanes. Data are expressed as fold increase in phosphorylation over basal. The dotted line designates basal = 1.

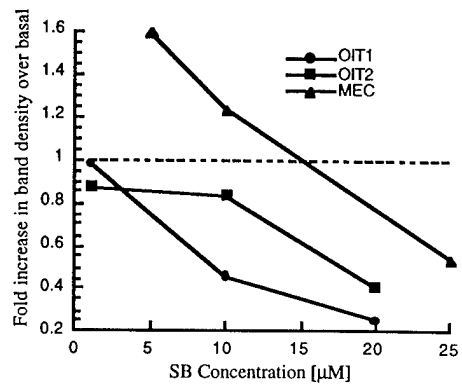
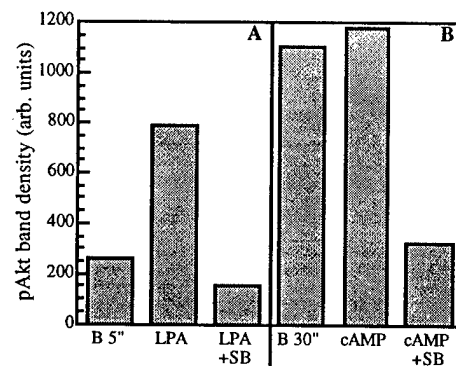


Fig. 11 Effect of SB202190 on Akt phosphorylation in the presence of LPA and cAMP in normal mammary epithelium. Cultured normal MEC were exposed to SB for 2 hrs. prior to the addition of LPA (0.05µM) for 5 min. or cAMP (100 µg/ml) for 30 min.. Cell lysates were subjected to western immunoblot analysis using antiphospho-Akt antiserum followed by ECL detection of bands and quantitation by scanning densitometry. Data are from a single representative experiment.



Research Accomplishments

1. Finished studies examining the effect of cAMP on MAP kinases and showed which MAP kinase pathways are not required for full cAMP mitogenesis in normal and tumor mammary epithelium.
2. Demonstrated that CREB/ATF-1 transcription factor phosphorylation plays no role in PT and PD98059 inhibition of cAMP-mediated proliferation.
3. Revealed novel and differential effects of pyridinyl-imidazole inhibitors on the proliferation of normal and tumor mammary epithelium and MAP kinase and transcription factor activation.
3. Revealed novel mitogenic effects of the protein kinase A inhibitor, H-89, on proliferation.
4. Extended studies on the lysolipid, LPA, to show its effects on the phosphorylation of cAMP responsive transcription factors and interactions with pyridinyl-imidazole inhibitors.

Reportable Outcomes

Manuscript: Xing, C and Imagawa, W (1999) Altered MAP Kinase (ERK-1,-2) Regulation in Primary Cultures of Mammary Tumor Cells: Elevated Basal Activity and Sustained Response to EGF. *Carcinogenesis* 20, 1201-1208

Abstract: Walter Imagawa, Charles Xing, Vadim Pedchenko (2000) Cyclic AMP Signaling During Mammary Tumorigenesis. Era of Hope DOD Breast Cancer Research Program Meeting, June 8-11, Atlanta, GA. Abst. no M-29.

{2 manuscripts in preparation}

Conclusions

1. Cyclic AMP stimulates proliferation by pathways not dependent upon the induction of CREB phosphorylation by PKA. One pathway is the ERK pathway which is also partially PT-dependent.
2. Lysophosphatidic acid can affect the cAMP signaling pathway by stimulating phosphorylation of the cAMP/PKA substrates CREB and ATF-2. Determination of the importance of cAMP-dependent PKA signaling in LPA or cAMP mitogenesis was confounded by the ability of the PKA inhibitor, H-89, to stimulate proliferation alone and potentiate LPA or cAMP mitogenesis. Determination of the pathways affected by this inhibitor will be useful to identify mitogenic

cAMP pathways.

3. The p38 pyridinyl-imidazole inhibitor, SB202190, alone stimulated proliferation and the phosphorylation of ERK, and most prominently, p38 and JNK in normal MEC. In ovarian-independent mammary tumors, SB202190 inhibited proliferation but its effects on MAP kinases differed from normal mammary epithelium only in not activating JNK. SB202190 stimulates and inhibits, respectively, ATF-2 and CREB phosphorylation. This inhibitor typically inhibits p38 activity which can occur independently of any effect on p38 phosphorylation. In new work we will examine the effect of this compound (and its relative PD16936) on p38 activity and determine which isoforms of p38 are present in normal and tumor mammary epithelial cells.
4. Both cAMP and LPA synergistically stimulate proliferation when combined with SB202190. Synergism is associated with an inhibition of CREB phosphorylation showing that CREB activation is not required for cAMP- or LPA-induced proliferation.
5. The p38 MAP kinase is known to be involved in the regulation of apoptosis. It appears that regulation of the Akt antiapoptotic pathways is not a primary response related to growth control by cAMP or LPA.
7. These findings contribute to our knowledge of the roles of MAP kinases in cAMP regulation of the growth in normal and tumor MEC. The identification of novel effects of kinase inhibitors and interactions with cAMP-mediated pathways raises cautions concerning the use of pathway inhibitors in the treatment of cancer. At the same time these inhibitors present opportunities to identify unknown interactions among kinase pathways that are altered during mammary tumorigenesis and that may prove to be useful therapeutic endpoints.

Bibliography

1. **Imagawa W, Bandyopadhyay G, Garcia M, Matsuzawa A, Nandi S** 1992 Pregnancy-dependent to ovarian-independent progression in mammary tumors delineated in primary culture: Changes in signal transduction, growth factor regulation, and matrix interaction. *Cancer Res.* 52: 6531-6538
2. **Imagawa W, Bandyopadhyay G, Nandi S** 1995 Analysis of the proliferative response to lysophosphatidic acid in primary cultures of mammary epithelium: Differences between normal and tumor cells. *Exp. Cell. Res.* 216: 178-186
3. **Richards J, Larson L, Yang J, Guzman R, Tomooka Y, Osborn R, Imagawa W, Nandi S** 1983 Method for culturing mammary epithelial cells in rat tail collagen matrix. *Journal of Tissue Culture Methods* 8: 31-36
4. **Hinegardner R** 1971 An improved fluorimetric assay for DNA. *Anal. Biochem.* 41: 477-487
5. **Daub H, Wallasch C, Lanckenau A, Herrlich A, Ullrich A** 1997 Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.* 16: 7033-7044
6. **Prenzel N, Zwick E, Daub H, Leserer M, Reimar A, Wallasch C, Ullrich A** 1999 EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* 402: 884-888
7. **Zwick E, Hackel P, Prenzel N, Ullrich A** 1999 The EGF receptor as a central transducer of heterologous signalling systems. *Trends Pharmacol. Sci* 20: 408-412
8. **Lali F, Hunt A, Turner S, Foxwell B** 2000 The pyridinly imidazole inhibitor SB203580 blocks phosphoinositide-dependent protein kinase activity, protein kinase B phosphorylation, and retinoblastoma hyperphosphorylation in interleukin-2-stimulated T cells independently of p38 mitogen-activated protein kinase. *J. Biol. Chem.* 275: 7396-7402
9. **Nemoto S, Xiang J, Huang S, Lin A** 1998 Induction of apoptosis by SB202190 through inhibition of p38 β mitogen-activated protein kinase. *J. Biol. Chem.* 273: 16415-16420