EXPLORATION OF A MAMMARY EPITHELIAL CELL MODEL FOR THE STUDY OF EPITHELIAL INFLAMMATION AND MECHANISMS OF ANTI-INFLAMMATORY ACTIVITY IN MEDICINAL PLANTS

Dissertation

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ABSTRACT

Failure to terminate the inflammatory response results in a state of chronic inflammation that might lead to cell transformation and cancer especially in epithelial cells. Hence, there is increased interest in the role of non immune epithelial cells in the regulation of inflammation to identify the link between chronic inflammation and cancer. The aim of this study was to investigate the regulation of inflammatory responses in non immune mammary epithelial cells and how this regulation is modulated by the cell microenvironment in order to establish an *in vitro* non immune cell model as a tool to understand inflammation in epithelial cells, and the mechanisms of action of potential anti-inflammatory agents in inflamed epithelia.

Bacterial endotoxin (ET) treated SCp2 cells showed sharp induction of both interleukin -6 (IL-6) secretion and nitric oxide (NO) production, but with unexpected delay in iNOS mRNA expression compared to that of IL-6 mRNA expression. ET also induced the activation of NFκB subunits p65 and p50 at 1 h after ET application (post-ET); however, p65 activation was transient, while that of p50 remained high throughout the experiment. Selective inhibition of NFκB activation pathway by inhibiting inhibitory kappa B kinase (IKK) α and IKKβ by Wedelolactone reduced ET-induced IL-6 mRNA expression and protein secretion but not that of iNOS mRNA

or NO production, suggesting that differences in regulation of ET-induced IL-6 and iNOS involve NFκB activation.

Interestingly, serum supplementation transiently up regulated cytokine protein secretion (IL-6) and mRNA expression (IL-6 and TNFα) in response to ET, but sharply reduced ET-induced iNOS mRNA expression and NO production, confirming the different modes of regulation of those inflammatory genes. Addition of exogenous extracellular matrix (EHS) to cultured SCp2 cells on plastic had no apparent effect on the temporal pattern of ET-induced IL-6 or iNOS mRNA expression.

Surprisingly, the coculture of SCp2 cells on a confluent monolayer of SCg6 mouse mammary myoepithelial cells induced a dramatic increase in IL-6 secretion to very high levels in the absence of ET exposure. Addition of ET to SCp2:SCg6 cell cocultures further increased IL-6 secretion to higher levels in sharp contrast to the meager induction of NO to maximum levels of ¼ to ½ that in SCp2 cells alone. These results showed that the microenvironment of the inflamed cell is important in the regulation of inflammation and for understanding the link between inflammation and cancer in epithelia.

Finally, the ET-induced inflammation in SCp2 cells was used to screen and identify the mechanism of action of anti-inflammatory fractions of methanol extracts of wild Lebanese *Centaurea ainetensis*, a plant used in Lebanese traditional folk medicine to treat inflammatory diseases. A partially purified fraction of *C. ainetensis* eluted by 60% methanol from solid phase extraction (SPE) columns followed by

methanol gradient elution on reverse phase high liquid performance chromatography (RP-HPLC) strongly inhibited ET-induced IL-6 secretion with a drastic reduction of the cytotoxicity observed in the crude methanol extract. The partially purified fraction of *C. ainetensis* also reduced ET-induced NO production by SCp2 cells, and inhibited the expression of ET-induced iNOS mRNA without affecting that of ET-induced IL-6 mRNA, suggesting an inhibitory role on the MAPK pathway but not on the NFκB pathway of inflammation. Concentration and partial purification of anti-inflammatory bioactivity from crude extracts of *C. ainetensis* by SPE columns followed by RP-HPLC suggest feasibility of purification and characterization. Moreover the use of non immune mammary epithelial cells for investigation of anti-inflammatory drugs presents a potential model to further investigate the link between chronic inflammation and cancers of epithelia.

To my parents: your love has not stopped nourishing me even miles away from home, your prayers were always answered.

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CHAPTER 1

INTRODUCTION

Inflammation is a coordinated physiological response elicited upon infection or trauma. The secretion of several pro-inflammatory cytokines and chemokines that signal immune cells to migrate to the site of infection and eliminate the infectious agent is elevated in inflamed cells. At the end of the inflammatory response, several anti-inflammatory cytokines and signaling molecules are secreted to stop inflammation and regain tissue homeostasis. Failure to terminate the inflammatory response results in a state of chronic inflammation leading to diseases such as inflammatory bowel disease (IBD), Crohn's disease, rheumatoid arthritis (RA), and many others. Furthermore, the persistence of pro-inflammatory cytokines and signaling molecules profoundly modulates the local microenvironment of epithelial, endothelial and mesenchymal cells, leading to cell transformation and several types of cancer especially in epithelial cells such as liver, intestine, and mammary gland [1-3]. As a result, there is a growing interest in inflammation and its regulation in epithelial cells to identify the link between inflammation, chronic inflammation, and cancer. Recent studies have suggested the

cytosolic transcription factor nuclear factor kappa B (NFκB) to be that missing link between inflammation and cancer since it plays a critical role not only during inflammation but also in regulating cell cycle, cell differentiation and other normal functions of the cell. Therefore, anti-inflammatory drugs that act via inhibition of NFκB activity could protect against cancer. Although a number of studies have investigated the role of epithelial cells in inflammation or inflammation-associated cancer in intestine [4, 5], liver [6, 7] and skin [8], an *in vitro* epithelial cell model to study inflammation under different conditions of cell states of growth and development and its association to cancer is still missing.

Mammary epithelial cells, unlike other epithelial cells such as intestinal or skin cells, are well defined in terms of responsiveness to proliferation signals (hormone signal), differentiation signals (hormone and matrix signals), and to dedifferentiation signals (inflammatory stimuli such as bacterial endotoxin (ET)) in the different stages of development of the mammary gland [9-12], and thus constitute an excellent model to monitor cell proliferation, differentiation and dedifferentiation in response to different external stimuli, and in relation to inflammation. Therefore, the focus of this study was to use mammary epithelial cells as a model to investigate the regulation of inflammatory responses in non immune epithelial cells and the modulation of this regulation by the cell microenvironment in order to establish an *in vitro* non immune epithelial cell model as a tool to further understand inflammation and inflammation-associated cell transformation in epithelial cells, and to study the mechanisms of action of potential anti-inflammatory agents in inflamed epithelia, especially those acting via inhibition of NFκB. Though major inflammatory respondents regulated by NFkB activation such as interleukin-6 (IL-

6), tumor necrosis factor alpha (TNFα), nitric oxide (NO), cycloxygenase-2 (Cox-2), and matrix metalloproteinases (MMPs) are recognized for their involvement in and coordination of the inflammatory and immune response; they also assert activities that affect epithelial cell development and function such as cell regulation (IL-6, TNFα) [13], intervention in cell signaling (NO) [14], and cell or tissue structural interaction critical to development and differentiation (MMPs) [15] at different stages of mammary gland development. Therefore, studying the regulation of these markers in the mammary epithelial system allows the characterization of an epithelial cell model that allows investigaton of the response of epithelial cells to external inflammatory stimuli under different growth and differentiation conditions, and their role in acute or chronic inflammation of epithelial tissues. The understanding of such innate immune responses of epithelia and linkage thereof to immune responses is crucial for understanding the link between chronic inflammation and cancer in epithelial tissues.

Mammary epithelial cells

Mammary epithelial cells, like other epithelial cells in the intestine or skin, form a physical barrier and constitute the first line of defense against bacterial infections. Therefore, they are often involved in acute and chronic inflammation that compromise function and health of the mammary gland tissue due to induction of cell damage, dedifferentiation and altered cell proliferation. However, unlike other epithelial cells, mammary epithelial cells are relatively easy to culture and exhibit inflammatory responses, differentiation and dedifferentiation, and proliferation in response to defined

cell regulators or growth conditions. They therefore provide a model for many tissues affected by inflammation.

Mammary epithelial cells form the ducts and alveoli in the mammary tissue. At the virgin state, the terminal end bud (TEB) structures at the end of the branching mammary ducts is comprised of an outer layer of cap cells and an inner layer of body cells that are highly sensitive to proliferation signals. During pregnancy, the body cells form a monolayer of secretory epithelial cells lining the ducts and alveolar lumena and are responsible for milk production and secretion during lactation. The cap cells give rise to myoepithelial cells that form a continuous layer on the duct exterior, and later form a discontinuous, spindle-shaped myoepithelial cell layer surrounding the luminal secretory epithelial monolayer and capable of contraction to expel milk into the mammary ducts. Electronmicrographs of the ultrastructure of the mammary gland in its lactating state [16] suggest that the ratio of secretory epithelial to myoepithelial cells is closer to unity in the duct but increases substantially with the dramatic increase in alveolar development during mammary gland development of pregnancy and lactogenesis, due mainly to the dense monolayer of secretory epithelial cells overlaid by the sparse myoepithelial cell network comprising the secretory alveolus. Both secretory epithelial and myoepithelial cells are separated from the connective tissue stroma of the mammary gland by a basement membrane that confers polarity to secretory/myoepithelial cell organization and the secretory epithelial cell structure and function, and is important for their functional differentiation for milk synthesis and secretion.

Cell shape is critical for the function of mammary secretory epithelial cells during development and differentiation. *In vivo*, undifferentiated mammary secretory epithelial

cells are marked by cuboidal shape with no polarity and large nuclei occupying the majority of the cytosol during gland development, while differentiation is distinguished by columnar polarization of epithelial cells with rounded basal nuclei, increased metabolic organelle content, and production of milk proteins (β-caseins, etc.) and fat droplets as during lactation. The cell shape and polarity of epithelial cells is maintained by junctions between neighboring cells or between cells and the extracellular matrix (ECM). For example, tight junctions and spot desmosomes mechanically couple epithelial cells to form a physical barrier against passage of molecules such as proteins through the intercellular space, while gap junctions confer metabolic coupling and developmental regulation of cells to insure intercellular passage to contiguous cells of small molecules responsible for intercellular communication. On the other hand, integrins and hemidesmosomes connecting the secretory epithelial cells to the basement membrane contribute to the induction of cell polarization and the machinery of milk synthesis [17-19]. Disruption of the polarized columnar shape of secretory epithelial cells either by stretching due to accumulation of milk in the alveolus or by disturbance of cell-cell and cell-ECM interaction as during inflammation induces initial loss of function and subsequent dedifferentiation in cells, leading to eventual apoptosis and involution of the mammary gland.

Inflammation of the mammary gland during mastitis is largely due to bacterial endotoxin (ET) that induces an innate immune response in the mammary tissue leading to chemo-attraction of immune cells to the site of inflammation as part of the injury response, obstruction of milk synthesis and secretion, increased cytokine production, and induction of matrix remodeling and apoptosis resulting in involution and restructuring of

the mammary gland tissue [20]. However, the repertoire of responses, particularly inflammatory responses, and their cellular origin and regulation are not well understood.

SCp2 mouse mammary secretory epithelial cell model

Mammary epithelial cells can differentiate in culture when provided with hormonal and ECM signals [9, 21]. COMMA-1D was the first mammary cell line to maintain the characteristics of normal mammary epithelial cells in culture [21]. They were shown to maintain a near diploid chromosomal karyotype [21] and expressed kertatin intermediate filaments characteristic of epithelial cells. When reinjected into mouse stroma, they were able to produce the mammary structure and did not induce tumors [22]. They also respond to hormonal and extracellular matrix and are able to produce β-casein and form dome structures indicative of secretion [21]. CID-9 cells isolated from COMMA-1D retained their ability to respond to lactogenic hormones (prolactin, insulin and hydrocortisone) and ECM by forming "alveolar-like" structures and producing β-casein [11]. However, CID-9 cells were a mixture of secretory epithelial, myoepithelial, and fibroblastic cells. Desprez et al (1993) separated two homogeneous subclones from the heterogeneous mammary CID-9 cell line [12]. The first, SCp2 (Subclone "petit"#2) is composed of small cuboidal epithelial cells that express keratin intermediate filament. They form a flat monolayer when cultured on plastic, tend to aggregate, and express β-casein when supplied with exogenous basement membrane and lactogenic hormones; characteristics of secretory epithelial cells. The second subclone is SCg6 (Subclone "grand" #6) comprised of large flat cells that exclusively express vimentine intermediate filaments and do not produce β-casein under any circumstances, characteristics of myoepithelial cells. When the two subclones are

cocultured, SCp2 cells are able to aggregate and produce β-casein in the presence of lactogenic hormones but without the need for exogenous sources of basement membrane [12]. Moreover, mammary epithelial cells can be monitored for inflammation by their ability to synthesize and secrete inflammatory mediators such as IL-6, TNFα, and MMPs, in addition to downregulation of the expression of β-casein [9, 11, 23-25] in response to ET. Collectively, these characteristics make the mammary gland an interesting model to study inflammation in cells of epithelial tissues.

Lipopolysaccharide induced inflammation

Bacterial lipopolysaccharide (LPS), also referred to as endotoxin (ET), is composed of a lipid A and a polysaccharide moiety attached via the oligosaccharide core present in the outer envelope of Gram negative bacteria. LPS is released upon bacterial cell death to cause inflammation in eukaryotic cells [26] exemplified by the acute inflammatory response of the mammary gland in bacterial infections of mastitis [27]. The binding of LPS to its receptor, Toll-like receptor 4 (TLR-4), on the surface of immune and certain non immune cells (epithelial, endothelial) [28-30] requires the assistance of co-receptor molecules Cluster of differentiation # 14 (CD14) [31], LPS binding proteins (LPB), and accessory protein MD2 present in the serum or on the cell membrane (Fig. 1.1) [32]. LPS complex (LPS/CD14/LBP/MD2) bound to TLR-4 activates a cascade of specific kinases either via a myeloid differentiation primary response protein 88 (MyD88)-dependent or MyD88-independent pathway leading to the activation of several transcription factors such as nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) (Fig. 1.1) involved in the expression of proinflammatory genes

(cytokines, NOS, and Cox-2, etc.) [29, 33-36]. Since NFκB is a highly suspected link between inflammation and cancer, we focused this study on the regulation of LPS induced genes via NFκB.

NFκB

NFκB is a key transcription factor that is activated by signaling associated with cell development and function [37], as well as by inflammatory stimuli such as LPS and TNFα to induce the expression of inflammatory genes such as IL-6, and iNOS [38] among others (Fig. 1.2). The mammalian NFκB family is comprised of five subunits, p65 (RelA), RelB, c-Rel, p50/p105 (NFκB1) and p52/p100 (NFκB2) that are classified into two groups based on their modes of synthesis and activation (reviewed in [39, 40]). Class I NFκB includes Rel A, Rel B and c-Rel that are synthesized in their mature form and are composed of an amino (N) terminal Rel homology domain (RHD) responsible for DNA binding activity, protein dimerization, and association with inhibitory kappa B (IκB) proteins, and a carboxy (C) terminus containing the transactivation domain. Class II NFκB (NFκB1 and NFκB2) are synthesized in an immature precursor forms p105 or p100 and are composed of RHD on the N terminus, and ankyrin repeats on the C terminus but lack a transactivation domain. Ubiquitin dependent proteolytic processing is required to degrade the ankyrin repeats and set the shorter forms p50 and p52, respectively, to either homodimerize (p50:p50) or heterodimerize (p50:p65, p52:RelB) with class I NFκB subunits. The heterodimer p65: p50 and the homodimer p50: p50 are the major forms of NFκB found in normal mammary epithelial cells; however, elevated RelB: p52 form of NFκB was found in mammary carcinoma cell lines and primary

tumors [41, 42]. Although most NFκB dimers are activators of transcription, homodimers p50/p50 and p52/p52 can repress the transcription of their target genes [43].

Regulation of NFκB activity

Mammalian IκB protein family is composed of 7 members: IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and NFκB1/p105 and NFκB2/p100 precursor proteins, responsible for binding NFκB transcription factor and holding it inactive in the cytosol (reviewed in [39, 40]). IκB kinase (IKK), considered the major activator of NFκB, phosphorylates IκB to label it for ubiquitin dependent degradation. Degradation of IκB unmasks the DNA binding domain and allows the free NFκB dimers to translocate to the nucleus and activate target genes [44]. IKK complex is comprised of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ) [40]. A third IKK form (IKKε) was also discovered [45] but its role and regulation are not well understood.

The regulation of NFκB activation is very intricate, leading to activation of specific genes in a stimulus specific and cell type specific manner [46]. IKK complex activates NFκB via two pathways; the classical or canonical pathway and the alternative pathway [40]. Microbial and viral infections (e.g., LPS) activate NFκB (RelA, p50 and c-Rel) via IKKβ through the canonical pathway, while members of the TNF cytokine family such as lymphotoxin β activate alternate NFκB forms (comprised of RelB:p52) via IKKα through the alternative pathway (reviewed in [39]). Although IKKβ is the major catalytic subunit in the canonical pathway of NFκB activation, IKKα was required for the activation of NFκB by receptor activator of NFκB ligand (RANKL) leading to cyclin D1 expression [47].

Both ET-induced MyD88-dependent and MyD88-independent pathways of NFκB activation are part of the canonical pathway leading to the activation of the IKK complex and subsequently NFκB [48]. The MyD88-dependent pathway involves MyD88 and a cascade of kinases such as insulin-1 receptor associated kinase 1 (IRAK1), IRAK4, TNF receptor associated factor 6 (TRAF6) and leads to rapid activation of NFκB and AP-1 transcription factors (Fig. 1.1). On the other hand, the MyD88-independent pathway is not well characterized but is known to involve kinases such as TRIF (Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β) resulting in activation of IRF3 (interferon (IFN) regulatory factor 3) and mediates slower activation of NFκB and AP-1 transcription factors [49]. The end result of either or both LPS induced pathways is activation of IKK complex that activates NFκB and subsequently induces the activation of NFκB dependent genes such as inflammatory cytokines (IL-6, IL-1, TNFα) [50, 51], chemokines IL-8 [52], extracellular matrix metalloproteases (MMPs), the inducible form of nitric oxide synthase (iNOS) responsible for NO production [53], and the inducible cyclooxygenase-2 (COX-2) that converts arachidonic acid to prostaglandins (PGs) [38, 54]. Inhibition of IKKα and IKKβ should inhibit the canonical pathway of NFκB activation.

Interleukin-6

IL-6 is a multifunctional cytokine produced by most immune cells as well as by many other non immune cells such as endothelial, fibroblast and epithelial cells [55, 56]. IL-6 is often a marker of acute or chronic inflammation in clinical diagnostic assays [57-

60]. Several factors such as LPS and other cytokines (e.g., IL-1β and TNFα) induce the expression of IL-6 mRNA [61-64]. In addition to cytokine responsive elements, the promoter of human IL-6 was shown to contain serum response elements (reviewed in [65]). The expression of IL-6 is mediated via the MyD88-dependent pathway of NFκB activation [66]. IL-6 mRNA contains AU rich element (ARE) in the 3' untranslated region [67] which suggest a possible regulation of IL-6 expression on the translational level. IL-6 affects the activity of several types of cells by either binding to its cell surface receptor to induce autocrine and/or paracrine signals or binding to its soluble receptor (sIL-6R) with the resultant IL-6:sIL-6R interacting with cells that lack the expression of IL-6R to induce systemic effects away from its site of production (reviewed in [55, 56, 58]). The binding of IL-6 to its receptor activates a janus activated kinase (JAK)-signal transduction and activator of transcription 3 (STAT-3) signaling pathway involved in cell apoptosis [68] and cell cycle arrest [69]. Moreover, IL-6 was suggested to have a role in the development of the mammary gland [70].

Nitric oxide

Nitric oxide is a pleiotropic molecule whose function varies based on its concentrations and sites of production [71]. At nanomolar concentrations, NO acts as a potent vasodilator and neurotransmitter, but at higher micromolar concentrations it induces pathogen death and tissue damage [72-75]. NO is produced by the conversion of L-arginine to L-citrulline [76] via three different types of nitric oxide synthase (NOS). Neuronal (nNOS or NOS-1) and endothelial (eNOS or NOS-3) are constitutively expressed and responsible for the small amounts of NO production, while, induced NOS

(iNOS or NOS-2) is induced by inflammatory stimuli such as ET and cytokines (interferon gamma (IFN-γ), IL-1β, etc..) to produce high amounts of NO [77-80]. Onoda and Inano in 1998 [81] showed all three forms of nitric oxide synthase present in the rat mammary gland. That same study described the distribution of the three forms of NOS as follows: the inducible nitric oxide synthase (iNOS) and the endothelial nitric oxide synthase (eNOS) were present in myoepithelial cells, while only the neuronal form (nNOS) was found in the secretory epithelial cells [81]. Other studies described the presence of eNOS in both secretory epithelium and vascular endothelium of goat and bovine mammary glands [82], and iNOS in tumor associated macrophages and nNOS in endothelial and myoepithelial cell of human breast tumors [83].

Traditional folk medicine and *Centaurea* species

Traditional folk medicine has been shown as a potential source of drugs for several illnesses, such as gastrointestinal disorders, wound healing, inflammation, and certain types of tumors. There is growing interest in medicinal botanicals with nearly 50% of the medicines used today being of plant origin. *Centaurea* species are widely distributed in different areas of the world, in particular in Europe and the Middle East, with medicinal value attributed to several species from many world regions of different climate, geography and cultures. Their successful use in traditional folk medicine to treat several illnesses, including inflammation, leads to an extensive literature on the different secondary compounds [84] isolated from different species and different parts of the plant (seed, floral part, etc) and their various biological activities such as anti-bacterial [85-87], anti-mycotic [88], anti-viral [89], and hypotensive [90]. Most of the bioactive

compounds were of the flavonoid family, in addition to polysaccharides (reviewed in [84]).

Lebanese *Centaurea* species.

Lebanon, famous for its rich flora, is the habitat for many plants characteristic of the Mediterranean region. Many of its plants are used as food or as remedies in Lebanese folk medicine. Several species of *Centaurea* were identified in different climates of Lebanon; *C. eryngioides* Lam. and *C. iberica* Trev. Var. hermois Boiss. [91], and *C. ainetensis* [86], the species used in this study. Several research groups at several universities in Lebanon have studied bioactivities of some of these plants [86, 88]; however, none of these studies focused on the potential uses of these plants in pharmacology as the foundation for the pharmaceutical industry. Preliminary survey studies at the American University of Beirut demonstrated that crude aqueous or solvent extracts of *Centaurea ainetensis* reduced inflammation in CID-9 cells by inhibiting the secretion of ET-induced inflammatory markers (IL-6, TNFα) and enzymes that regulate remodeling of the extracellular matrix environment (matrix metalloproteinases; MMP) in mouse mammary cells (El- Jouni, WM., 2003, "Characterization of Potential Anti-Inflammatory Bio-Activities In Selected Indigenous Medicinal Plants Of Lebanon", MSc Thesis, The American University of Beirut, Beirut, Lebanon).

Suggested model of inflammation

Based on the literature, LPS generates an inflammatory response in the cell via the activation of transcription factors (NFκB and AP-1) that leads to the expression of

cytokines (such as IL-6), nitric oxide (NO) production, and the activation of enzymes such as MMPs and Cox-2 (Fig. 1.2). In turn, these inflammatory markers would induce a cascade of signaling pathways (e.g., IL-6, NO) or catalytic reactions (e.g., MMPS) leading to either positive or negative modulation of the inflammatory response, as well as regulation of other physiological processes in the cell such as cell cycle and apoptosis, in addition to modulation of the ECM and subsequently cell-ECM interactions leading to modulation of cellular responses in the inflamed tissue (Fig. 1.2). Since NFκB is suspected to be the major culprit in the link between chronic inflammation and cancer [92], understanding the role of NFκB in orchestrating the expression of the different inflammatory markers is essential in identifying critical points of regulation of the inflammatory response in epithelial tissues.

Purpose of the Study.

The regulation of the inflammatory response by both immune and epithelial cells is very intricate because of the multiple roles that inflammatory molecules play in normal as well as inflamed tissues. As a result, the task of searching for an effective anti-inflammatory drug has proven challenging because anti-inflammatory drugs that target one inflammatory respondent (e.g., Cox-2 inhibitors) were found to also induce serious adverse collateral side effects [93, 94]. Therefore, we propose in this study the use of mammary epithelial cells as a model for ET-induced inflammation in *non immune* epithelial cells. An epithelial model of inflammation would help us understand the signaling pathways of inflammation in non immune cells and can be used to screen and identify potential anti-inflammatory targets for chronic inflammation and potential cancer

therapies. Moreover, the use of mammary epithelial cells allows study of the role of the environment and cell-cell interaction on the inflammatory pathway and responses in the absence of immune cells. Additionally, the system can be modified to include co-cultures of immune and epithelial cells to investigate the role of chronic inflammation on early processes of tissue development or function and perhaps on cell transformation.

For this purpose we chose the mammary epithelial cell system as a model to investigate key marker inflammatory respondents interleukin-6 (IL-6) and nitric oxide (NO) induced in response to bacterial endotoxin (ET; also referred to as lipopolysaccharide (LPS)) via the regulation of NFκB activity and how the changes in the mammary cell microenvironment modulate its responses to inflammation. Furthermore, we used this model to screen and purify the anti-inflammatory fraction of *Centaurea ainetensis*, a Lebanese medicinal plant used in Lebanese traditional folk medicine to treat several types of inflammation and other diseases; and further investigate its potential targets in the ET-induced inflammation in mammary epithelial cells.

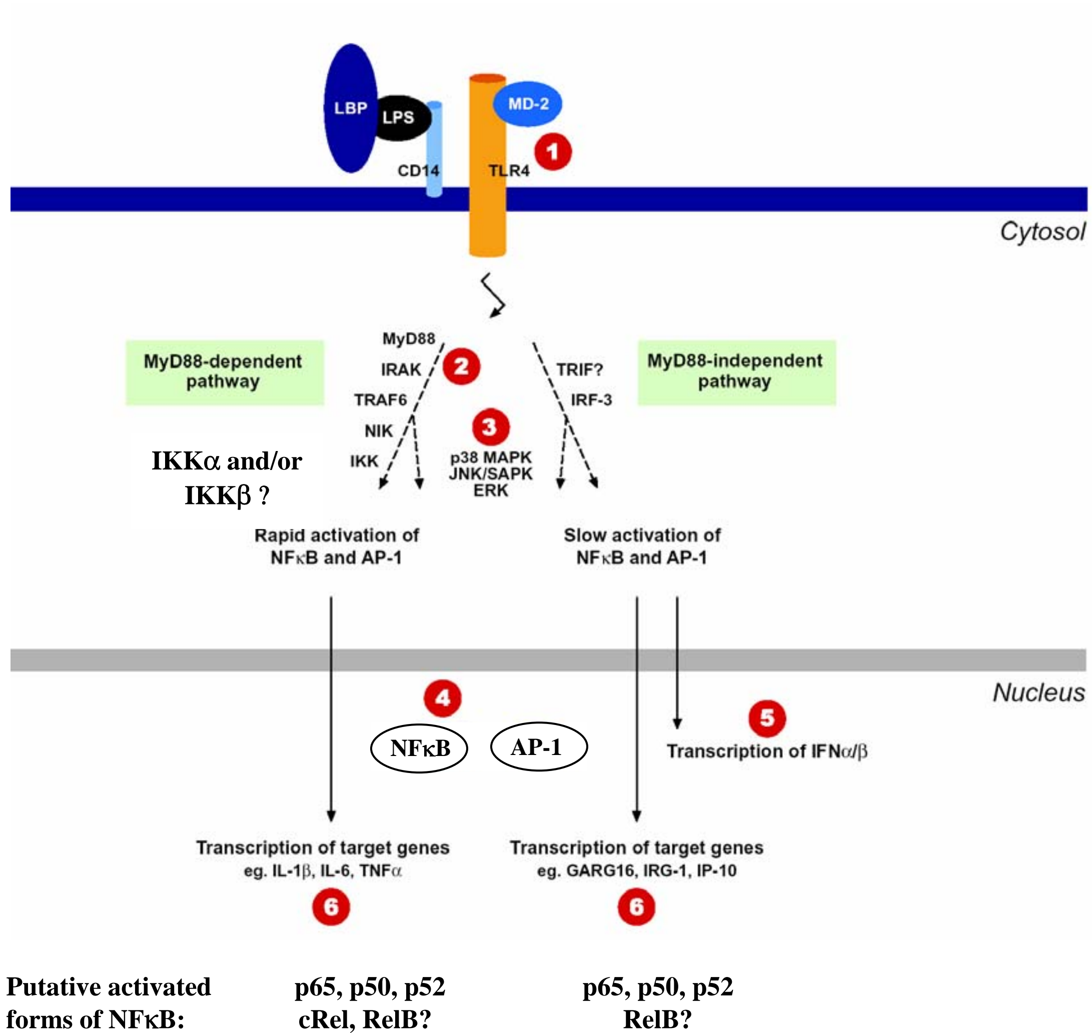

Figure 1.1: Illustration of the signaling pathway of lipopolysaccharide (LPS) adapted from Schroder et. al. (2004) [49].

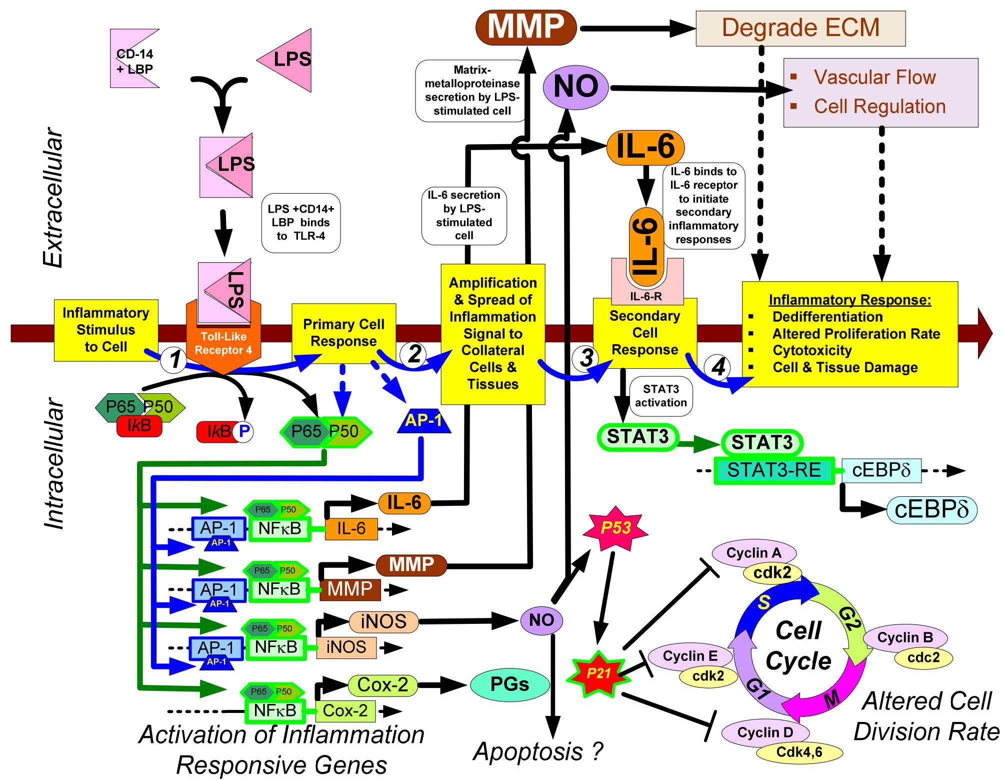

Figure 1.2: Schematic illustration of inflammatory respondent induced via NFκB and AP-1 in response to ET, and their roles in normal and inflamed tissues.

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CHAPTER 2

ENDOTOXIN-INDUCED IL-6 SECRETION AND INOS/ NO PRODUCTION ARE DIFFERENTIALLY REGULATED IN MOUSE MAMMARY EPITHELIAL CELLS

ABSTRACT

Recent findings link sustained inflammation to chronic disease or cancer in epithelia. Although inflammation in immune cells shows coordinate regulation of inflammatory interleukin-6 (IL-6) secretion and nitric oxide (NO) production by nuclear factor kappa B (NFκB), little is known about such inflammatory responses in epithelial cells. We investigated the co-regulation of IL-6 and iNOS mRNA expression and products by bacterial endotoxin (ET) via NFκB in SCp2 mouse mammary secretory epithelial cells. SCp2 cells were grown with differentiating hormones on plastic before inducing inflammation with bacterial endotoxin (ET; 10 μg/ml). ET-induced inflammatory responses were monitored by IL-6 secretion (ELISA immunoassay), NO production (Griess reaction assay), IL-6 and iNOS mRNA expression (real-time PCR), NFκB activation (Trans AM binding assay), as well as global cytokine secretion (immuno dot blot cytokine array). The results showed that ET induced IL-6

and iNOS but not eNOS or nNOS in mouse mammary secretory epithelial cells *in vitro*. Moreover, IL-6 and iNOS were both sharply induced by ET but with an unexpected delay in iNOS/NO induction compared to that of IL-6. ET also induced the activation of p65 and p50 NFκB subunits at 1 h after ET application (post-ET); however, while p65 activation was transient that of p50 remained high throughout the experiment suggesting that the observed difference in the temporal pattern of induction of NO vs. IL-6 might involve different pathways of NFκB activation. The selective inhibition of IKKα and IKKβ by Wedelolactone inhibited only ET-induced IL-6 mRNA expression and protein secretion but not that of iNOS/NO production, suggesting that the difference in the regulation of the ET-inflammatory respondents (IL-6 and iNOS) is on the level of NFκB activation. In addition to NO and IL-6, ET induced an array of chemokines such as RANTES, MCP-1 and GCSF similar to immune cells. In conclusion, we suggest mammary secretory epithelial cells, the first cells in contact with bacterial infection of the mammary gland, as a model to study the role of epithelial cells during inflammation and in innate immunity. The understanding of such innate immunity responses of epithelia and linkage thereof to immune responses is crucial for understanding the link between chronic inflammation and cancer in epithelial tissues.

INTRODUCTION

Although immune cells play a main role in inflammation, epithelial cells are often involved in acute or chronic inflammation that compromises function and health of the involved organ system due to induction of cell damage, dedifferentiation, and altered cell proliferation. Recent studies have linked failure to terminate an inflammatory response

to increased risk of chronic diseases that may lead to cancer in epithelial tissues such as colon or mammary [1-3]. As a result, there is a growing interest in inflammation and its regulation in epithelial cells [2, 4-6].

The mammary gland is highly sensitive to inflammatory insult from microbial infection and/or endotoxin exposure, but also shows evidence of inflammation in the absence of bacterial infection during developmental transitions such as development or involution [7]. Inflammation of the mammary gland results in cessation of milk synthesis and secretion, increased pro-inflammatory cytokine production, and activation of extracellular matrix (ECM) remodeling enzymes leading to involution [8]. However, the repertoire of inflammatory responses and their regulation are not well understood for epithelia such as the mammary gland in the absence of immune cells. This seems especially important since epithelial cells are often the first tissue contact for invading bacteria.

Mammary epithelial cells can be grown in culture and monitored for induced inflammation by their ability to secrete inflammatory mediators such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), nitric oxide (NO) and activated matrix metalloproteases (MMPs), and their downregulation of expression of the major milk protein, β-casein [6, 9-12]. Collectively, these characteristics make the mammary gland an interesting model for the study of inflammation in epithelial tissues and cells.

The SCp2 mammary secretory epithelial cell line is a subculture of CID-9, a mixed mouse mammary cell line (fibroblast, secretory epithelial cells, and contractile myoepithelial cells) which in turn is derived from the COMMA 1D cell line [13, 14]. SCp2 secretory epithelial cells in culture are capable of response to exogenous ECM and

lactogenic hormones by cell three dimensional clustering and induction of β-casein expression [15], thus mimicking the differentiation and normal function of mammary epithelial cells *in vivo.* Moreover, upon treatment with bacterial endotoxin (ET; also referred to as lipopolysaccharide; LPS), SCp2 cells in culture were shown to upregulate the expression and secretion of pro-inflammatory cytokines IL-6 and TNF-α, and reduce β-casein expression ([6] and Talhouk, RS; personal communication). Therefore, SCp2 cells present a good model to study the inflammatory response in mammary secretory epithelial cells and its regulation by the cell environment and intracellular signaling.

IL-6 and NO are two important inflammatory mediators reported to be also involved in normal functions of the mammary gland such as involution [16, 17]. IL-6 is a multifunctional cytokine produced by immune cells as well as non immune cells such as endothelial, fibroblast and epithelial cells [18, 19]. The expression of IL-6 is upregulated during acute inflammation mainly by NFκB and is regulated by other cytokines (IL-1β and TNFα) [20-23]. IL-6 is often a marker of acute or chronic inflammation in clinical diagnostic assays [24-27]. IL-6 binds to its cell surface receptor to induce autocrine and/or paracrine signals or binds to its soluble receptor to induce systemic effects remote from its site of production (reviewed in [18, 19, 25]). IL-6 has been described as one of the activators of apoptosis during involution of the mammary gland via its paracrine signaling pathway [28].

Nitric oxide, a companion to IL-6 in the inflammatory response, is a pleiotropic molecule whose function varies based on its concentrations and sites of production [29]. At nanomolar concentrations NO acts as a potent vasodilator and neurotransmitter, but at

higher micromolar concentrations it induces pathogen death and tissue damage [30-33]. NO is produced by the conversion of L-arginine to L-citrulline [34] via three different types of nitric oxide synthase (NOS). Neuronal (nNOS or NOS-1) and endothelial (eNOS or NOS-3) are constitutively expressed and responsible for the small amounts of NO production, while induced NOS (iNOS or NOS-2) is activated by inflammatory stimuli such as ET and cytokines (interferon gamma (IFN-γ), IL-1β, etc..) to produce high amounts of NO [35-38]. At least one or more of the three forms of NOS have been reported in the mammary glands of goats, cows and rats as well as in normal and cancerous human breast tissues [12, 39, 40].

NFκB is a key transcription factor that is regulated not only by signaling associated with cell development and function [41], but also by inflammatory stimuli such as ET and TNFα to induce the expression of inflammatory genes, such as IL-6, and iNOS [42]. Without stimulation, NFκB is found inactive in the cytosol due to binding to inhibitory kappa B (IκB). IκB kinase (IKK) phosphorylates IκB and labels it for ubiquitin-dependent degradation, thereby releasing NFκB to its active state. The free NFκB then translocates to the nucleus to activate target genes [43]. The mammalian NFκB family is comprised of five subunits: p65 (RelA), RelB, c-Rel, p50/p105 (NFκB1) and p52/p100 (NFκB2) that combine in different homo and hetero dimers to form active NFκB. NFκB can be activated by two pathways; the rapid MyD88-dependent pathway and the slow MyD88-independent pathway [44]. While the MyD88-independent pathway is poorly understood, the rapid MyD88-dependent pathway involves activation of the IKK complex which is comprised of two catalytic subunits (IKKα and IKKβ) and

a regulatory subunit (IKKγ) [44]. Bacterial ET binds to Toll-like receptor-4 (TLR4) and activates NFκB (p65/p50) via both rapid MyD88-dependent and slow MyD88-independent activation pathways [45-49]. Inhibition of IKKα and IKKβ modulates the rapid MyD88-dependent NFκB activation pathway.

IL-6, iNOS, MMP, and Cox-2 are all inflammatory respondents coregulated by NFκB that in turn is the primary intracellular mediator of ET-induced inflammation via TLR-4 [50]. Therefore, ET-induced inflammation should induce closely coordinated expression of each of the NFκB-regulated respondents. In this study, we show that ET induces IL-6 and iNOS but not eNOS or nNOS in mouse mammary secretory epithelial cells *in vitro*. Moreover, IL-6 and iNOS are both sharply induced by ET but with unexpected differences in timing of their induction for both product and mRNA. The lag in induction of iNOS vs. IL-6 suggests their differential control by NFκB. We also show that the IKKα/IKKβ inhibitor, Wedelolactone, inhibits protein and mRNA expression of IL-6 but not of iNOS. Those studies show SCp2 mammary secretory epithelial cells to be a good model to study the mechanism of regulation and coordination of induced inflammatory responses in epithelial cell systems, and especially for the mammary epithelium.

MATERIALS AND METHODS

Cell line and materials. Mouse mammary epithelial cells SCp2 were kindly provided by Dr. Pierre Desprez, (Geraldine Brush Cancer Research Institute; San Francisco, CA). Dulbecco's modified Eagle's medium/Ham F12 (DMEM/F12; 1:1), bovine insulin, ovine

prolactin, hydrocortisone, endotoxin (ET, as *Salmonella typhosa* lipopolysaccharide >500,000 EU (ET units) / mg), dimethyl sulfoxide (DMSO) and mouse monoclonal IgG1 against β-actin (A5441) were purchased from Sigma (St. Louis, MO). Heat-inactivated fetal bovine serum (FBS), Hanks's balanced salt solution (HBSS), and gentamicin were purchased from Cambrex Bio Science (Walkersville, MD). BD Falcon cell culture flasks (75 cm^2), 6-well plates and 100 mm dishes along with Englebreth-Holm-Swarm (EHS)-Matrix growth-factor-reduced BD Matrigel™ (extracellular matrix, ECM) were purchased from BD Biosciences (Bedford, MA). Complete™ protease inhibitor tablets containing specific and broad-spectrum multi-protease inhibitors were purchased from Roche Diagnostics (Mannheim, Germany). Wedelolactone, selective inhibitor of IKKα and IKKβ was purchased from EMD Biosciences (La Jolla, CA). Tetramethyl benzidine (TMB) peroxidase substrate was purchased from BioFX Laboratories (Owings Mills, MD). Rabbit polyclonal affinity purified primary IgG antibodies against iNOS (sc 650), eNOS (sc653) and nNOS (sc8309) were purchased as 200μg / ml solutions from Santa Cruz Biotechnology, Inc. Antibodies (Santa Cruz, CA), while rabbit polyclonal affinity purified primary IgG antibodies against phospho-eNOS (ser 1177; cat. # 9571) and phospho-eNOS (Thr 495; cat. # 9574) were purchased from Cell Signaling Technology, Inc (Danvers, MA). HRP conjugated anti-rabbit and anti-mouse IgG and enhanced chemiluminescence reagent (ECL) were purchased from General Electric (GE) Healthcare (Buckinghamshire, UK).

Cell culture. SCp2 cells were maintained in growth medium (GM) comprised of DMEM/F12 containing 5% FBS, insulin (5 μg/ ml) and gentamicin (50 μg/ ml) (5% FBS-GM) in a 37°C humidified atmosphere with 5% CO_2. To screen for an optimal ET dose, SCp2 cells were plated at $3x10^4$ cells / cm^2 in 96-well plates with triplicate wells / treatment. However, for all subsequent studies, SCp2 cells were plated at $4x10^4$ cell / cm^2 either in 6-well plates (~35 mm diameter / well) for RNA extraction or in 100 mm dishes for protein isolation, in duplicate wells or dishes per treatment unless otherwise specified. To ensure predominance of secretory epithelial cell type in SCp2 cell cultures, SCp2 cells were maintained at low passage number (13 through 15) and were regularly selected for secretory epithelial cells by selective trypsinization: plating freshly trypsinized cells for 1 h to allow attachment of fibroblast-like cells followed by transfer of the unattached secretory epithelial cells into a fresh plate for either maintenance or expansion for future experiments. To confirm secretory epithelial cell presence and functionality, SCp2 cells plated on plastic in 5% FBS-GM were induced to differentiate by change to differentiation medium (0%FBS-DM) comprised of serum-free DMEM/F12 containing gentamicin (50μg/ml), lactogenic hormones (insulin (5μg/ml), prolactin (3μg/ml), and hydrocortisone (1 μg/ml)), and Matrigel™ (1.5% v/v) [51]. Reverse transcribed polymerase chain reaction (RT-PCR) was used to assay for β-casein expression in differentiated SCp2 cells using the following primer set:

forward (F)= 5'- GTGGCCCTTGCTCTTGCAAG -3'

reverse (R)= 5'- AGTCTGAGGAAAAGCCTGAAC -3' [52].

ET-induced inflammation in SCp2 mouse mammary secretory-epithelial cells. A stock solution of ET was prepared at 1 mg / ml in 0% FBS-DM. SCp2 cells were plated in 5% FBS-GM for cell attachment and proliferation to achieve 50% confluence after 24 h. Cells were then washed twice with HBSS before feeding with 0% FBS-DM. After incubation for 24 h, inflammation was induced by application of a non-toxic dose of ET (10 μg / ml) in 1% FBS-DM, determined from our preliminary studies in SCp2 cells as the optimum dose able to induce a maximal inflammatory response (defined by IL-6 secretion) without cytotoxicity (measured by LDH release (Cytotox 96® Non-Radioactive cytotoxicity assay)). ET added in 1% FBS-DM remained on the cells until harvest time. The medium was collected from each well at 0, 1, 3, 6, 12, 24 and 48 h after ET application (post-ET) and supplemented with Complete™ protease inhibitor solution (1 Complete tablet/ 2 ml deionized water) [53] added at 40 μl/ ml to collected medium before storing at -80°C for later analysis. Cells were immediately washed and either frozen at -80°C for later RNA extraction or processed immediately for nuclear and cytosolic protein extraction.

Inhibition of NFκB activation by Wedelolactone, a selective inhibitor of IKKα and IKKβ. Wedelolactone is the common name for 7-Methoxy-5, 11, 12 –trihydroxy-coumestan, the naturally isolated active ingredient of herbal medicine *Eclipta alba*. Wedelolactone is a cell permeable selective and irreversible inhibitor of IKKα and IKKβ kinase activity (IC_{50} 10μM) that inhibits NFκB mediated gene transcription by blocking the phosphorylation and degradation of IκBα [54]. Wedelolactone is described as having

no effect on the activities of p38 MAPK or Akt by the provider company (EMD Biosciences). SCp2 cells were plated as above for ET-induced inflammation; however, on the day of ET treatment, Wedelolactone (5 mg / ml in DMSO) was added at 10 μM to the cells in 1% FBS DM for 1 h only before removal of Wedelolactone medium (to avoid cytotoxicity with longer exposure) and addition of ET (10 μg / ml 1% FBS-DM) [54]. Medium was collected for analysis of IL-6 and NO, and cells were harvested for either RNA or protein extraction at 0, 1, 3, 6, 12, 24, and 48 h post–ET treatment.

Immunoassay of Interleukin-6. To measure IL-6 secretion in response to ET in SCp2 cells, medium collected at various times post-ET treatment was assayed by enzyme linked immunosorbent assay (ELISA) for IL-6 (DuoSet kit; R&D Systems Inc, Minneapolis, MN) according to the manufacturer's protocol. The IL-6 ELISA was performed in Immunolon flat bottom 96 well plates (Thermo; Milford, MA) and relied on rat anti-mouse IL-6 antibody, blocking with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 5% sucrose (Sigma, St. Louis, MO), mouse IL-6 standards dissolved in either 5% FBS-GM or 1% FBS-DM as for experimental samples, and detection with biotinylated goat anti-mouse antibody followed by streptavidin conjugated to horseradish-peroxidase (HRP). Addition of HRP chromophoric substrate TMB yielded color intensity (A_{690}) in proportion to the amount of IL-6 present in the sample. Samples were assayed in duplicate and data is represented as the average pg IL-6 / ml of duplicate samples ± standard error of the mean (SEM).

Griess reaction assay of NO for NOS activity. The analysis of NO was accomplished by the Griess assay for nitrite (the spontaneous oxidation product of NO) using a Griess Reagent Kit [55] (Molecular Probes, Eugene, OR) involving addition of 20 μl of Griess Reagent (0.05% N-(1-napthyl) ethylenediamine dihydrochloride, 0.5% sulfanilic acid in 2.5% phosphoric acid) to 150 μl of nitrite-containing sample or standard and 130 μl deionized water, per the manufacturer's instructions. The color developed by azo dye formation in proportion to nitrite concentration (μM) in solution was quantified by A_{550}. Assays of nitrite in cell culture medium used sodium nitrite ($NaNO_2$) standards diluted in appropriate medium for that experiment. Samples were assayed in duplicate and data is represented as the average concentration of NO_2^- (μM) of duplicate samples $\pm$ SEM.

RNA extraction, reverse-transcription and quantitative real time polymerase chain reaction analysis. Total RNA was scrape harvested from cells grown in 6-well plates using Qiagen RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Isolated RNA was quantified by A_{260}, and only RNA with a A_{260} / A_{280} ratio $\geq$ 1.6 was used for reverse transcription (RT) reactions. A defined amount of RNA (1μg) was treated with DNAse I (Promega, Madison, WI) before being reverse transcribed by incubation with random primer and avian myeloblastosis virus (AMV) reverse transcriptase at 42°C for 30 min in a 40 μl reaction using the Promega reverse transcription system (Promega, Madison, WI). Quantitative real time PCR (qPCR) was performed by adding 2 μl of undiluted RT product to 18 μl of Qiagen Hot start SyBR Green PCR master mix (Qiagen, Valencia, CA) containing 0.15 μM each of the sense (or forward; F) and anti-sense (or reverse; R) primers for each of IL-6, iNOS, nNOS, and eNOS target genes and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) as a reference gene (Table 2.1). A no cDNA template blank reaction was included in each PCR plate. SybrGreen fluorescence was detected with an MJ Research Opticon 2 reader (BioRad, Hercules, CA); amplified products were quantified relative to an appropriate standard curve from autonomous qPCR assay reactions. Primer pairs were either adopted from the literature or designed using Primer_3 primer design software [56] and synthesized by Operon Biotechnologies Inc (Huntsville, AL). Target amplicons spanned an intron to exclude amplification of genomic DNA. The standard curves for each of IL-6, iNOS and GAPDH genes of interest were generated by serial dilutions of the purified (QIAquick clean up kit; Qiagen, Valencia, CA (per manufacturer's instructions)) and sequenced RT-PCR product amplified from total RNA isolated from ET-treated SCp2 cells. Trials to amplify nNOS and eNOS RT-PCR products were not successful in either control or ET treated SCp2 cells. Quantitative PCR conditions were, in sequence: a hot start at 94°C, 15min; followed by initial denaturation at 94°C, 5min, and subsequent annealing at 58°C, 30 sec (Table 2.2). Each sample was analyzed in triplicate qPCR reactions with the triplicate average for each gene of interest normalized against the average for the reference GAPDH gene. The results of qPCR analysis are presented as the average for duplicate samples $\pm$ SEM.

Intracellular protein isolation. Total proteins were extracted by scraping SCp2 cells in lysis buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 1% v/v Triton X) supplemented with 0.5% sodium orthovandate and 40 µl of Complete^{TM} protease inhibitor solution (1 tablet/ 2 ml deionized water) per 1 ml lysis buffer immediately added before use. Separate nuclear and cytosolic proteins were selectively extracted using a commercial Nuclear

Extract kit (Active Motif; Carlsbad, CA) according to the manufacturer's protocol. Cells were washed and gently scraped in PBS containing phosphatase inhibitor before centrifuging at 45 x g for 10 min. After cell membrane permeabilization in a proprietary 1X hypotonic buffer supplemented with 5% (v/v) detergent solution, the released cytosolic proteins were collected in the supernatant after centrifugation at 14,000 x g for 30 s at 4°C. Sedimented nuclei were then lysed with a proprietary lysis buffer in the presence of protease inhibitor cocktail (provided in the kit) and nuclear proteins were collected in the supernatant after centrifugation at 14,000 x g for 10 min at 4°C. Total protein concentration of nuclear and cytosolic extracts was determined using the Bradford dye binding protein assay [57] (BioRad, Hercules, CA) with bovine serum albumin as standard. Protein extracts were stored at -80°C until analyzed.

Western immunoblot analysis. Equal amounts of either total or cytosolic proteins were mixed with sodium dodecyl sulfate (SDS) loading buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue) freshly supplemented with 10% β-mercaptoethanol before boiling for 5min. The denatured samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) along with Rainbow protein molecular weight markers (General Electric (GE); Buckinghamshire, UK). Resolved proteins were transferred to Polyvinylidene Fluoride transfer membrane (PVDF, GE) by electroeluion. To block non-specific binding, the blotted membranes were incubated with 5% fat-free powdered milk in 20mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween 20 (TBST) for 1h at room temperature. Blocked membranes were then incubated with specific primary

antibodies overnight at 4°C. The following day, after extensive washing with TBST, immunoprobed membranes were incubated for 1h with HRP-conjugated anti-rabbit IgG antibody (1:10,000). Signal detection was evaluated using enhanced chemiluminescence reagent (ECL) and exposure to Kodak x-omat AR film (Kodak; New Haven, CT). The primary antibodies were used per each manufacturer's recommendation: anti-iNOS (1: 1,000), anti- eNOS (1:500), and anti-nNOS (1:500) were diluted in 5% milk TBST while anti-phospho eNOS (Ser 1177) (1:1,000) and (Thr 495) (1:1,000) were diluted in 5% BSA Tris-buffered solution supplemented with 0.1% Tween 20. To verify equal loading, developed immunoblot membranes were reprobed with mouse IgG1 anti-β-actin (1:1,000) for 1 h at room temperature before incubating with HRP conjugated sheep anti-mouse secondary IgG antibody (1:10,000) for 30 min at room temperature. Molecular weights of target bands were verified by their migration relative to both Rainbow protein molecular weight markers and positive signal bands from total protein extracts of primary bovine aortic endothelial cells (BAEC) provided as a positive control by Cell Signaling Technology.

Immunobinding assay for NFκB activity. The NFκB activity in nuclear proteins was determined by using the NFκB family Trans-AM NFκB binding assay kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The primary antibodies, provided as 2μg / μl solution, were known to cross react with human p65, p50, p52, c-Rel, and RelB, as well as with mouse and rat p65 but not to p50, with cross reaction to the remaining NFκB subunits untested. Moreover, the primary antibodies used to detect

NFκB recognize an epitope that is present only when NFκB is activated and bound to target DNA. Briefly, 5 μg of nuclear proteins from control or ET-treated SCp2 cells were added to each well of the 96-well plate pre-coated with oligonucleotide containing target NFκB consensus binding sequence (5'- GGACTTTCC -3) and incubated for 2 h at room temperature. The provided primary antibodies were then added to assigned wells at the recommended dilutions (1:1,000) for 1 h before removal, washing, and addition of HRP conjugated anti-rabbit IgG (1:1,000 of 0.25 μg / μl stock solution) and incubation for an additional 1 h. Wells were washed three to four times with washing buffer (provided) before adding 100 μl of developing solution which yielded color intensity (A_{690}) in proportion to the activity of NFκB subunits present in the nuclear protein extract samples. Positive and negative controls were assayed simultaneously to verify response specificity. Each sample was assayed in duplicate and data is represented as the average of duplicate samples ± SEM.

Immuno dot blot Cytokine protein Array analysis. RayBio™ Mouse Cytokine Array I was purchased from RayBiotech, Inc (Norcross, GA). Conditioned medium collected from control and ET-treated SCp2 cells (1 ml / well of 6 well plate) at 1, 3, 6, and 12 h post-ET was incubated with the cytokine array membranes according to RayBiotech protocols. Signal was detected using a provided ECL Plus detection system (Amersham Phramacia Biotech) and exposed to Kodak x-omat AR film (Kodak; New Haven, CT).

Statistical analysis. Significant differences between different groups were determined using Proc Mixed analysis of SAS 9.1 (SAS Institute Inc., Cary, NC). For each of the

experiments studying the effect of ET alone or ET in the presence or absence of Wedelolactone (IKK inhibitor) on IL-6, iNOS/NO and / or NFκB, the statistical model included time, treatment (ET, Wedelolactone, or ET and Wedelolactone), and time by treatment interactions. The effect of treatment within each time point was tested using the slice option by time. Results were expressed as mean ±SEM, and significance was defined as $p<0.05$, unless noted otherwise.

RESULTS

ET induced IL-6 secretion and NO production in SCp2mouse mammary epithelial cells. In order to study the co-induction of IL-6 and NO by ET in mouse mammary epithelial cells, initial dose response studies were done to determine an optimal ET dose able to induce maximal inflammation, as measured by IL-6 secretion, without cytotoxicity. ET doses (0-100 μg of ET / ml 5% FBS-GM) were applied to SCp2 cells at 50% confluence (generally 24 h after plating). After exposure to ET for 24h, the medium was collected and analyzed for IL-6 secretion by ELISA. The optimum ET dose for induction of inflammation without cytotoxicity was determined to be 10μg ET/ ml (data not shown) which generally agrees with doses used by others to induce inflammatory responses *in vitro* in mammary epithelial cells [6]. The optimum ET dose of 10 μg / ml was used in the remainder of this study.

To study the temporal pattern of ET-induced IL-6 secretion and NO production, SCp2 cells were plated and grown in 5% FBS-GM for 24 h before treatment with a single application of 0 or 10μg ET / ml of 1% FBS-DM for 0, 1, 3, 6, 12, and 24 h. The

collected medium was analyzed for IL-6 and nitrite concentrations at each time point. Fig. 2.1 shows the resulting temporal pattern of ET-induced IL-6 secretion and nitrite production. IL-6 secretion significantly increased by 3 h post-ET and continued to increase up to 12 h post-ET before the rate of secretion decreased at 24 h post-ET (Fig. 2.1 A). The rate of IL-6 secretion (calculated as increase in concentration per hour for each time interval) showed an abrupt increase from near zero at 0-1 h post-ET to a maximum between 1 and 3 h post-ET before sharply decreasing at 3-6 h to remain near zero thereafter (data not shown). In contrast, nitrite concentrations in the medium of SCp2 cells, reflecting NOS activity, did not increase until 6 h post-ET treatment and continued to increase for the duration of the experiment (Fig. 2.1B). The rate of nitrite production was near zero from 0-3 h, increased sharply between 3-6h, and started to decrease between 6 and 12 h to return to near zero thereafter (data not shown).

Co-induction of IL-6 and iNOS mRNA expression by ET treatment in SCp2 cells. To further investigate the temporal patterns of ET-induced expression of IL-6 and iNOS mRNA, total RNA was isolated from SCp2 cells treated with ET for 0, 1, 3, 6, 12 and 24 h, and analyzed by qPCR. The amplified mRNA of genes of interest were normalized to mRNA of the GAPDH housekeeping gene. The results (Fig. 2.2) show a sharp induction of IL-6 mRNA expression as early as 1 h after ET treatment which increased to peak at 3 h before sharply decreasing to control concentration by 6 h post-ET treatment. Interestingly, a transient increase in IL-6 mRNA expression was observed at 1 h after change of medium from 0% FBS-DM to 1% FBS-DM with 0 μg ET/ml (control medium) (Fig. 2.2A), suggesting a role for serum in IL-6 mRNA expression. Also, a slow rise in

the expression of IL-6 mRNA was observed with time between 12 and 24 h post-ET in both ET-treated and control cells (Fig. 2.2 A). In contrast, iNOS mRNA expression was not induced at 1 h post-ET but showed a sharp increase at 3 h post-ET from near zero to peak at a maximum level 10-15 fold higher than the maximum induction of IL-6 mRNA expression at the same time point. At 6 h post-ET, iNOS mRNA expression decreased sharply and was maintained near control concentrations for the remainder of the experiment (Fig. 2.2B).

Though ET is known to upregulate the expression of iNOS [12, 37, 58], we also investigated the expression of eNOS and nNOS mRNA since there are conflicting reports on the NOS forms in the different cell types comprising the mammary gland [12, 16, 39]. Despite testing different primers and varying the PCR conditions as described in Table 2.1 and 2.2, we were unable to conclusively show eNOS or nNOS mRNA expression in SCp2 mammary cells (data not shown), in contrast to the readily amplified iNOS mRNA. Hence, it appears that iNOS is the major NOS in SCp2 mouse mammary secretory epithelial cells after ET treatment.

Immunoblot analysis of the protein expression of the three isoforms of NOS. To investigate possible activation of NOS by post-translational phosphorylation, western immunoblotting analysis was used to examine the temporal pattern of protein expression for iNOS, eNOS and nNOS in SCp2 cells following ET treatment. Immunoblots of iNOS showed no target bands in control untreated cells. However, a target 130 kDa band appeared at 3 h post-ET treatment, and persisted at 6 h before starting to disappear after 12 h (Fig. 2.3A), matching the temporal pattern of ET-induced IL-6 secretion (Fig. 2.1A).

An inexplicable lower, but apparently non-specific unidentified band at ~120 kDa appeared in all samples assayed independent of ET treatment, including in the bovine aortic endothelial cells (BAEC) protein extract used as positive control. Immunoblots of total eNOS showed an expected specific upper band at ~140 kDa and a second slightly lower band at ~130 kDa. Both bands appeared in the BAEC positive control, with the upper band more intense (Fig. 2.3A). Moreover, immunoblots for the phosphorylated forms of eNOS (Fig. 2.3B) (phosphoserine (Ser 1177) and phosphothreonine (Thr 495)) showed no positive bands except in the BAEC positive control. Though eNOS showed as a ~ 140 kDa band, there was no clear pattern of regulation of eNOS protein expression or its phosphorylation after ET treatment in SCp2 mammary cells. Immunoblots for nNOS showed no positive reaction with the antibody used, even in the positive BAEC control (Fig. 2.3A).

NFκB activity in SCp2 cells in the presence or absence of ET. We undertook studies to confirm NFκB activation in response to ET, define the temporal pattern of its activation, and compare that pattern to the temporal pattern of ET-induced IL-6 and iNOS mRNA expression and product formation. The nuclear proteins isolated from SCp2 cells treated with 0 or 10 μg of ET/ ml of 1% FBS-DM for 0, 1, 3, and 6 h were analyzed for the different forms of NFκB using the NFκB family binding assay (described in methods). Of the 5 subunits of NFκB tested, two forms (p52 and c-Rel) were known not to be reactive with the human primary antibodies provided in the assay kit. The other three forms p65, RelB and p50 showed a specific signal in response to their respective

provided primary antibodies, although anti-human p50 was not expected to react with mouse p50. Upon ET treatment, only p65 and p50 increased in response to ET compared to the basal concentrations in SCp2 cell nuclear protein extracts. Fig. 2.4A shows an abrupt and significant increase in ET-induced p65 activity to peak at 1 h post-ET treatment then decrease to 1/3 the peak level by 3 hrs and continue to decrease at 6 h, but remaining well above the basal level seen in non-ET treated cells at 3 and 6 h. In contrast, p50 activity increased at 1 h post–ET, remained significantly high at 3h post-ET then slightly decreased at 6 h post-ET (Fig. 2.4B). Surprisingly, non-ET treated SCp2 cells showed transient and significant ($p<0.01$) elevation of p65 activity at 1 h after change of medium from 0% FBS-DM to 1% FBS-DM with 0 μg ET/ml (control medium), peaking at about 1/3 lower than ET-induced 1h p65 peak activity, suggesting that a change in serum concentration alone might modulate NFκB for ET treated cells at 1 h post-ET (Fig. 2.2A) and its respondents. In contrast, p50 was not activated in SCp2 cells in the absence of ET (Fig. 2.2B). These results clearly show how regulation of NFκB activity via differential regulation of its subunits by ET, and by serum, and that NFκB is activated very early after exposure of cells to ET and before or commensurate with ET-induced expression of both IL-6 and iNOS.

The effect of IKK Inhibitor on ET induction of IL-6 and iNOS mRNA. To determine if the expression of ET-induced IL-6 and iNOS was via NFκB in non immune mouse mammary epithelial cells, Scp2 cells were treated with Wedelolactone, an irreversible inhibitor of IKKα and IKKβ activities [54], thus inhibiting IKK-dependent NFκB

activation and its respondents. SCp2 cells were incubated with Wedelolactone (10μM) in 1% FBS-DM for 1 h prior to ET treatment (described in methods). Cells were collected at 0, 1, 3, and 6 h post-ET treatment for total RNA extraction. qPCR analysis showed that treatment with Wedelolactone significantly reduced IL-6 mRNA expression at 3 h after ET (by ~70%, $p<0.05$) compared to that of cells treated with ET but without Wedelolactone treatment (Fig. 2.5A). However, the expression of IL-6 mRNA in response to ET in the presence of Wedelolactone remained significant at 1 and 3 h post-ET (Fig. 2.5A). By 6 h, ET-induced IL-6 mRNA expression had decreased to non-ET control levels (at 0 h) independent of Wedelolactone treatment (Fig. 2.5A). In surprising contrast, Wedelolactone did not inhibit iNOS mRNA expression in SCp2 cells at 3 h post-ET (Fig. 2.5B). By 6 h after ET treatment, iNOS mRNA decreased to near pre-ET control levels but remained significantly higher from control iNOS mRNA levels (Fig. 2.5B) irrespective of whether or not SCp2 cells had been pre-treated with Wedelolactone (Fig. 2.5).

The effect of IKK Inhibitor on ET induction of IL-6 secretion and NO production. IL-6 mRNA expression was significantly ($p<0.05$) inhibited by Wedelolactone only at 12 h post-ET. Surprisingly, despite strong inhibition of IL-6 mRNA expression (~70% inhibition) by Wedelolactone, ET-induced IL-6 secretion was only inhibited by 38 % compared to control cells treated with ET alone in the absence of Wedelolactone (Fig. 2.5A). In contrast, ET-induced NO production was not affected by Wedelolactone pretreatment (Fig. 2.6B), consistent with the lack of effect of Wedelolactone on ET-induced iNOS mRNA expression (Fig. 2.5B).

ET induces an array of cytokines in SCp2 mouse mammary cells. We also investigated the secretion of other cytokines in the medium of SCp2 cells after 1, 3, 6 and 12 h of ET application. The medium collected at the designated times after ET treatment was analyzed by RayBio Mouse cytokine Antibody Array I. Of the 22 cytokines tested on the cytokine antibody array, only IL-6, granulocyte colony stimulating factor (GCSF), regulated on activation, normal T expressed and secreted (RANTES), and monocyte chemotactic protein-1 (MCP-1) were secreted into the medium of ET-treated but not control cells not treated with ET (Fig. 2.7). Both GCSF and IL-6 protein were secreted starting at 3 h post-ET and appeared to increase up to 12 h post-ET. RANTES seemed to increase slowly starting at 6 h and stained darker at 12 h post-ET. MCP-1 was high at 1 h in both control and ET-treated cells and increased with time in both groups, however, the intensity of MCP-1 in ET-treated cells was higher at 12 h post-ET (Fig. 2.7). The additional regulatory factors induced by ET treatment, together with the differential regulation of IL-6 and iNOS (NO) shown here, and MMP regulation shown previously by Safieh-Grabedian et al (2004) [6] indicate a complex inflammatory response by mammary secretory cells, and likely for other epithelia, in the absence of immune cells.

DISCUSSION

We investigated the coregulation of IL-6 and NO by ET in SCp2 mouse mammary secretory epithelial cells. Though IL-6 and NO are predicted to be induced by ET from studies in immune cells [59, 60], they showed a difference in the temporal patterns of their production in response to ET in SCp2 mouse mammary secretory

epithelial cells. The difference in timing was observed in both product and mRNA expression (Fig. 2.1 & 2.2). While, IL-6 secretion was significantly induced early at 3h post-ET, and continued to increase with a slower rate until 24 h post-ET (Fig. 2.1A), NO production began later, not increasing until 6 h post-ET ($p<0.05$) and continued to increase steadily until 24h post-ET. Although the observed delay in NO production might be partly explained by the time necessary for iNOS to synthesize NO and the rate of the conversion of the latter to a more stable nitrite (NO_2^-) form, the observed delay in expression of iNOS mRNA not induced before 3 h post-ET (Fig. 2.2B) compared to that of IL-6 mRNA induced at 1 h post-ET (Fig. 2.2A) supports a difference in the regulation of both ET-induced respondents.

Although ET-induced IL-6 secretion and NO production continued to increase with time (Fig 2.1), the computation of rate of IL-6 secretion or NO production per cell (data not shown) showed a similar temporal pattern to that observed for the respective mRNA expressions (Fig. 2.2). ET-induced IL-6 secretion peaked between 1 and 3 h post-ET, while that of NO production peaked between 3 and 6 h post-ET (data not shown).

On the other hand, the delay of ET-induced iNOS mRNA expression and NO production compared to that of IL-6 expression and secretion also suggested that IL-6 protein might induce the expression of iNOS mRNA. However, treatment of SCp2 cells with different doses of exogenous mouse recombinant IL-6 protein at concentrations (0.1, 1, and 10 nM) representing respectively 1/10, 1, and 10 X the concentrations of secreted IL-6 protein found in the medium of ET-treated SCp2 cells, did not induce the expression of iNOS when applied alone in the absence of ET (data not shown), suggesting that the

expression of iNOS mRNA was induced in response to ET alone. In contrast, comparing the sharp decrease in ET-induced IL-6 rate of secretion at interval 3 - 6 h (data not shown) to the sharp increase in ET-induced rate of NO production during the same time interval (data not shown) suggests possible negative regulation of ET-induced IL-6 secretion by ET-induced NO production in SCp2 mammary cells, in agreement with reports describing inhibition of IL-6 by NO donors in mouse macrophages [61] and in human endothelial cells [62].

We also investigated the effect of ET on the binding activity of p65 and p50, the major NFκB subunit types known to be involved in ET signaling via the rapid MyD88-dependent pathway (reviewed in [63]). Fig. 2.4 showed a rapid activation of both p65 and p50 at 1h post-ET; however, while p65 activation was transient, activation of p50 was sustained through 6 h post-ET. Although others (reviewed in [63]) described the activation of p65/p50 NFκB via the rapid MyD88-dependent pathway involving IKK-dependent IκB phosphorylation and ubiquitin-dependent degradation, other reports suggested that the regulation of p50 might involve additional pathways other than IκB degradation [64]. In any case, the difference in p65 and p50 activation suggests that the difference between ET-induced IL-6 and iNOS mRNA expression might involve different pathways of NFκB activation, one that is IκB degradation dependent while the other is not, therefore, inhibition of the IKK complex that phosphorylates IκB and labels it for degradation would modulate only the IκB dependent NFκB activation pathway and its target genes.

SCp2 cells were treated with Wedelolactone, a selective and irreversible inhibitor

of IKKα and IKKβ that inhibits both IκB phosphorylation and ubiquitin-dependent degradation[54] and therefore should inhibit IκB dependent NFκB activation. Consistent with the difference in regulation of both IL-6 and iNOS mRNA expression, Wedelolactone treatment inhibited only ET-induced IL-6 mRNA expression but not that of iNOS. These results suggest that while IL-6 mRNA expression is likely dependent on the rapid MyD88-dependent NFκB activation pathway, iNOS mRNA expression might involve the delayed MyD88-independent NFκB activation pathway. Interestingly, Although ET-induced IL-6 mRNA showed a short half life by peaking at 3 h post-ET and decreasing to control levels thereafter (Fig. 2.2A and 2.5A), ET-induced IL-6 protein secretion into the medium of SCp2 cells started to increase at 3 h post-ET and remained high at 12 h to start to plateau (Fig. 2.1A) or slowly decrease (Fig. 2.6A) by 24 h post-ET depicting a longer half life of the protein. This observation explains the discrepancy between the observed ~70% inhibition of ET-induced IL-6 mRNA by Wedelolactone at 3 h post-ET (Fig. 2.5A) and limited 38% inhibition of ET-induced IL-6 secreted protein in the medium of cells treated with Wedelolactone at 12 h post-ET (Fig. 2.6A).

In an attempt to investigate the temporal pattern of other NFκB subunits in response to ET, we found that RelB binding activity was not modulated by ET at any time point post-ET treatment. Unfortunately, we were unable to detect c-Rel and p52 from mouse SCp2 cells with the available assay kits relying on antibodies to human NFκB subunits. In contrast, when similar studies by Safieh-Garabedian et al, (2004) [6] investigated the activation of NFκB subunits in the mixed cell CID-9 mammary cells at 1 h post-ET, only p52 but not p50 nor p65 were activated, suggesting analytical differences

or that cell-cell interaction might affect the activation pattern of NFκB, the resolution of which requires further investigation.

Interestingly, serum addition without ET treatment induced the binding activity of p65 but not p50 at 1 h post addition of 1% FBS-DM (the vehicle for ET addition) in the absence of ET (Fig. 2.4). In addition to demonstrating a difference in the activation of different NFκB subunits in the ET-signaling pathway in mammary epithelial cells, this also demonstrates that the environment of cells in culture (i.e. addition of serum) is capable alone of influencing NFκB activity in the absence of inflammatory stimuli. It is suggested, therefore, that serum addition plays a role in modulating p65-containing NFκB dimers and their target genes such as IL-6 (Fig 2.2A). In addition, others demonstrated the requirement of serum in ET-induced signaling as a source of CD14 (cluster of differentiation 14) and LPS binding protein as co-receptors for ET binding to its TLR4 receptor [65, 66]. However, it remains to be determined as to how serum modulates NFκB activation and its target gene expressions in the absence of ET. Analysis of serum used in these studies showed undetectable ET, and therefore was free of ET at concentrations capable of eliciting ET-induced inflammatory responses.

Kawai et al (2000) showed that ET-induced IL-6 production was impaired in MyD88$^{-/-}$ mouse macrophages [67], while Schilling et al (2002) reported that expression of iNOS but not that of IL-6 mRNA was enhanced by ET-induced IFNγ in mouse macrophages [68]. These studies, together with the present results suggest that the differential regulation of ET-induced IL-6 and iNOS by NFκB, might involve different kinetics and alternate pathways of NFκB activation for each, whereby IL-6 mRNA

expression is induced by the rapid MyD88-dependent activation pathway of NFκB but that of iNOS mRNA expression might involve the slower MyD88-independent activation pathway of NFκB. Furthermore, the observed delay in expression of iNOS compared to that of IL-6 mRNA coincides with reports describing a role for IL-6 in acute inflammation and in the transition to chronic inflammation [25], in contrast to NO that was proposed to limit inflammation [69, 70].

Though iNOS is the induced form of NOS upregulated in response to ET, several studies described a possibility for induction of mRNA expression of supposedly constitutive eNOS and nNOS in disease states of cardiovascular, skin and lung systems [71-74]. Others also showed that the constitutive activity of eNOS can be modulated by phosphorylation of amino acid residues serine 1177 (Ser 1177) and/or threonine 495 (Thr 495) in rat heart and endothelial cells [75-78]. In addition to that, rat mammary epithelial cells were reported to express nNOS both in mammary tissues and cells *in vitro* [12, 79]. Therefore, to investigate the presence and role of eNOS and nNOS in the ET-induced NO production in SCp2 mammary secretory epithelial cells, we assayed for both eNOS and nNOS mRNA as well as for proteins and phosphorylated forms of eNOS. We were unable to show conclusive qPCR amplification of either eNOS or nNOS mRNA expression in response to ET (data not shown). Moreover while total eNOS protein appeared to be expressed in SCp2 cells, we were unable to detect nNOS proteins in SCp2 mammary epithelial cells (Fig. 2.3A) with the antibodies available. Moreover, there was no evidence of modulation of eNOS activity by either its total concentration or by phosphorylation (Fig. 2.3 A&B). Thus, these data suggest that ET-induced NO in SCp2 mammary epithelial cells is due mainly to iNOS expression and activity; contrasting with

a report by Onoda and Inano (1998) of iNOS and eNOS immunlocalization only to myoepithelial and endothelial cells, and nNOS (trace amount) to epithelial cells in the rat mammary gland [12]. The absence of nNOS by immunoblot and mRNA analysis, the immunoblot analysis showing presence but not regulation of total or phospho-eNOS, and the inconclusive data of eNOS suggest that iNOS is the major NOS form upregulated in response to ET treatment in mouse mammary SCp2 cells.

Also interesting, mammary epithelial cells apparently are less sensitive than immune cells to ET-induced inflammation [80-83]. While nanomolar ET was able to induce an adequate inflammatory response in macrophages and monocytes [80-83], micromolar concentrations were needed to induce an inflammatory response in SCp2 cells, as shown here and in agreement with results for CID-9 mouse mammary cells described by Safieh-Grabedian et al (2004) [6]. Moreover, by more global immunodot-blot assay for ET-induced secretion of cytokines, we found that SCp2 mammary secretory epithelial cells secreted an array of chemokines similar to immune cells. The major chemokines secreted were GCSF, MCP-1 and RANTES in addition to IL-6. Those chemokines are involved in monocyte and leukocyte chemotaxis as well as cell differentiation, suggesting that mammary secretory epithelial cells, likely to be the first cells in contact with infecting bacteria of the mammary gland, play an important role in innate immunity and enhanced signaling involved in induction of the inflammatory response, antimicrobial defense, and the immune response. Understanding of the innate immune response of epithelia and linkage thereof to immune responses are crucial for understanding the link between chronic inflammation and cancer in epithelial tissues.

In conclusion, we have shown that ET induces IL-6 and iNOS but not eNOS or

nNOS mRNA expression in SCp2 mammary epithelial cells. ET-induced IL-6 and iNOS mRNA expression occurs likely via different regulatory mechanisms as shown by a delay in the temporal pattern of ET-induced iNOS mRNA expression and NO production compared to that of IL-6 mRNA expression and protein secretion. The inhibition of ET-induced IL-6 but not iNOS mRNA expression by the IKK inhibitor Wedelolactone suggests that the different regulation of IL-6 and iNOS by ET likely involves a rapid vs. a slow NFκB activation pathway. Therefore, SCp2 mammary epithelial cells present a good model to study the mechanism of ET-induced inflammation in epithelial cells and their modulation by anti-inflammatory agents, as well as a model to identify mechanisms of action of potential anti-inflammatory drugs, and especially if acting via NFκB is suggested to be the link between chronic inflammation and cancer.

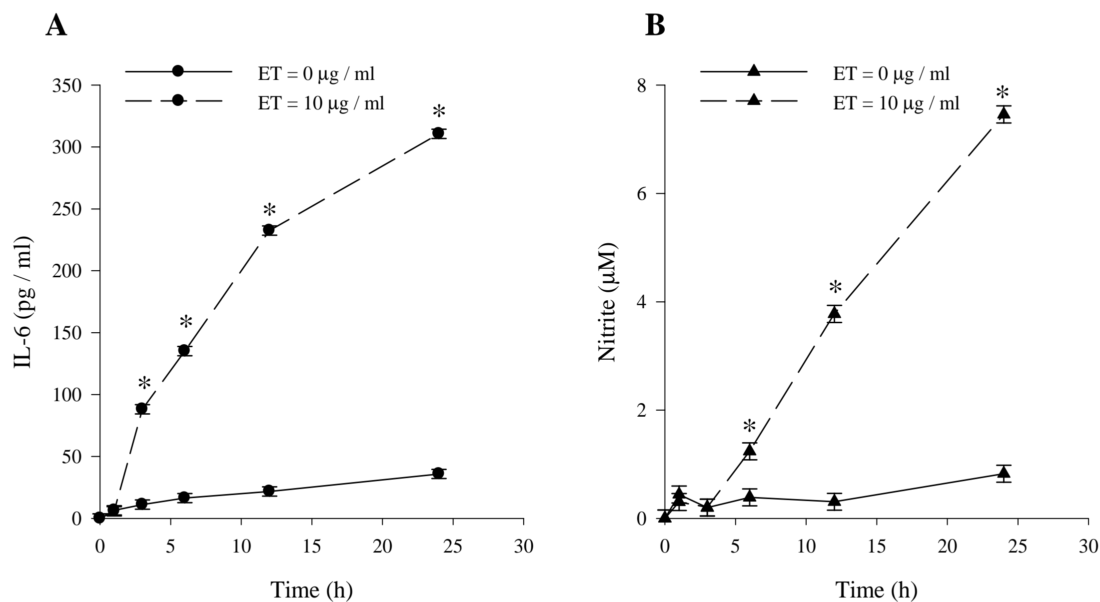


Figure 2.1: Temporal pattern of ET-induced IL-6 secretion and NO production in SCp2 mouse mammary epithelial cells. Subconfluent SCp2 cells were treated with ET (0 or 10 μg / ml) in 1% FBS DM for 0, 1, 3, 6, 12, and 24 h, and medium collected at each time point was assayed for (A) IL-6 secretion by ELISA (closed circle) or (B) NO production by Griess reaction (closed triangle). Solid-line represents the results for control samples from cells not treated with ET, while dashed-line represents the results for samples from ET-treated cells. This experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in duplicate analyses by either ELISA or Griess reaction assay. Data represent the average for IL-6 protein or nitrite concentrations for duplicate samples $\pm$ SEM of a representative experiment with (*) denoting significant differences from non-ET control within each time point ($p<0.05$).

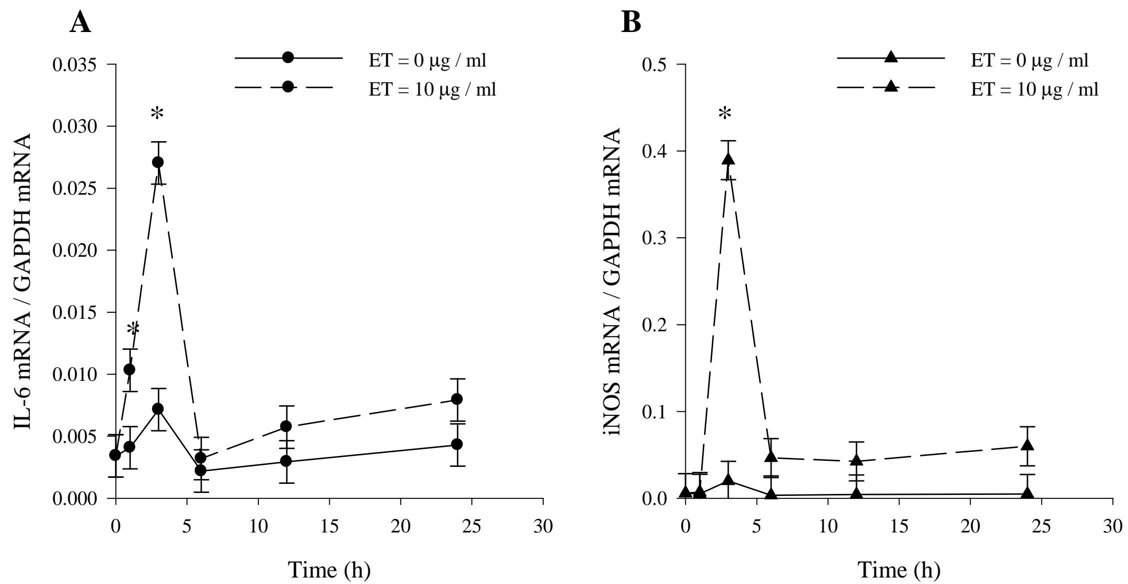


Figure 2.2: qPCR analysis of ET-induced IL-6 and iNOS mRNA expression in SCp2 cells. Subconfluent SCp2 cells were treated with ET (0 or 10μg/ml) for 0, 1, 3, 6, 12 and 24 h, and RNA harvested at each time was analyzed by RT-qPCR for ET-induced (A) IL-6 mRNA (circle) or (B) iNOS mRNA (triangle) and quantified relative to GAPDH mRNA for each. Solid line represents results for control cells not treated with ET; while dashed-line represents results for samples from ET-treated cells. This experiment was repeated at least three times. Each experiment was performed in duplicate samples / treatment / time point. Each sample was analyzed in triplicate qPCR reactions. Data represents the average for duplicate samples ± SEM of a representative experiment. (*) denotes significant differences among treatment within each time point ($p<0.05$).

Figure 2.3: ET induced expression of iNOS protein but not eNOS expression or phosphorylation in SCp2 cells. Subconfluent SCp2 cells were treated with ET (0 or 10 μg/ ml) for 1, 3, 6, 12, 24 and 48h, and intracellular proteins were harvested at each time point. Western immunoblots of total cellular protein extracts were probed for (A) iNOS, eNOS and nNOS with expected molecular weights of 130, 140 and 150 kDa, respectively, (specific bands indicated by arrow), or (B) for the phosphorylated forms of eNOS (phospho Ser 1177 and phosphor Thr 495) with expected molecular of 140 kDa. Bovine aortic endothelial cell (BAEC) total protein extract (+C) was included as a positive control. Equal loading of proteins was verified by probing for 42 kDa β-actin. The molecular weights of specific bands were verified by comparing their migration distance to rainbow protein molecular weight markers included on each gel.

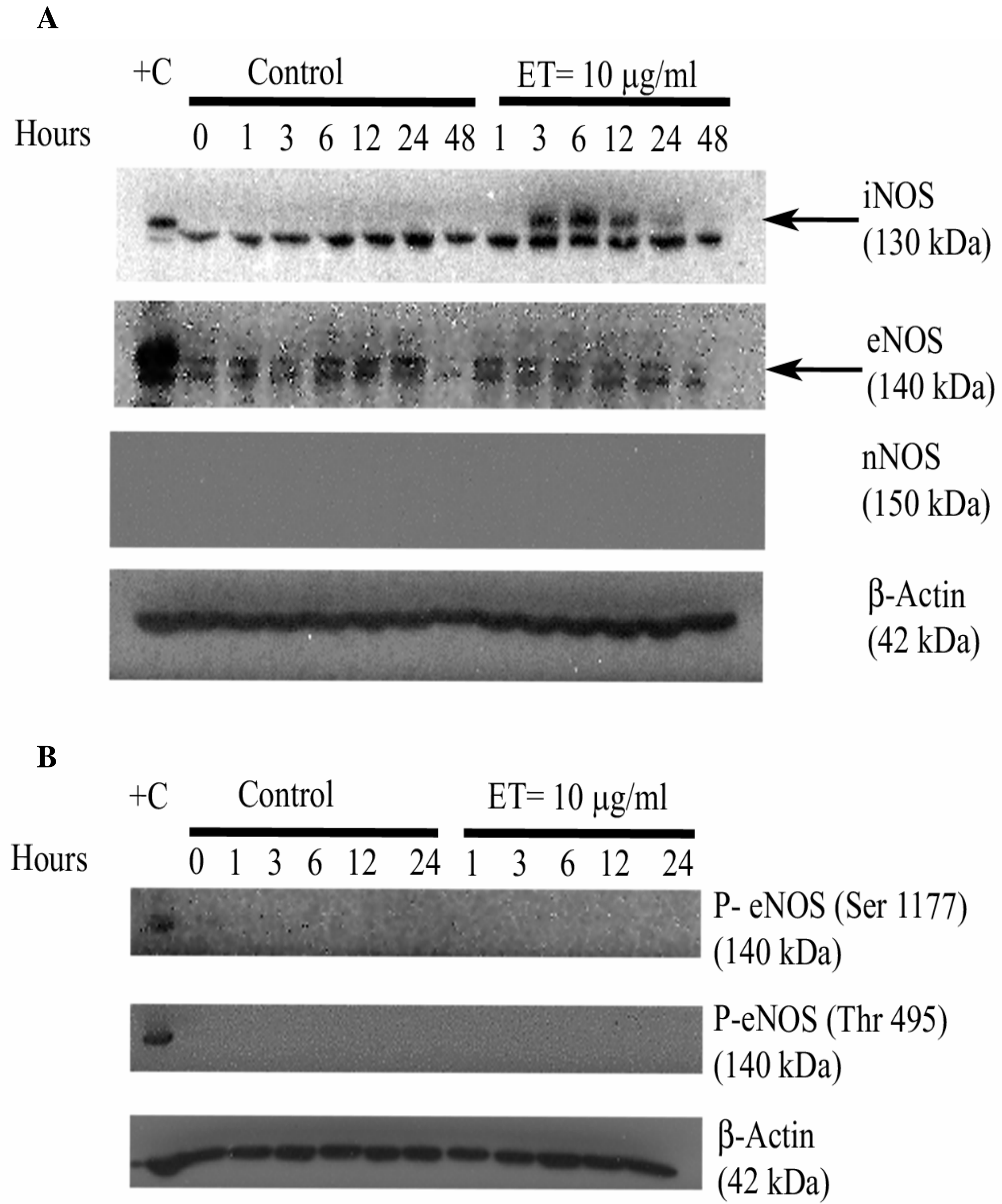


Figure 2.3

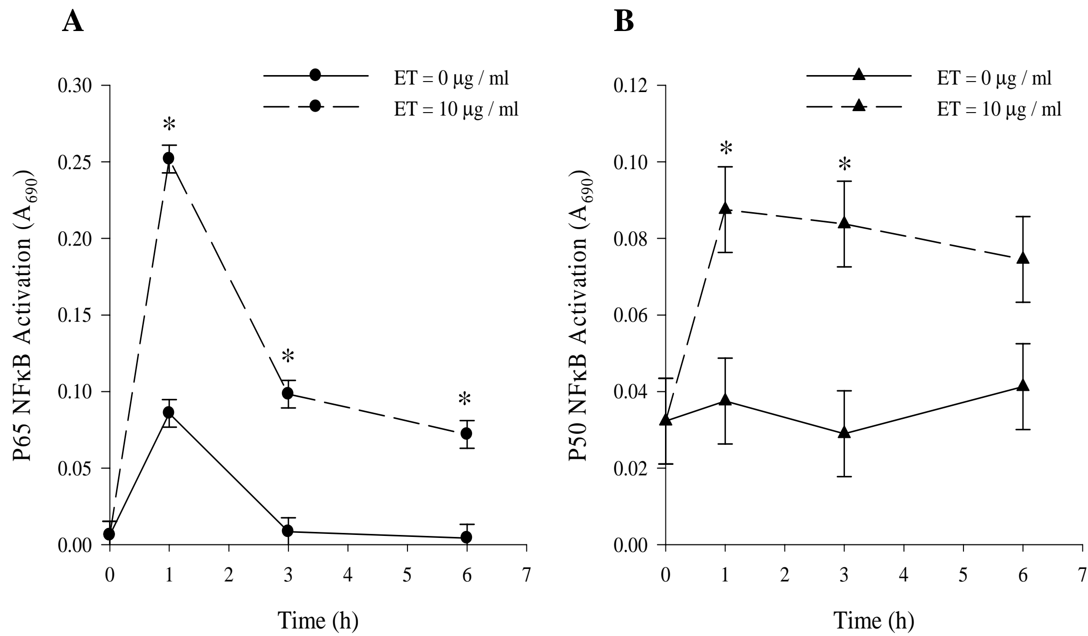
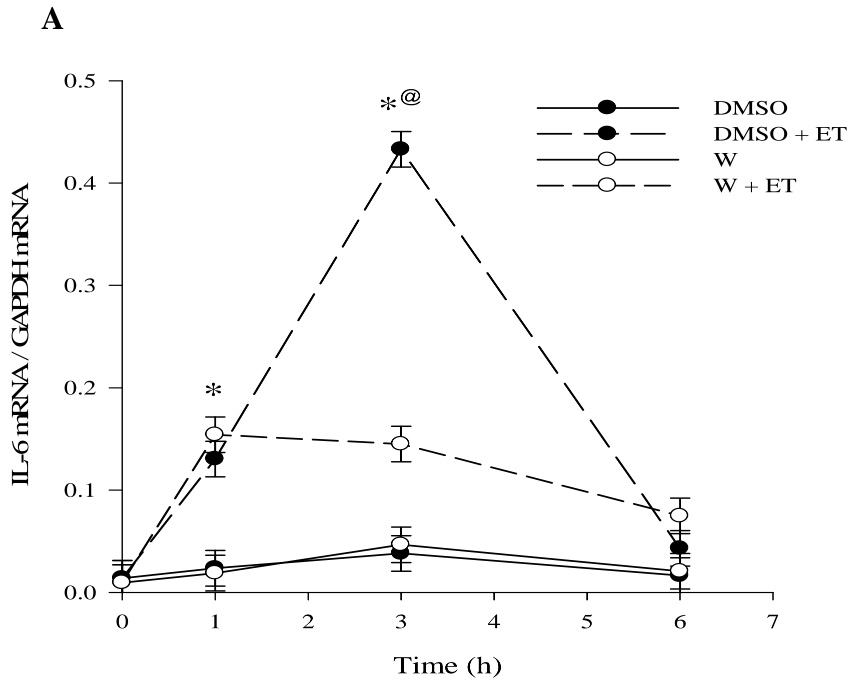


Figure 2.4: ET activation of NFκB p65 and p50 in SCp2 cells. SCp2 cells were treated with 0 or 10 µg of ET per ml of 1% FBS-DM for 0, 1, 3, and 6 h. Isolated nuclear proteins of control (0 µg ET) and ET-treated (10 µg ET) SCp2 cells were analyzed for (A) NFκB p65 or (B) NFκB p50 activities by immunobinding assays, with relative binding activity shown as A_{690} above blank. This experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in duplicate analysis by NFκB Trans AM immunobinding assay. Data represent the average for duplicate samples $\pm$ SEM of a representative experiment. (*) denotes significant difference among treatment within each time point ($p<0.05$).

Figure 2.5: Wedelolactone inhibits ET-induced IL-6 but not iNOS mRNA. Total RNA was extracted from SCp2 cells treated with 0 or 10 μM Wedelolactone (W) in DMSO (vehicle) for 1 h before ET treatment at 0 or 10 μg ET/ml for 0, 1, 3, and 6 h. Aliquots of 1μg total RNA from cells at each time point after ET application were reverse transcribed before amplification by real-time qPCR for ET induced (A) IL-6 and (B) iNOS mRNA. Amplified mRNA concentrations of IL-6 and iNOS were normalized to those of constitutive GAPDH mRNA. Amplified IL-6 (circle) and iNOS (triangle) mRNA levels shown for: control cells treated with vehicle alone (DMSO) for 1h then replaced with 1% FBS-DM only for 1, 3, or 6 h (closed symbols, solid line); cells treated with DMSO for 1h then replaced with ET treatment (10 μg / ml 1% FBS-DM) for 1, 3, or 6 h, (closed symbol, dash-line); cells treated with 10 μM IKK inhibitor (IKKI) wedelolactone, followed by 1% FBS-DM only for 1, 3, or 6 h (open symbols, solid line); and cells treated with IKKI for 1h before replacing it with ET treatment for 1, 3, or 6 h (open symbols, dash-line). This experiment was performed in duplicate samples / treatment / time point. Each sample was analyzed in triplicate qPCR reactions. Data represents the average for duplicate samples $\pm$ SEM. (*) denotes significant differences between ET treatment and non-ET treated control within each time point ($p<0.05$), and (@) denotes significant differences between W+ET treatment and ET treated control within each time point ($p< 0.05$).

A



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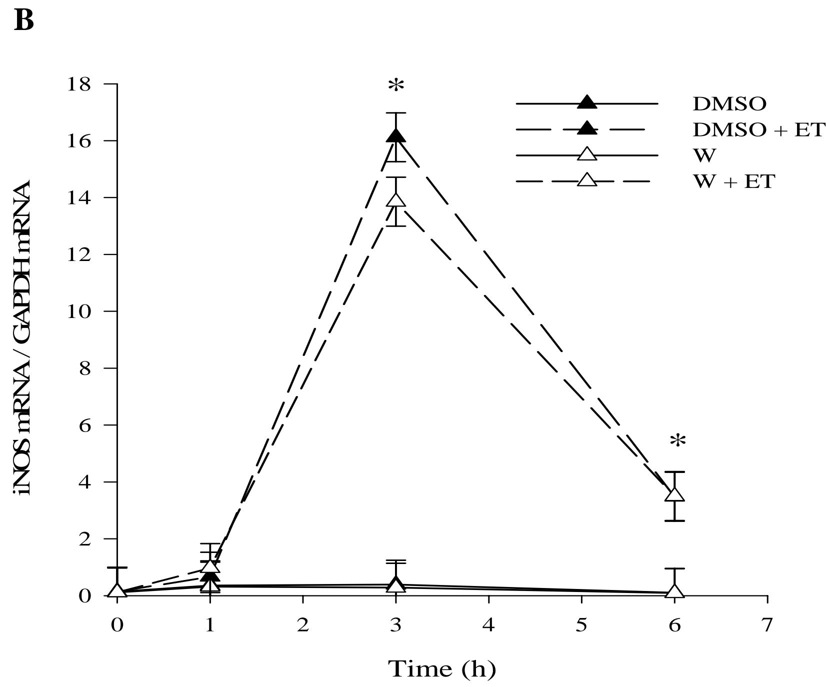
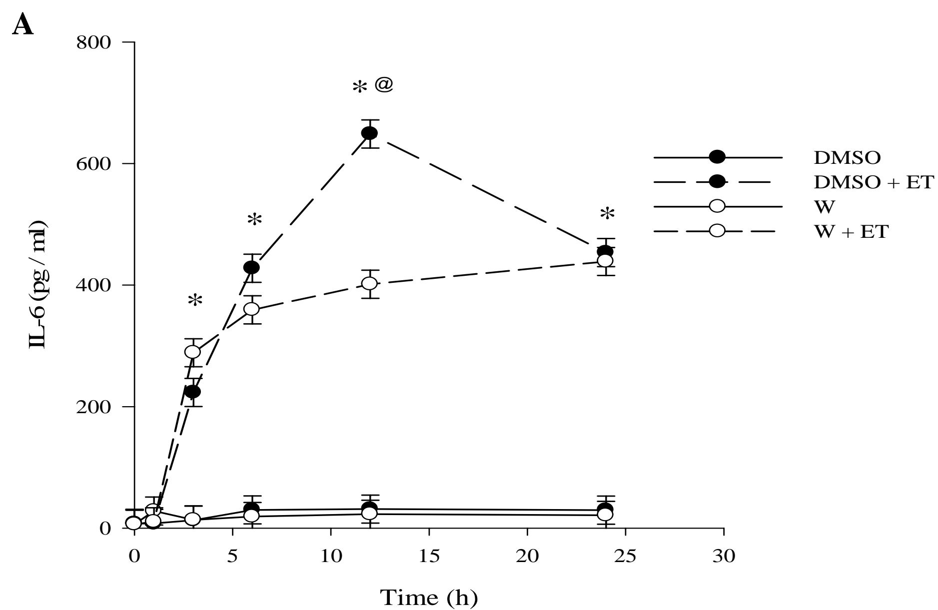


Figure 2.5

Figure 2.6: Wedelolactone reduces ET-induced IL-6 secretion but does not affect ET-induced NO production. Medium collected from cells treated with 0 or 10 μM Wedelolactone (W) in DMSO (vehicle) for 1h before replacing it with ET-treatment medium for 1, 3, 6, 12, and 24 h as in Fig. 2.5, were assayed for ET-induced (A) IL-6 secretion and (B) NO production. Control cells treated with vehicle alone (DMSO) for 1h then replaced with 1% FBS-DM only for 1, 3, or 6 h (closed symbols, solid line); cells treated with DMSO for 1h then replaced with ET treatment (10 μg / ml 1% FBS-DM) for 1, 3, or 6 h, (closed symbols, dash-line); cells treated with 10 μM IKK inhibitor (IKKI) wedelolactone, followed by 1% FBS-DM only for 1, 3, or 6 h (open symbols, solid line); and cells treated with IKKI for 1h before replacing it with ET treatment for 1, 3, or 6 h (open symbols, dash-line). This experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in duplicate ELISA or Griess reaction analysis. Data represents the average for duplicate samples ± SEM. (*) denotes significant differences between ET treatment and non-ET treated control within each time point ($p<0.05$), and (@) denotes significant differences between W+ET treatment and ET treated control within each time point ($p< 0.05$).



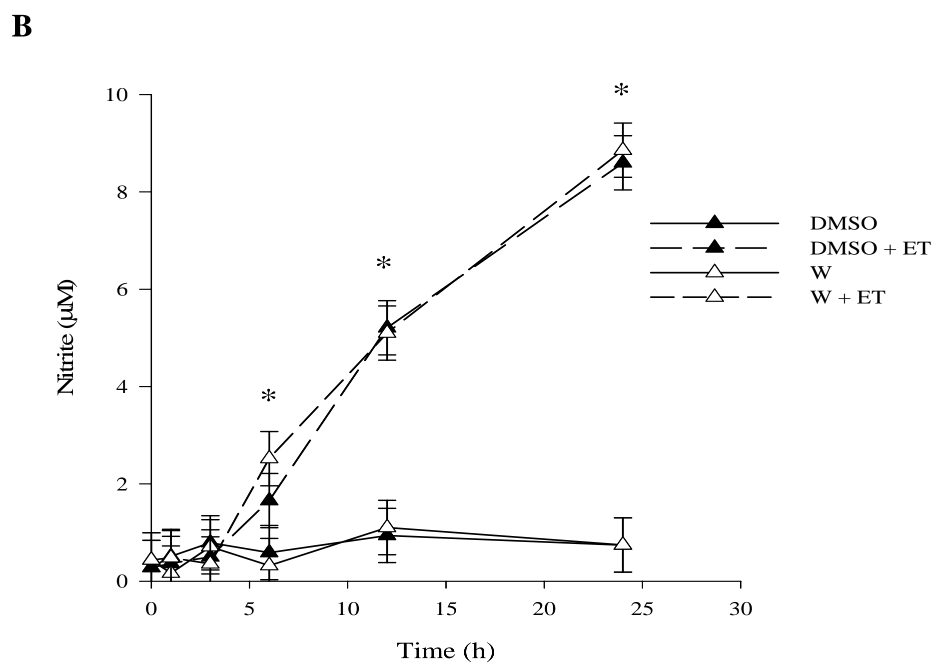
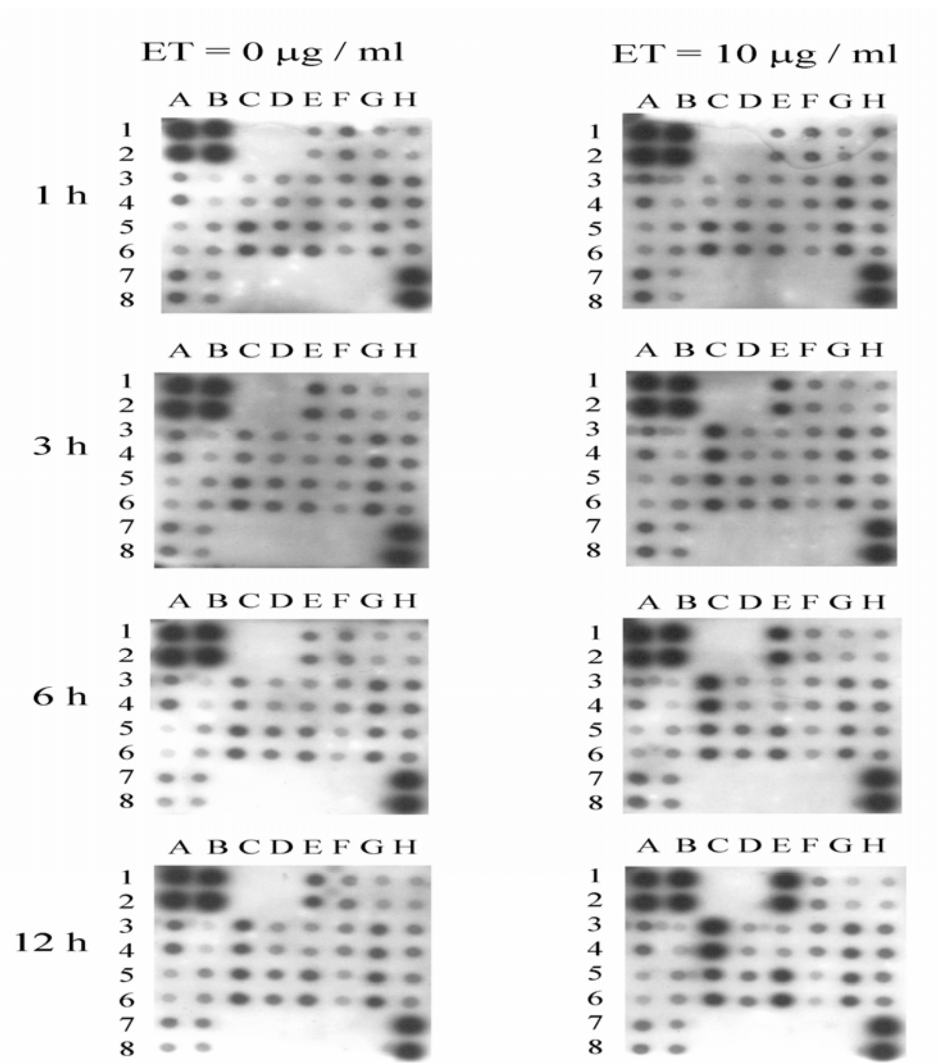


Figure 2.6

Figure 2.7: Mouse cytokine protein array of SCp2 cells following treatment with ET. Subconfluent SCp2 cells were treated with ET (0 or 10 μg / ml) in 1% FBS DM for 1, 3, 6, and 12h, and medium collected at each time point was assayed by the RayBio Mouse Cytokine Antibody Array I for different inflammatory cytokines enumerated in the table according to their position on the membrane. Abbreviations POS- positive control, NEG- negative control. GCSF- granulocyte colony stimulating factor, GM-CSF- granulocyte macrophage colony stimulating factor, IL- interleukin, IFN-γ- interferon gamma, MCP- monocyte chemotactic protein, RANTES- regulated on activation, normal T expressed and secreted, SCF-stem cell factor, sTNFR- soluble tumor necrosis factor α receptor, TNFα- tumor necrosis factor alpha, VEGF-vascular endothelial growth factor.



POS	POS	NEG	NEG	GCSF	GM-CSF	IL-2	IL-3
POS	POS	NEG	NEG	GCSF	GM-CSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNFα
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNFα
Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	POS
Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	POS

Figure 2.7

Gene (Genbank accession #)	Primer Pairs 5’→3’	Ref.
IL-6 (NM_031168)	F: 5’- GTTCTCTGGGAAATCGTGGA-3’ R: 5’- GGAAATTGGGGTAGGAAGGA-3’	[56]
iNOS (NM_010927)	F: 5’-CCCTTCCGAAGTTTCTGGCAGCAGC -3’ R: 5’- GGCTGTCAGAGCCTCGTGGCTTTGG -3’	NA
nNOS (NM_008712) Set 1 Set 2	 F: 5’- AGCACCTACCAGCTCAAGGA-3’ R: 5’- ATAGTGATGGCCGACCTGAG-3’ F: 5’- AGCACCTACCAGCTCAAGGA-3’ R: 5’-ATGGGTACTTCCAGCACCAG-3’	[56]
eNOS (NM_008713) Set 1 Set 2	 F: 5’-AGCTGCAGGTATTTGATGCTCG-3’ R: 5’-CAGCCACATCCTCAAGTATG-3’ 5’-TCTTCGTTCAGCCATCACAG-3’ 5’- CACAGGGATGAGGTTGTCCT -3’	NA [56]
GAPDH (BC094037)	F: 5’- ACCACAGTCCATGCCATCAC -3’ R: 5’- TCCACCACCCTGTTGCTGTA -3’	[84]

Table 2.1 Nucleotide sequences of primer pairs used for real time PCR amplification and analysis.

Gene Primer set	Product Size (bp)	Cycle No.	$MgCl_2$ (mM)	Annealing T^oC
IL-6	339	40	2.5 mM	58^oC
iNOS	497	40	2.5 mM	58^oC
nNOS set1	209	40	varied	varied
set 2	464	40	varied	varied
eNOS set 1	599	40	varied	varied
set 2	513	40	varied	varied
GAPDH	452	30	2.5 mM	58^oC

Table 2.2 Conditions of real time PCR for cDNA amplication.

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CHAPTER 3

SERUM, EXTRACELLULAR MATRIX, AND CELL-CELL INTERACTION DIFFERENTIALLY REGULATE ET-INDUCED IL-6 SECRETION AND INOS/NO PRODUCITON IN MOUSE MAMMARY SCP2 CELLS

ABSTRACT

Recent studies linking chronic inflammation to the risk of cell transformation and cancer may apply to the mammary gland and breast cancer. Mammary secretory epithelial and myoepithelial cells reside in close contact in the ducts and alveoli of the mammary gland and are the origin of most malignant proliferations likely initiated by an inflammatory response. Mouse mammary secretory epithelial cells (SCp2) and myoepithelial cells (SCg6) were used separately and together as representatives of the two types of epithelial cells present in the mammary gland. We describe here the effect of environmental factors such as serum, exogenous extracellular matrix (ECM), and coculture of secretory SCp2 with myoepithelial SCg6 mammary cells (mimicking cell-cell interactions in the mammary epithelium), on the ET-induced inflammatory respondents interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and nitric oxide (NO). SCp2 cells were either cultured on plastic +/- EHS and treated with ET (0 or 10 μg / ml) in the presence or absence of serum, or cultured on a monolayer of SCg6 cells and

treated + / - ET in the presence of serum. ET-induced IL-6 secretion and NO production were monitored by ELISA immunoassay and Griess reaction assay, respectively, and IL-6 and iNOS mRNA expression by real time PCR. While serum supplementation positively regulated expression of mRNA and secretion of pro-inflammatory cytokines (IL-6 and TNFα) in response to ET, it sharply reduced ET-induced iNOS mRNA expression and NO production, confirming the different modes of regulation previously described for IL-6 and iNOS in SCp2 cells (Chapter 2). EHS addition to SCp2 cells cultured on plastic had no apparent effect on the temporal pattern of ET-induced IL-6 or iNOS mRNA expression. However, the interaction in the coculture system between SCp2 secretory epithelial cells and SCg6 myoepithelial cells induced a surprising and dramatic increase in IL-6 secretion to very high levels in the absence of ET exposure, suggesting that cell-cell interaction between the two mammary epithelial cell types can dramatically modulate spontaneous inflammation in mammary cells in the absence of ET. Also surprising, addition of ET to SCp2:SCg6 cells in coculture further increased IL-6 secretion to even higher levels but caused only a meager induction of NO to maximum levels less than ¼ to ½ of that in SCp2 cells alone. These results show that the context of the inflamed cell, especially the microenvironment, likely is important in regulation of inflammation and for understanding the link between inflammation and cancer, in epithelia, for which mouse mammary epithelial cells present a good model for investigation.

INTRODUCTION

The microenvironment of the cell is very important in the regulation of its growth and function. This is demonstrated in the mammary gland, which is composed of several types of cells that not only can proliferate and differentiate, but also are able to dedifferentiate in response to coordinated signals from hormones, extracellular matrix (ECM), and cell-cell interactions [1-4] and show sharp inflammatory responses not only to bacterial insult (mastitis) [5, 6] but also to normal differentiation and developmental transitions such as involution at the cessation of lactation [7, 8]. Mammary epithelial cells are of two types: the secretory or luminal epithelial cells, organized into a monolayer of polarized columnar cells lining the acinus and responsible for milk production and secretion; and the myoepithelial cells surrounding the basal side of the secretory epithelial cell layer, and arranged in a discontinuous mesh-like contractile layer able to eject milk from the alveolar lumen into the ducts [9].

Mammary epithelial cells can differentiate in culture when provided with hormonal and ECM signals [10, 11]. In the presence of lactogenic hormones prolactin, insulin and hydrocortisone, together with exogenous Engelbreth-Holm-Swarm (EHS) ECM, CID-9 cells [12], derived from the mouse mammary COMMA-1D cell line [10] and composed of a mixture of secretory epithelial and myoepithelial mammary cells, and fibroblast cells, were shown to differentiate *in vitro* as characterized by formation of focal 3-dimensional cellular structures and expression of β-casein. SCp2 and SCg6 cell lines [13] are, respectively, the secretory epithelial and myoepithelial cells isolated from the CID-9 cell line. SCp2 cells retain the parental capability to differentiate in response to lactogenic hormones and EHS by forming three dimensional cell clusters and expressing

β-casein [13], while myoepithelial SCg6 cells do not [13].

Inflammation of mammary epithelial cells in response to exposure to bacterial endotoxin or at involution is characterized by secretion of pro-inflammatory respondents such as cytokines (interleukins (IL-1, IL-6), tumor necrosis factor alpha (TNFα), nitric oxide (NO), metalloproteases and others [7, 14-17] responsible for changes in both cell microenvironment and state of differentiation.

IL-6 is a pleiotropic cytokine produced by immune as well as non immune cells such as endothelial, fibroblast and epithelial cells. Expression of IL-6 is upregulated during acute inflammation by two transcription factors; nuclear factor kappa B (NFκB) and activator protein-1 (AP-1). In addition, IL-6 expression was shown to be regulated by cytokine (IL-1 and TNFα) and serum response elements in epithelial cells [18-22]. Although IL-6 is a pro-inflammatory cytokine, it is also involved in normal cell physiology such as cell growth and proliferation (reviewed in [23-25]).

Nitric oxide (NO) is another multifunctional molecule whose function varies based on its concentration and site of production [26]. At nanomolar concentrations, NO acts as a potent vasodilator and neurotransmitter, whereas at higher micromolar concentrations it induces pathogen death and tissue damage [27-30]. Three forms are known for nitric oxide synthase (NOS) that synthesizes NO by the conversion of L-arginine to L-citrulline [31]. Neuronal NOS (nNOS or NOS-1) and endothelial NOS (eNOS or NOS-3) are constitutively expressed forms; while, induced NOS (iNOS or NOS-2) expression is regulated by inflammatory stimuli such as bacterial endotoxin (ET) and cytokines (such as: interferon gamma (IFNγ), IL-1β, etc.) [32-34].

Endotoxin treatment of CID-9 cells in the presence of fetal bovine serum (FBS) [35] resulted in induction of pro-inflammatory cytokine expression (IL-1, IL-6, and TNFα), activation of matrix metalloproteases, and downregulation of β-casein expression. Our previous studies in SCp2 cells (Chapter 2) demonstrated that in addition to induced IL-6 expression and secretion, ET-treatment in the presence of FBS induced NO production by upregulating the expression of iNOS mRNA. Although both IL-6 and iNOS are expected to be upregulated via NFκB during an inflammatory response, our previous studies (Chapter 2) showed a different temporal pattern of expression of IL-6 mRNA compared to that of iNOS. Moreover, inhibition of IKK-dependent NFκB activation inhibited ET-induced IL-6 but not iNOS mRNA expression (Chapter 2) showing different regulation of both inflammatory markers. Both findings suggest that factors other than or in concert with NFκB activation are also involved in the regulation of the expression of either inflammatory respondents (IL-6 and iNOS). Also surprising was that NFκB was activated, in the absence of ET, in response to addition of serum alone to the medium of SCp2 cells (Chapter 2).

The aim of the present study was to investigate whether microenvironment elements such as serum, ECM, and cell-cell interactions, influence ET-induced inflammatory responses (IL-6 and NO) in mouse mammary epithelial cells as suspected from our previous studies. We tested the effects of serum and EHS on the inflammatory response of SCp2 secretory mammary epithelial cells alone and the effect of SCp2 cell interaction with SCg6 myoepithelial cells on their inflammatory response, as measured by IL-6 secretion and NO/iNOS production. Differential regulation of ET-induced IL-6

expression and secretion compared to iNOS expression and NO production was observed in response to serum and/or EHS. Furthermore, differential and opposite regulation of IL-6 secretion and NO production by SCp2 cells in response to coculture on an SCg6 monolayer in the absence of ET was observed, with dramatic upregulation of IL-6 secretion by interaction of the two mammary epithelial cell types that is further increased by ET exposure; suggesting that cell-cell interaction between the two mammary epithelial cell types can dramatically modulate spontaneous or induced inflammation in mammary cells in the absence of ET.

MATERIALS AND METHODS

Cell lines and materials. Mouse mammary epithelial cell lines SCp2 (secretory) and SCg6 (myoepithelial) were kindly provided by Dr. Pierre Desprez, (Geraldine Brush Cancer Research Institute; San Francisco, CA). Dulbecco's modified Eagle's medium/Ham F12 (DMEM/F12; 1:1), bovine insulin, ovine prolactin, hydrocortisone and endotoxin (ET, as *Salmonella typhosa* lipopolysaccharide, >500000EU (ET units) / mg) were purchased from Sigma (St. Louis, MO). Heat-inactivated fetal bovine serum (FBS), Hanks's balanced salt solution (HBSS), and gentamicin were purchased from Cambrex Bio Science (Walkersville, MD). BD Falcon cell culture flasks (75 cm^2) and 6 well (9.6 cm^2 / well) plates along with Englebreth-Holm-Swarm (EHS)-Matrix growth-factor-reduced BD Matrigel™ (extracellular matrix, ECM) were purchased from BD Biosciences (Bedford, MA). Complete™ protease inhibitor tablets containing specific and broad-spectrum multi-protease inhibitors were purchased from Roche Diagnostics (Mannheim, Germany). Tetramethyl benzidine (TMB) peroxidase substrate was

purchased from BioFX Laboratories (Owings Mills, MD).

Cell culture. SCp2 and SCg6 cells were maintained in growth medium (5% FBS-GM) comprised of DMEM/F12 containing 5% FBS, insulin (5 μg/ ml) and gentamicin (50 μg/ ml) in a 37°C humidified atmosphere with 5% CO_2. To ensure predominance of secretory epithelial cell type in SCp2 cell cultures, SCp2 cells were maintained at low passage number (13 through 15) and were regularly selected for secretory epithelial cells by selective trypsinization: plating freshly trypsinized cells for 1 h to allow attachment of fibroblast-like cells, followed by transfer of the unattached secretory epithelial cells into a fresh plate for either maintenance or expansion for future experiments. To confirm the secretory epithelial cell presence and functionality, SCp2 cells plated on plastic in 5% FBS-GM were induced to differentiate by change to differentiation medium (0%FBS-DM) comprised of serum-free DMEM/F12 containing gentamicin (50μg/ml), lactogenic hormones (insulin (5μg/ml), prolactin (3μg/ml), and hydrocortisone (1 μg/ml)), and Matrigel™ (1.5% v/v). Reverse transcribed polymerase chain reaction (RT-PCR) was used to assay for β-casein expression in differentiated SCp2 cells using the following primer set:

forward (F)= 5'- GTGGCCCTTGCTCTTGCAAG -3'

reverse (R)= 5'- AGTCTGAGGAAAAGCCTGAAC -3' [36].

SCp2 cells cultured on Plastic or in the presence of EHS: Depending on the experimental design (Fig. 3.1), SCp2 cells were plated at 4 x 10^4 cells / cm^2 or 8 x 10^4

cells / cm^2 on plastic in 5% FBS-GM. SCp2 cells designated for EHS treatment were plated at the higher plating cell density to ensure optimum confluence at the time of EHS application. At target confluence, cells were washed twice with HBSS and fed with 0% FBS-DM supplemented with 0 or 1.5 % (v/v) exogenous Matrigel™ (EHS) for another 24 h before addition of ET (0 or 10µg / ml) in 1% FBS-DM.

SCp2 / SCg6 coculture system: SCg6 cells were plated on plastic at 4 x 10^4 cells / cm^2 in 5% FBS-GM and grown to 80-90% confluence, generally at 24 h post plating, before addition of SCp2 cells to the same well at 4 x 10^4 cells / cm^2, except for the last experiment (Fig. 3.9) when different SCp2 cell densities (0, 1, 2, 3, 4, and 8 x 10^4 cell / cm^2) were plated on confluent SCg6 monolayers. After 24 h the cocultured cells were washed twice with HBSS and fed with serum free DM for another 24 h before induction of inflammation by addition of an optimum dose of ET (10µg / ml in 1% FBS-DM) to the cells. Wells of either SCg6 or SCp2 (both at 4 x 10^4 cells / cm^2) alone plated on plastic were used as controls for the SCp2-SCg6 coculture response to ET treatment.

ET-induced inflammation in mouse mammary cells. A stock solution of ET was prepared at 1 mg / ml in 0% FBS-DM. SCp2, SCg6, or cocultured cells were plated at 4x10^4 cell / cm^2 (unless otherwise specified for SCp2 cells) in 5% FBS-GM for cell attachment and proliferation to achieve 50% confluence, generally after 24 h. Cells were then washed twice with HBSS before feeding with 0%FBS-DM. After incubation for 24 h, inflammation was induced by application of a non-toxic dose of ET (10 µg/ml

medium), determined from our preliminary studies in SCp2 cells as the optimum dose able to induce a maximal inflammatory response (defined by IL-6 secretion) without cytotoxicity (measured by LDH release (Cytotox 96® Non-Radioactive cytotoxicity assay)). ET was added in 1% FBS-DM and remained on the cells until harvest time. The medium was collected from each well at 0, 1, 3, 6, 12, 24 and 48 h post-ET treatment and supplemented with Complete™ protease inhibitor solution (1 Complete™ tablet/ 2 ml deionized water) added at 40 μl/ ml to collected medium before storing at -80°C for later analysis. Cells were washed *in situ* and frozen at -80°C for later RNA extraction.

Immunoassay of Interleukin-6. To measure IL-6 secretion in response to ET in SCp2 cells, medium collected at various times post-ET treatment was assayed by enzyme linked immunosorbent assay (ELISA) for IL-6 (DuoSet kit; R&D Systems Inc, Minneapolis, MN) according to the manufacturer's protocol. The IL-6 ELISA was performed in Immunolon flat bottom 96 well plates (Thermo; Milford, MA) with rat anti-mouse IL-6 antibody, blocking with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 5% sucrose (Sigma, St. Louis, MO), mouse IL-6 standards dissolved in either 0% FBS-DM or 1% FBS-DM as for experimental samples, and detection with biotinylated goat anti-mouse antibody followed by streptavidin conjugated to horseradish-peroxidase (HRP). Addition of HRP substrate TMB yielded color intensity (A_{690}) in proportion to the amount of IL-6 present in the sample. Samples were assayed in duplicate and data is represented as the average pg IL-6 / ml of duplicate samples ± standard error of the mean (SEM).

Griess reaction assay of NO for NOS activity: The analysis of NO was accomplished by the Griess assay for nitrite (the spontaneous oxidation product of NO) using a Griess Reagent Kit [37] (Molecular Probes, Eugene, OR) involving addition of 20 µl of Griess Reagent (0.05% N-(1-napthyl) ethylenediamine dihydrochloride, 0.5% sulfanilic acid in 2.5% phosphoric acid) to 150 µl of nitrite-containing sample or standard and 130 µl deionized water, per the manufacturer's instructions. The color developed by azo dye formation in proportion to nitrite concentration (µM) in solution was quantified by A_{550}. Assays of nitrite in cell culture medium used sodium nitrite ($NaNO_2$) standards diluted in appropriate medium from that experiment. Samples were assayed in duplicate and data is represented as the average µM NO_2^- of duplicate samples $\pm$ SEM.

RNA extraction, reverse-transcription and quantitative real time polymerase chain reaction analysis. Total RNA was isolated from scrape harvested cells grown in 6-well plates using Qiagen RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Isolated RNA was quantified by A_{260}, and only RNA with A_{260} / A_{280} ratio $\geq$ 1.6 was used for reverse transcription (RT) reactions. A defined amount of RNA (2µg) was treated with DNAse I (Promega, Madison, WI) before being reverse transcribed by incubation with random primer and avian myeloblastosis virus (AMV) reverse transcriptase at 42°C for 30 min in a 40 µl reaction using the Promega reverse transcription system (Promega, Madison, WI). Quantitative real time PCR (qPCR) was performed by adding 2 µl of undiluted RT product to 18 µl of Qiagen Hot start SyBR Green PCR master mix (Qiagen, Valencia, CA) containing 0.15 µM each of the sense (or forward; F) and anti-sense (or

reverse; R) primers for each of IL-6, iNOS, and TNFα target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene (Table 3.1). SybrGreen fluorescence was detected with an MJ Research Opticon 2 reader (BioRad, Hercules, CA); amplified products were quantified relative to appropriate standard curve in autonomous qPCR analyses. Primer pairs were either adopted from the literature or designed using Primer_3 primer design software [38] and synthesized by Operon Biotechnologies Inc (Huntsville, AL). Target amplicons spanned an intron to exclude amplification of genomic DNA. The standard curves for each of IL-6, iNOS and GAPDH genes of interest were generated by serial dilutions of the purified (QIAquick clean up kit; Qiagen, Valencia, CA (per manufacturer's instructions)) and sequenced RT-PCR product amplified from total RNA isolated from ET-treated SCp2 cells. Quantitative PCR conditions were, in sequence: a hot start at 94°C, 15min; followed by initial denaturation at 94°C, 5min, and subsequent annealing at 58°C, 30 sec. Each sample was analyzed in triplicate qPCR reactions with the triplicate average for each gene of interest normalized against the average for the reference GAPDH gene. The results of qPCR analysis are presented as the average for duplicate samples $\pm$ SEM.

Statistical analysis. Significant differences between different groups were determined using Proc Mixed analysis of SAS 9.1 (SAS Institute Inc., Cary, NC). For each of the experiments, the statistical model included time, treatment (ET, matrix, FBS), and time by treatment interactions. The effect of treatment within each time point was tested using the slice option by time. Results were expressed as mean +SEM, and significance was defined as $p<0.05$, unless noted otherwise.

RESULTS:

The effect of serum on ET-induced IL-6 secretion and NO production in SCp2 cells cultured on plastic. To determine the effect of serum on ET-induced inflammation, SCp2 cells cultured on plastic were treated with endotoxin (0 or 10 μg/ ml) in either 0% or 1% FBS-DM according to the experimental design shown in Fig. 3.1A. The ET treatment remained on cells in each well until the medium was collected at 0, 1, 3, 6, 12, or 24 h post-treatment and assayed for secreted IL-6 and NO production. Results in Fig. 3.2A & B show that serum addition enhanced significantly ($p<0.05$) IL-6 secretion in response to ET in SCp2 cells. In the absence of serum, secreted IL-6 concentrations increased slowly in response to ET, with a near linear increase in IL-6 secretion from 3 h through 24 h after ET application (post-ET) with an overall 5-fold increase in IL-6 secretion by 24 h post-ET (Fig. 3.2A). However, in the presence of serum, ET induced a dramatic 8 fold increase in IL-6 secretion as early as 3 h post-ET with a slower increase thereafter to 10-fold above basal concentrationl by 24 h post-ET (Fig. 3.2B). In contrast, ET-induced NO production was not significantly affected by the absence or presence of serum (Fig. 3.2C vs. D). In the absence of serum, ET-treated SCp2 cells showed a near-linear increase from 3 to 24 h, with a slight decrease in rate of NO production from 12 to 24 h compared to 3 -12 h (Fig. 3.2C). In the presence of serum, ET induced a near linear increase of NO production from 6 h through 24 h with overall 10% less NO produced (not statistically significant) in the presence of serum compared to the level of production in the absence of serum (Fig. 3.2D vs. C). These results show that ET-induced IL-6 secretion was enhanced but NO production was unaffected by the addition of serum to the medium.

The effect of serum on ET-induced IL-6 and iNOS mRNA expression in SCp2 cells cultured on plastic. The effect of serum on IL-6 and iNOS mRNA expression was investigated at different times post-ET treatment. Total RNA was isolated from SCp2 cells on plastic treated with ET in the presence or absence of FBS and analyzed by RT-qPCR (Fig. 3.3). In the absence of serum, ET appeared to induce a slight increase in IL-6 mRNA expression but it was not significantly different from the basal IL-6 mRNA expression in control non-ET treated cells (Fig. 3.3A). However, the temporal pattern of expression showed a slight increase in IL-6 mRNA expression over time and independent of ET treatment (Fig. 3.3A). Addition of serum along with ET treatment induced a sharp increase in ET-induced IL-6 mRNA expression at 1 h post-ET, with further increase to peak ($p<0.05$) at 3 h post-ET before sharply decreasing after 6 h of ET exposure followed by an apparent transient rise ($p<0.05$) at 12 h post-ET (Fig. 3.3B). In contrast to IL-6 mRNA expression, ET-induced iNOS mRNA expression peaked at 3 h post-ET independent of the presence of serum (Fig. 3.3C & D), however, the temporal pattern after 3 h post-ET was different (Fig. 3.3C vs D). In the absence of serum, iNOS mRNA expression increased sharply at 3 h post ET ($p<0.05$), remained high at 6 h ($p<0.05$) then decreased to one third its peak induction by 12 h and decreased further at 24 h post-ET to slightly above the basal level of iNOS mRNA expression in control non-ET treated cells (Fig. 3.3C). However, in the presence of serum, the expression of iNOS mRNA increased sharply to peak at 3 h post-ET ($p<0.05$), followed immediately by a sharp decrease in expression by 6 h post-ET to levels only slightly higher than basal levels of expression, and decreased further to basal levels by 24 h post-ET (Fig. 3.3D). AT 6 h

post-ET, iNOS mRNA remained significantly high in the absence of serum (Fig.3.3C) but decreased sharply ($p<0.05$) in the presence of serum (Fig. 3.3D). In contrast to IL-6, ET-induced iNOS mRNA expression showed no secondary expression peak at any time after the primary peak at 3 h post-ET. It is interesting to note also that ET-induced iNOS mRNA expression was about 10-fold higher than ET-induced IL-6 mRNA expression regardless of the presence or absence of serum. In summary, serum greatly enhanced the level of ET-induced IL-6 mRNA expression but had little effect on the time or magnitude of ET-induced iNOS mRNA expression, although serum addition appeared to significantly decrease iNOS mRNA half life ($t_{1/2}$) based on the rate of reduction of its mRNA after the peak at 3 h in Fig. 3.3D (1% FBS) vs. the rapid decay of the ET-induced iNOS mRNA in the absence of serum (Fig. 3.3C).

The effect of serum on ET-induced TNFα mRNA expression in SCp2 cells. Although IL-6 is a commonly used marker of inflammation [25, 39, 40], and was previously shown to be a responsive inflammatory marker in the SCp2 cell model (Chapter 2), we also examined TNFα, a similarly well known pro-inflammatory cytokine, in SCp2 cells for its coordination with the other inflammatory markers (IL-6 and NO/iNOS) of this study. RT-qPCR analysis showed TNFα mRNA expression to be modulated by both serum and ET in SCp2 cells (Fig. 3.4). In the absence of serum, a sharp increase in the expression of TNFα mRNA was observed to peak by 1 h in response to ET but not in control non-ET treated SCp2 cells (Fig. 3.4A). ET-induced TNFα mRNA expression peaked sharply at 1 h post-ET and began to decrease by 3 h

post-ET but stabilized to maintain a relatively high level of expression between 6-12 h before slightly decreasing afterwards (Fig. 3.4A). In contrast, serum addition without ET treatment also induced a sharp but smaller ($p<0.05$) increase in TNFα mRNA expression at 1 h after addition of medium without ET but with 1% FBS (Fig. 3.4B). ET treatment in the presence of serum induced a sharp and higher but more sustained increase in TNFα mRNA expression than in response to serum alone (Fig. 3.4B). ET-induced TNFα expression peaked at 1 h and remained high at 3 h post-ET before decreasing by 6 h to maintain a relatively high level of expression between 6-12 h and decreasing slightly afterwards (Fig. 3.4B). There was no significant difference between the levels of ET-induced TNFα mRNA both in the absence or presence of serum (Fig.3.4). Also notable was the transient elevation of expression of TNFα at 12 h, regardless of +/- serum and +/- ET-induction (Fig. 3.4A & B), similar to the 12 h transient increase seen for IL-6 but not NO mRNA expression in Fig. 3.3. Efforts to define TNFα protein expression with commercially available ELISA, western immunoblots, or cytokine immunondot arrays were not successful; therefore, TNFα regulation in response to ET or serum was not pursued further in this study.

Effect of EHS and cell-cell interaction on SCp2 cell organization in culture. The microenvironment of epithelial cells is very critical for their organization, differentiation, and function. Therefore, we investigated the effects of EHS soluble extracellular matrix and epithelial -myoepithelial cellular interaction (in SCp2:SCg6 cells in cocultures) on the SCp2 cell organization and differentiation in culture. SCp2 cells grown under non-

differentiation conditions (in 5% FBS-GM on plastic) formed a near-uniform cell monolayer on plastic (Fig. 3.5A) and did not express β-casein mRNA (data not shown), reflecting their undifferentiated state. In contrast, addition of exogenous EHS extracellular matrix along with serum withdrawal and supplementation with lactogenic hormones (0% FBS-DM) induced differentiation of Scp2 cells, characterized by rearrangement of SCp2 cells with focal alterations in the cell monolayer suggestive of three dimensional structures as early as 24 h after medium change (Fig. 3.5B) and up-regulation of the mRNA expression of β-casein after 4 days or more in culture (data not shown), in agreement with previous reports [10, 12, 13]. SCp2 cells cultured on a monolayer of SCg6 mammary myoepithelial cells also showed formation of cell foci (presumably SCp2) with even more pronounced putative three dimensional structuring suggestive of acinar formation *in vitro* (Fig. 3.5C), while SCg6 cells alone grew into a uniform monolayer of elongated cells on plastic (Fig. 3.5D) and did not express β-casein mRNA under any condition (data not shown) as expected for myoepithelial cells. Having shown the effect of EHS and cell-cell interaction on the morphology of SCp2 cells, we next tested whether these modifications in the microenvironment affected the response of SCp2 cells to ET-induced inflammation. Since this study focused on the short term responses, we did not pursue the effect of ET on β-casein expression that was not observed until 4 days after induction of differentiation, longer time than these experiments allowed.

Effect of exogenous EHS on ET-induced IL-6 secretion and NO production in the presence or absence of serum in SCp2 cells. After demonstrating its role in cell organization and function (β-casein expression), we tested whether addition of soluble ECM substratum (EHS) modulates ET-induced inflammation in mammary secretory epithelial cells. SCp2 cells were grown in 5% FBS-GM on plastic for 24 h before shifting to 0% FBS-DM +/- EHS to induce SCp2 cell differentiation. At 24 h after induction of differentiation, SCp2 cells were treated with ET (0 or 10 μg / ml) in either 0% FBS-DM or in 1% FBS-DM with the ET treatment remaining on cells for 0, 1, 3, 6, 12, 24, or 48 h. The medium collected at the specified time points was assayed by ELISA for IL-6 secretion and Griess reaction assay for NO production, and the quantified IL-6 and nitrite concentrations were normalized to cell number (Fig. 3.6). The results show that SCp2 cells grown in the presence of EHS induced similar temporal patterns and levels of IL-6 secretion (Fig. 3.6A & B) when treated with ET in the presence (Fig. 3.6A & B) or absence (data not shown) of 1% serum. SCp2 cells grown in the presence of EHS also show similar temporal pattern of NO production in response to ET, however, NO production was significantly ($P<0.05$) increased (by 25%) in cells treated with ET in the presence (Fig. 3.6C &D) but not in the absence (data not shown) of serum at 12, 24 and 48 h post-ET.

Effect of exogenous EHS on ET-induced expression of IL-6 and iNOS mRNA in the presence or absence of serum. Similar to ET-induced IL-6 protein secretion, EHS had no effect on ET-induced IL-6 mRNA expression whether in the presence (Fig. 3.7A vs.

B) or absence (data not shown) of 1% serum. SCp2 cells grown on plastic (Fig. 3.7A) or in the presence of EHS (Fig. 3.7B) treated with ET in the presence of serum showed a sharp increase in IL-6 mRNA expression at 1h post-ET that peaked higher at 3 h post-ET and decreased sharply to starting levels by 6-12 h post-ET, but with a subsequent 2-fold transient increase at 24 h post-ET in both. The transient and significant ($p<0.05$) increase in IL-6 mRNA expression in Fig 3.7 A and B at 12 h post-ET is reminiscent of the 12 h transient peak in IL-6 mRNA expression for SCp2 cells on plastic (Fig. 3.3B). In the absence of serum, SCp2 cells grown either on plastic or in the presence of EHS showed markedly reduced IL-6 mRNA expression in response to ET that only slightly increased with time (data not shown), similar to the pattern observed earlier on plastic in Fig. 3.3A.

ET-induced expression of iNOS mRNA showed similar patterns of induction for SCp2 cells cultured on plastic (Fig. 3.7C) or in the presence of EHS (Fig. 3.7D). The expression of iNOS mRNA was not induced until 3 h post-ET when it peaked sharply to a maximum, decreased sharply by 6 h post-ET to slightly above starting levels and maintained that level for the remainder of the experiment (Fig, 3.7C) or decreased to control levels by 48 h post-ET (Fig. 3.7D). The 3 h peak of ET-induced iNOS mRNA expression was significantly higher ($P<0.01$) in SCp2 cells on plastic (Fig. 3.7C) than in those cultured in the presence of EHS (Fig. 3.7D), in contrast to the observed higher ET-induced NO production in SCp2 cells in response to EHS (Fig. 3.6C vs. D). We did not investigate further the reason for the observed disparity in the ET-induced NO production and iNOS mRNA expression in response to EHS. In the absence of serum, no difference in patterns of ET-induced iNOS mRNA expression was observed in SCp2 cells cultured in the presence or absence of EHS (data not shown).

Effect of cell-cell interaction on ET-induced IL-6 secretion and NO production in SCp2 cells. Since myoepithelial cells are important in the differentiation process of secretory epithelial cells in the mammary gland [36], we studied their effect on ET-induced inflammation in secretory epithelial cells. SCg6 myoepithelial mammary cells were plated on plastic in 5%FBS-GM to form a monolayer, and SCp2 secretory mammary epithelial cells were then either plated on plastic or on the monolayer of SCg6 cells for 24 h in 5%FBS-GM before induction of differentiation by shifting to 0% FBS-DM. At 24 h later, ET (0 or 10 µg / ml) was added in 1% FBS-DM and the medium was collected at 24 h post-ET and analyzed for IL-6 secretion and NO production (Fig. 3.8). Surprisingly, cell-cell interaction alone in the absence of ET induced a dramatic increase ($p<0.05$) in the concentration of secreted IL-6 in the medium of cocultured SCp2:SCg6 cells (Fig. 3.8A) that was independent of cell number in the individual vs. cocultures (Fig. 3.8 A, inset). ET treatment strongly increased IL-6 secretion by 4 fold or more ($p<0.05$) in SCp2 and SCg6 cultures on plastic. And in SCp2:SCg6 cocultures, ET treatment even increased IL-6 secretion at least 2 fold ($p<0.05$) above the strong basal IL-6 secretion in the absence of ET (Fig. 3.8A).

In contrast, SCp2:SCg6 coculture had little effect on NO production in control non-ET treated cells (Fig. 3.8B), and increased in response to ET but only to half the level of NO produced in ET treated SCp2 cells on plastic (Fig. 3.8B). SCg6 cells alone showed no significant increase in NO production in response to ET (Fig. 3.8B). From these results, not only did SCp2:SCg6 coculture induce unexpectedly high IL-6 secretion

in the absence of ET but also differentially modulated IL-6 secretion and NO production in response to ET.

Effect of SCp2:SCg6 cell ratio in coculture vs. SCp2 cell plating density on plastic on ET-induced IL-6 secretion and NO production. To test the effect of SCp2:SCg6 cell ratios and to control for increased SCp2 plating density therein on IL-6 secretion and NO production in control and ET-treated cells, SCp2 cells were plated at different cell densities (0, 1, 2, 4, and 8 x 10^4 cell / cm^2) either on plastic or on a confluent SCg6 cell monolayer in 5% FBS-GM as for Fig. 3.8. After 24 h incubation, SCp2 cells on plastic and in coculture were induced to differentiate by shifting to 0% FBS-DM and incubating for another 24 h. Later, ET was added in 1% FBS-DM and the medium was collected at 24 h post-ET and analyzed for both basal and ET-induced IL-6 secretion and NO production (Fig. 3.9). In SCp2 cells plated on plastic, basal IL-6 secretion (without ET induction) was low but increased with increasing SCp2 cell plating density, while ET-induced IL-6 secretion was greater and increased with increasing SCp2 cell plating density (Fig. 3.9A), with ET-induced IL-6 secretion being significantly increased ($p<0.05$) in response to ET at the two highest SCp2 cell densities (0.4 x10^4 and 0.8 x10^4 cell / cm^2) (Fig. 3.9A). In SCp2:SCg6 cell cocultures, the basal IL-6 secretion (without ET) increased dramatically ($p<0.05$) and was progressively higher with increasing SCp2 cell plating density (Fig. 3.9B). Interestingly, ET-induced IL-6 secretion was even greater ($p<0.05$) and increasingly higher than basal levels, but with the relative increase over basal decreasing at higher SCp2 plating densities. Basal IL-6 secretion was 5 to 9 fold higher ($p<0.05$) for SCp2 cells plated on SCg6 cells (Fig. 3.9B) compared to those

on plastic (Fig. 3.9A), while ET-induced IL-6 secretion was 3 to 4-fold higher ($p<0.05$) from SCp2:SCg6 cell cocultures (Fig. 3.9B) vs SCp2 cells on plastic (Fig. 3.9A) independent of cell plating density. SCg6 alone in the absence of SCp2 showed a 5 fold increase ($p<0.05$) in IL-6 secretion in response to ET in comparison to non-ET treated cells (Fig. 3.9B, 0 SCp2 plating density).

In contrast to IL-6 secretion, basal NO production did not significantly increase with SCp2 cell plating density, nor differed between SCp2 cells on plastic or on an SCg6 monolayer in coculture (Fig. 3.9C & D). Furthermore, ET induced a strong increase in NO production that increased in proportion to the increase in SCp2 plating cell density significantly ($p<0.05$) for cells plated at 0.4 $x10^4$ and 0.8 $x10^4$ cell / cm^2 on plastic (Fig. 3.9C) or for all cell plating densities on SCg6 monolayer for cocultures (Fig. 3.9D). The basal levels of NO were not significantly different in SCp2 plated on plastic or in coculture with SCg6 for all cell plating densities, however, ET-induced NO levels were significantly ($p<0.05$) attenuated in SCp2:SCg6 cocultures (Fig. 3.9 D) compared to ET-induced NO levels in SCp2 cells on plastic but only for 0.4 $x10^4$ and 0.8 $x10^4$ cell / cm^2 cell plating densities (Fig. 3.9C), opposite the response of IL-6 in coculture (Fig. 3.9B). In contrast to SCp2 cells, SCg6 cells alone induced little NO production in response to ET (Fig. 3.9D, 0 SCp2 plating density).

DISCUSSION:

The present study demonstrates that ET-induced IL-6 secretion and NO production are differentially regulated by the microenvironment of SCp2 cells in culture. This is in contrast to our initial prediction of their parallel regulation by ET via NFκB, a

regulator of both [17, 21, 41]. We investigated the role of serum supplementation as well as two types of extracellular substratum, EHS and a monolayer of SCg6 myoepithelial cells, on ET-induced inflammation in SCp2 cells. EHS matrix is a solubilized basement membrane with reduced growth factor content, isolated from EHS mouse sarcoma that is rich in ECM proteins shown to stimulate differentiation of mammary cells *in vitro* as a tissue ECM surrogate [42]. Mammary myoepithelial cells growing on the basal side of the secretory cell monolayer and in contact with ECM are also known for their synthesis of basement membrane, and likely work cooperatively with ECM to confer polarity to secretory epithelial cells in the mammary alveolus.

Both EHS and SCp2:SCg6 coculture were capable of inducing SCp2 mouse mammary secretory epithelial cells to differentiate in culture in the absence of serum and upon addition of lactogenic hormones (Fig. 3.5), as expected [10-13, 35, 43]. SCp2 cells grown under differentiation conditions rearranged their monolayer growth into cell clusters with apparent three dimensional structure that coincided with upregulated β-casein expression (Fig. 3.5), similar to *in vitro* differentiation of CID-9 or COMMA-D cells [10, 12].

Serum is known to enhance ET-induced responses by providing soluble CD14, and LBP required for ET binding to its TLR-4 [44, 45] and by enhancing the expression of pro-inflamamtory cytokines such as IL-6 [18-20]. This study showed that serum supplementation indeed increased ET-induced IL-6 secretion by ~2.5 fold (Fig. 3.2B) when compared to ET-induced IL-6 secretion in the absence of serum in SCp2 cells on plastic (Fig. 3.2A). Control levels of IL-6 remained unchanged in response to serum addition, suggesting that the observed increase in the ET-induced IL-6 secretion is not

attributed to serum-induced increase in cell number; although, confirmation of this explanation must await cell count data. Contrary to IL-6 results, serum addition slightly decreased ET-induced NO production without affecting the basal concentrations of NO in control cells on plastic (Fig. 3.2 C vs. D). The expression of iNOS mRNA was maximally induced at 3 h post-ET independent of the presence of serum. However, while the presence of serum reduced ET-induced iNOS mRNA expression to near control levels by 6 to 12 h post-ET (Fig. 3.3 D), in the absence of serum iNOS mRNA expression peaked at 3 h, remained high at 6 h, and decreased at 12 h post-ET (Fig. 3.3 C). The longer persistence of iNOS mRNA in the absence of serum most likely explains the higher levels of NO production observed in Fig. 3.2C. Differences in peak expression patterns in Fig. 3.3C vs. D suggest that serum may decrease the half-life of iNOS mRNA in mammary cells.

The effect of serum on the expression of TNFα mRNA in response to ET was interesting for SCp2 cells on plastic or in the presence of EHS. Changing the medium from 0% FBS-DM to 1%FBS-DM was enough to sharply induce a transient peak of TNFα mRNA expression at 1 h post medium change in the absence of ET (Fig. 3.4 B). ET induced higher expression of TNFα mRNA that peaked between 1-3 h post-ET independent of serum (Fig. 3.4). Therefore, while serum supplementation positively regulated pro-inflammatory cytokine expression (IL-6 and TNFα) in response to ET, it negatively regulated that of iNOS, confirming different modes of regulation for IL-6 and iNOS in SCp2 cells as suggested in the previous study (Chapter 2) and that the effect of serum on the biochemical nature of cells and their responses has to be considered when

investigating signaling mechanisms. Further, cells *in vivo* are bathed in plasma and normally are not exposed to serum except during wound healing processes, an event known for its inflammation. On the other hand, the transient increase in mRNA expression observed at 12 h post-ET for both IL-6 (Fig. 3.3B) and TNFα (Fig. 3.4B) might suggest an oscillating regulation of the inflammatory response, possibly by oscillating patterns of NFκB activation as described by Covert et al (2005) [46], and the waves of activation and inactivation of the different NFκB family members depending on the signaling pathway involved [47, 48].

In the second part of the study we investigated the role of EHS and SCp2-SCg6 interaction on IL-6 secretion and NO production in SCp2 cells alone on plastic or in SCg6 cocultures with or without ET-induced inflammation. While EHS had no effect on ET-induced IL-6 secretion, SCp2 cells cultured in the presence of EHS showed a slight but significant increase (~25%) in NO production in response to ET compared to SCp2 cells on plastic (Fig. 3.6C & D). The differential regulation of IL-6 vs NO was only observed when ET was applied in the presence of serum (Fig. 3.6), since application of ET in 0% FBS-DM showed no difference in IL-6 secretion and NO production in SCp2 regardless of EHS addition (data not shown). Despite differential regulation of the secreted products, ET-induced iNOS mRNA had similar temporal patterns of expression regardless of substratum: plastic (Fig. 3.7C) vs. EHS (Fig. 3.7D). Whether the observed increase in medium NO levels was due in part to increased chemical stability of NO in the presence of EHS was not investigated. Similarly, IL-6 mRNA expression patterns in response to ET were not affected by EHS (Fig. 3.7A & B).

This study also investigated the role of SCg6 mouse mammary myoepithelial cells in modulating ET-induced inflammatory responses of SCp2 cells (IL-6 secretion and NO production). Most surprisingly, when SCp2 cells were cultured on a monolayer of SCg6 cells, IL-6 concentrations increased dramatically to several-fold higher than for either SCp2 or SCg6 cells alone, independent of ET treatment and cell number (Fig 3.8A and inset). The concentration of IL-6 in control coculture cells was higher than the sum of IL-6 secreted in both control SCp2 and SCg6 cells cultured independently, showing a strong synergistic effect of SCg6–SCp2 interaction on IL-6 secretion even in the absence of ET (Fig. 3.8A). These results are interesting in light of reports in the literature of a role for IL-6 in cell-cell association [18]. In marked contrast, growth of SCp2 cells on an SCg6 monolayer seemed to reduce ET-induced NO response although NO basal levels were not affected (Fig. 3.8B), confirming that the previously shown differential regulation of IL-6 and NO extends to SCp2 cells in coculture. Despite the intriguing effect of SCp2:SCg6 coculture on spontaneous and induced inflammation, it is not known how the effects of SCp2:SCg6 interaction would relate to the mammary gland *in vivo* since SCg6 cells were described as a malignant mammary cell line having lost their responsiveness to ECM regulation and were shown to induce tumors when injected into athymic nude mice [49, 50].

Preliminary investigations testing the temporal pattern of ET-induced IL-6 and NO in CID-9 cells, the mixed cell population and common parent cell line of SCg6 and SCp2 cells, showed that IL-6 secretion and NO production varied in different cultures depending on the passage number of these cells (data not shown). Low passage numbers (3-4) resulted in similar basal and ET-induced levels of IL-6 secretion and NO production

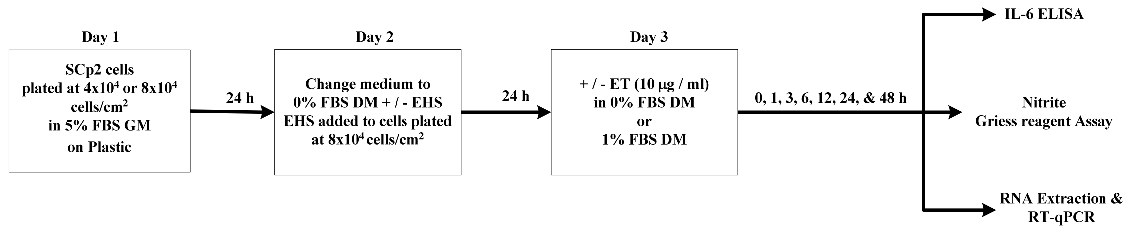
as those for SCp2 cells on plastic, while higher passage number CID-9 cells increased IL-6 secretion and reduced NO production in both control and ET-treated cells (data not shown), similar to the SCp2:SCg6 coculture (Fig. 3.9) and consistent with known changes in the ratio of secretory epithelial, myoepithelial and fibroblast cells in high vs. low passages of CID-9 cells [35].

The ratio of secretory epithelial to myoepithelial cells varies in ductular vs. alveolar mammary epithelia [51], with the ratio of secretory epithelial to myoepithelial cells low in ductal tissue and higher (>1) in the alveolus with dominance therein by the monolayer of secretory epithelial cells surrounded by a discontinuous layer of myoepithelial cells [51]. Therefore, we varied the ratio of SCp2 to SCg6 cells in the coculture by plating SCp2 cells on a monolayer of SCg6 cells at SCp2:SCg6 cell plating ratios of 1:4, 1:2, 1:1, and 2:1. SCp2:SCg6 coculture induced a dramatic 5 to 9-fold increase in basal levels of IL-6 secretion in control non-ET treated cells compared to that in SCp2 or SCg6 cells alone on plastic (Fig. 3.9A vs. B). Furthermore, SCp2 cells on plastic showed basal IL-6 secretion that was proportional to cell number except at the highest plating density of SCp2 cells (Fig. 3.9 A). The increase in IL-6 secretion in response to ET was proportional to SCp2 cell plating density on plastic (Fig. 3.9 A), while ET-induced IL-6 secretion in SCp2:SCg6 cocultures increased more slowly with increasing SCp2 plating density in high SCp2 plating density cultures (Fig 3.9B), most likely due to cell confluence. Interestingly, the net increase of IL-6 secretion from ET-induction was independent of cell number in SCp2:SCg6 cocultures, indicating that cell-cell interaction affected only basal secretion of IL-6 proteins but not that induced by ET (Fig. 3.9 B).

In contrast to IL-6, while basal NO levels varied little in SCp2 cells on plastic (Fig. 3.9C), ET-induced NO production increased significantly (by 15 to 20-fold) only at high SCp2 cell plating density (4 and 8 x 10^4 cell / cm^2) (Fig. 3.9D) compared to basal non-ET treated levels. However, SCp2:SCg6 cocultures showed induction of NO by ET that was only ¼ that of SCp2 cells on plastic, but increased significantly and proportionally at all SCp2 cell plating densities (Fig. 3.9 D); emphasizing the differential regulation of IL-6 and NO in mammary epithelial cells. Further studies are warranted on the effect of serum on SCg6 and SCp2:SCg6 cocultures for ET-induced secretion and expression of IL-6 and NO/iNOS to understand better the coregulation of these inflammatory respondents in this epithelial cell model.

In conclusion, we show here that serum in the culture medium, cell-ECM, and cell-cell interactions between the two resident cell types of the mammary epithelium differentially modulate ET-induced IL-6 and NO inflammatory responses. Surprisingly, cell-cell interaction alone induced remarkable secretion of IL-6 but not NO production. These results suggest that the microenvironment context of the inflamed cell likely is important for understanding the regulation of inflammation and the link between inflammation and cancer.

A. SCp2 mammary secretory epithelial cells on Plastic + or - EHS



B. SCp2 mammary secretory epithelial cells on Plastic or on a monolayer of SCg6 mammary myoepithelial cells

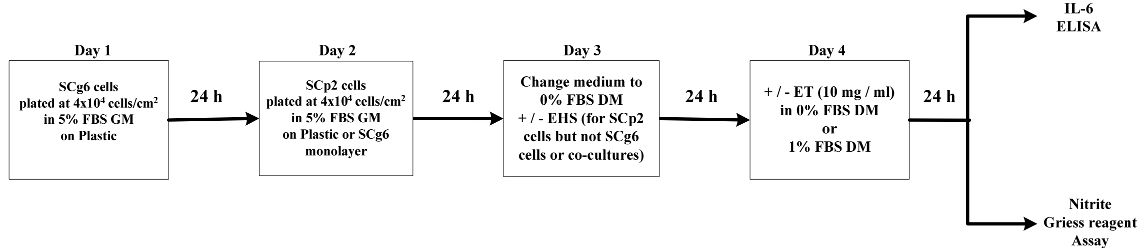


Figure 3.1: Schematic illustrations of time-line, treatments, and bioassays performed in this study.

Figure 3.2: The effect of serum on ET-induced IL-6 secretion and NO production over time in SCp2 cells cultured on plastic. SCp2 cells were plated at 4 x 10^4 cell / cm^2 in 5% FBS-GM. At 50% confluence, the 5% FBS-GM was replaced with 0% FBS-DM. After 24 h, ET (0 or 10 μg /ml) was applied in either 0% FBS-DM or 1% FBS-DM for different time durations (0, 1, 3, 6, 12, and 24 h) after which the medium was collected and assayed for (A & B) IL-6 secretion (pg/ ml; closed circle) and (C & D) NO production (μM; closed triangles) in the absence (A,C) or presence (B,D) of serum, respectively. Control levels of secreted IL-6 and nitrite are represented by a solid line, while those in the medium of ET-treated cells are represented by a dashed line. The experiment was performed in triplicate samples / treatment / time point. Each sample was assayed separately in duplicate analysis by both ELISA and Griess reaction assay. Data represents the average for IL-6 protein or nitrite concentrations for triplicate samples $\pm$ SEM with (*) denoting significant differences between treatment within each time point ($p<0.05$) and (#) denoting significant differences between ET-induced IL-6 (A vs. B) or nitrite (C vs. D) in the absence or presence of serum at each time point ($p<0.05$).

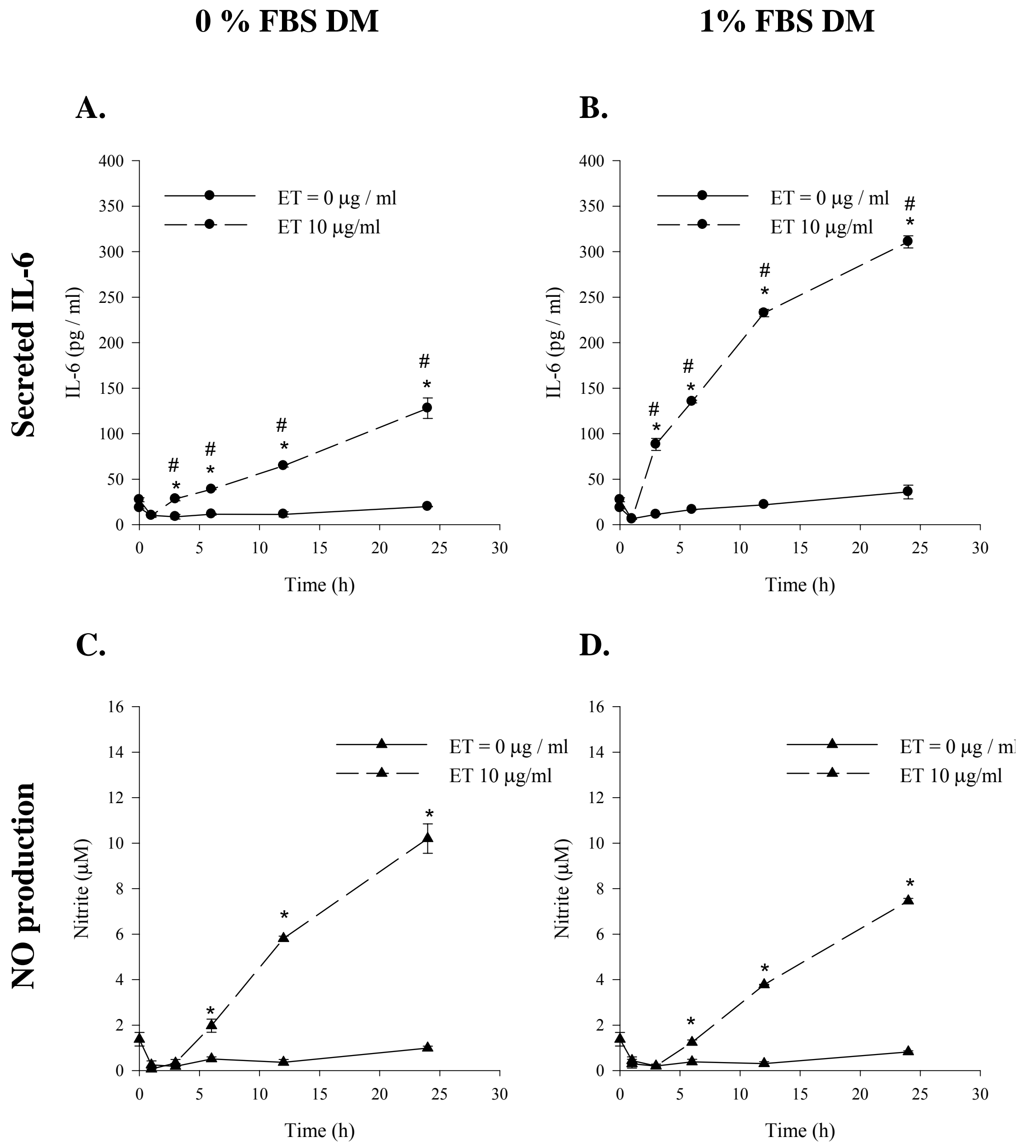


Figure 3.2

Figure 3.3: The effect of serum on the temporal pattern of mRNA expression of IL-6 and iNOS in response to ET in SCp2 cells on plastic. SCp2 cells were plated at 4 x 10^4 cell / cm^2 in 5% FBS-GM. At 50% confluence, the 5% FBS-GM was replaced with 0% FBS-DM for 24 h before ET (0 or 10 µg /ml) was applied in either 0% FBS-DM or 1% FBS-DM for different time durations (0, 1, 3, 6, 12, and 24 h) after which total RNA was extracted and quantified by RT-qPCR. (A & B) IL-6 mRNA (closed circle) and (C & D) iNOS mRNA (closed triangle) expression are presented relative to GAPDH mRNA in SCp2 cells. Relative mRNA level in control cells is depicted by a solid line, while that in ET-treated cells are depicted by a dashed line. The experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in triplicate qPCR reactions. Data represents the average for relative IL-6 or iNOS mRNA expression for duplicate samples $\pm$ SEM with (*) denoting significant differences between ET and non-ET treated cells within each time point ($p<0.05$). IL-6 mRNA expression at 3 h and 12 h in the presence of serum (B) were significantly higher ($p<0.05$) than the levels at 3 h and 12 h in the absence of serum (A). Similarly, the levels of iNOS mRNA induced at 6 h post-ET in the absence of serum (C) were significantly higher ($p<0.05$) than their corresponding levels at 6 h post-ET in the presence of serum (D).

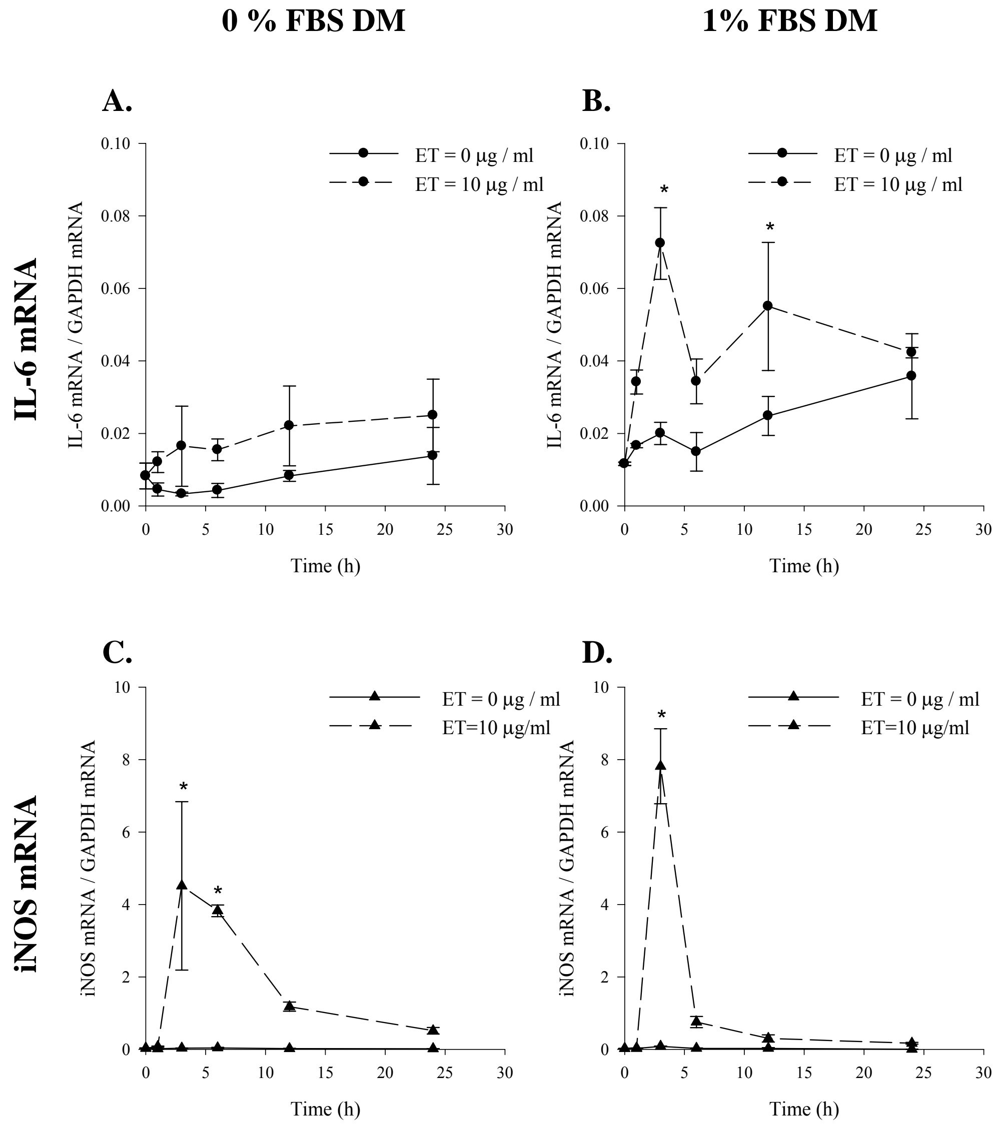


Figure 3.3

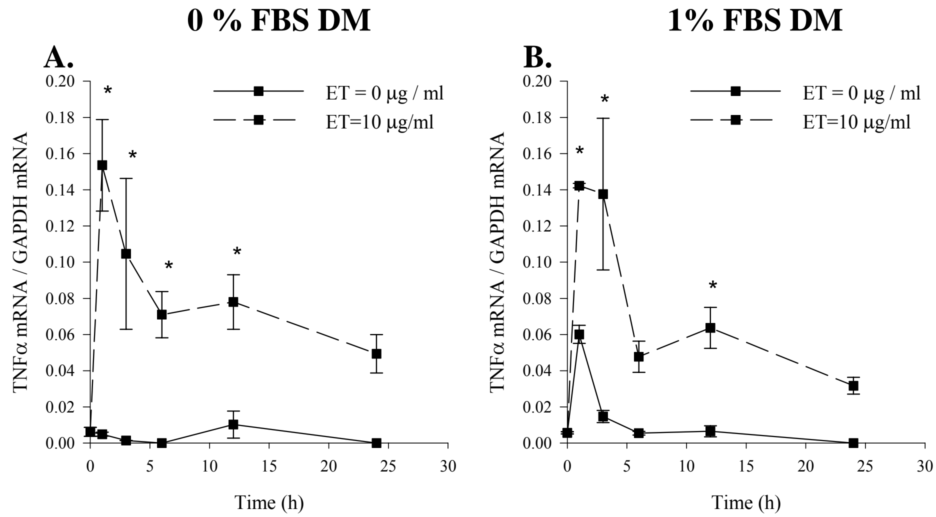


Figure 3.4: The effect of Serum on mRNA expression of TNFα over time in response to ET in SCp2 cells on Plastic. SCp2 cells were (plated at 4 x 10^4 cell / cm^2) grown in 5% FBS-GM to 50% confluence when the 5% FBS-GM was replaced with 0% FBS-DM for 24 h before ET (0 or10 µg /ml) was applied in either 0% FBS-DM or 1% FBS-DM for different time durations (0, 1, 3, 6, 12, and 24 h) after which total RNA was extracted and quantified by RT-qPCR. TNFα mRNA (closed square) expression in the (A) absence or (B) presence of serum are presented relative to GAPDH mRNA in SCp2 cells. Relative mRNA in control cells are depicted by a solid line, while those in ET-treated cells are depicted by a dashed line. The experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in triplicate qPCR reactions. Data represents the average for relative TNFα mRNA expression for duplicate samples $\pm$ SEM with (*) denoting significant differences between ET vs. non-ET treatment within each time point ($p<0.05$).

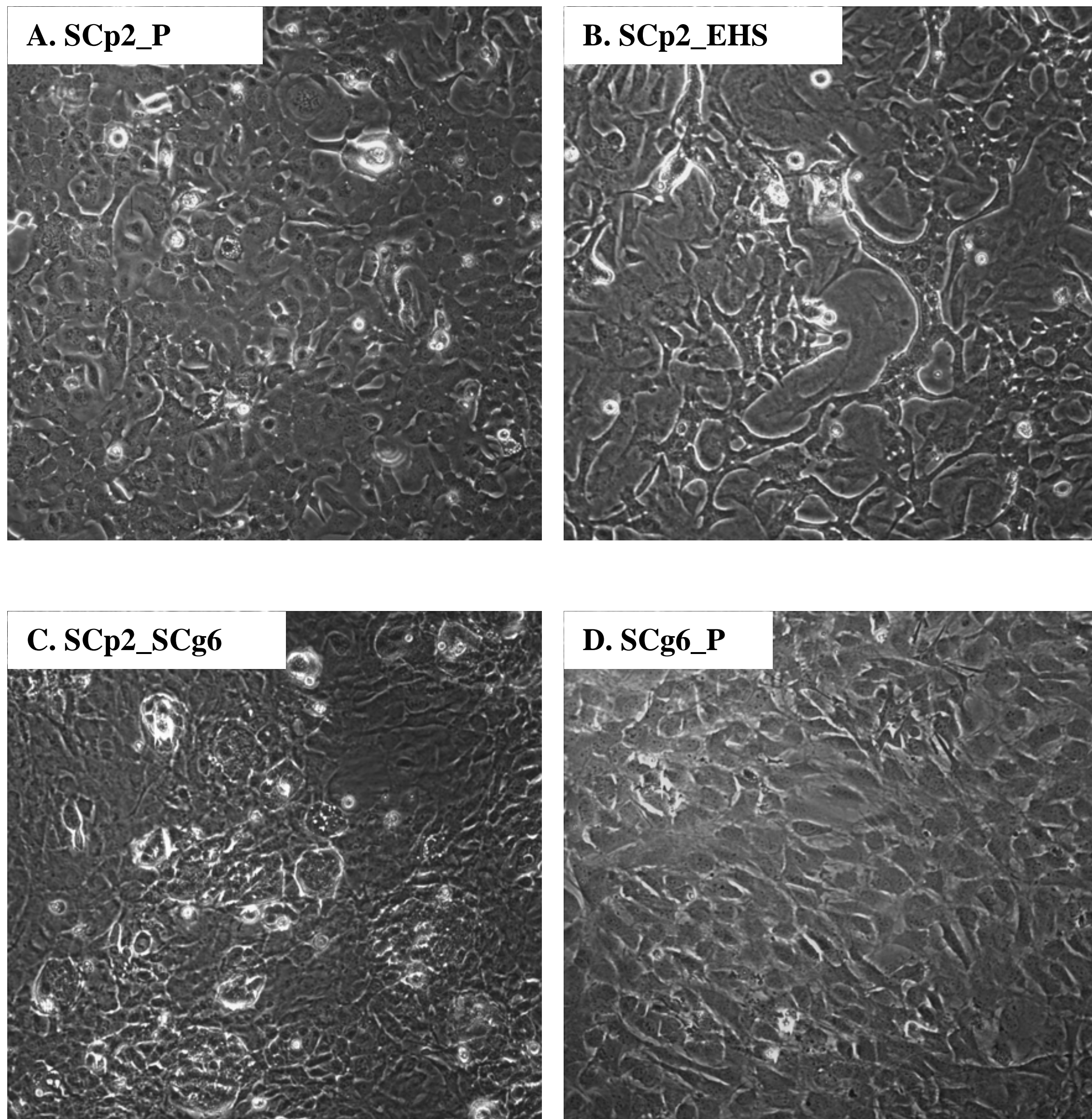


Figure 3.5: The effect of different substrata on SCp2 cells morphology. Phase contrast photomicrographs (100X) of SCp2 cells (plated at 4 x 10^4 cell / cm^2) on day 3 of culture on (A) plastic (SCp2_P), (B) in the presence of EHS (SCp2_EHS), or (C) on a monolayer of SCg6 mammary myoepithelial cells (SCp2_SCg6) and (D) SCg6 cells on plastic (SCg6_P).

Figure 3.6: The effect of EHS addition on ET-induced IL-6 secretion and nitrite production in SCp2 cells. SCp2 cells were plated at 4 x 10^4 cell / cm^2 in 5% FBS-GM. At 50% confluence, the 5% FBS-GM was replaced with 0% FBS-DM alone or supplemented with EHS. After 24 h, ET (0 or10 µg /ml) was applied in 1% FBS-DM for different time durations (0, 1, 3, 6, 12, 24, and 48 h) after which the medium was collected and assayed for IL-6 secretion (pg/ ml) and nitrite production (µM) normalized to cell number. Circles depict secreted IL-6 concentrations from SCp2 cells cultured (A) on plastic (closed circle) or (B) in the presence of EHS (open circle). Triangles depict nitrite production from SCp2 cells cultured (C) on plastic (closed triangle) or (D) in the presence of EHS (open triangle). Control levels of secreted IL-6 and nitrite are represented by a solid line, while those in the medium of ET-treated cells are represented by a dashed line. The experiment was performed in triplicate samples / treatment / time point. Each sample was assayed separately in duplicate analysis by either ELISA or Griess reaction assay and cells were counted using a hemocytometer. Data represents the average for IL-6 protein or nitrite concentrations per cell number for triplicate samples $\pm$ SEM with (*) denoting significant differences between ET and non-ET treatment within each time point ($p<0.05$) and (#) denoting significant differences between ET-induced IL-6 (A vs. B) or nitrite (C vs. D) in the absence or presence of EHS ($p<0.05$).

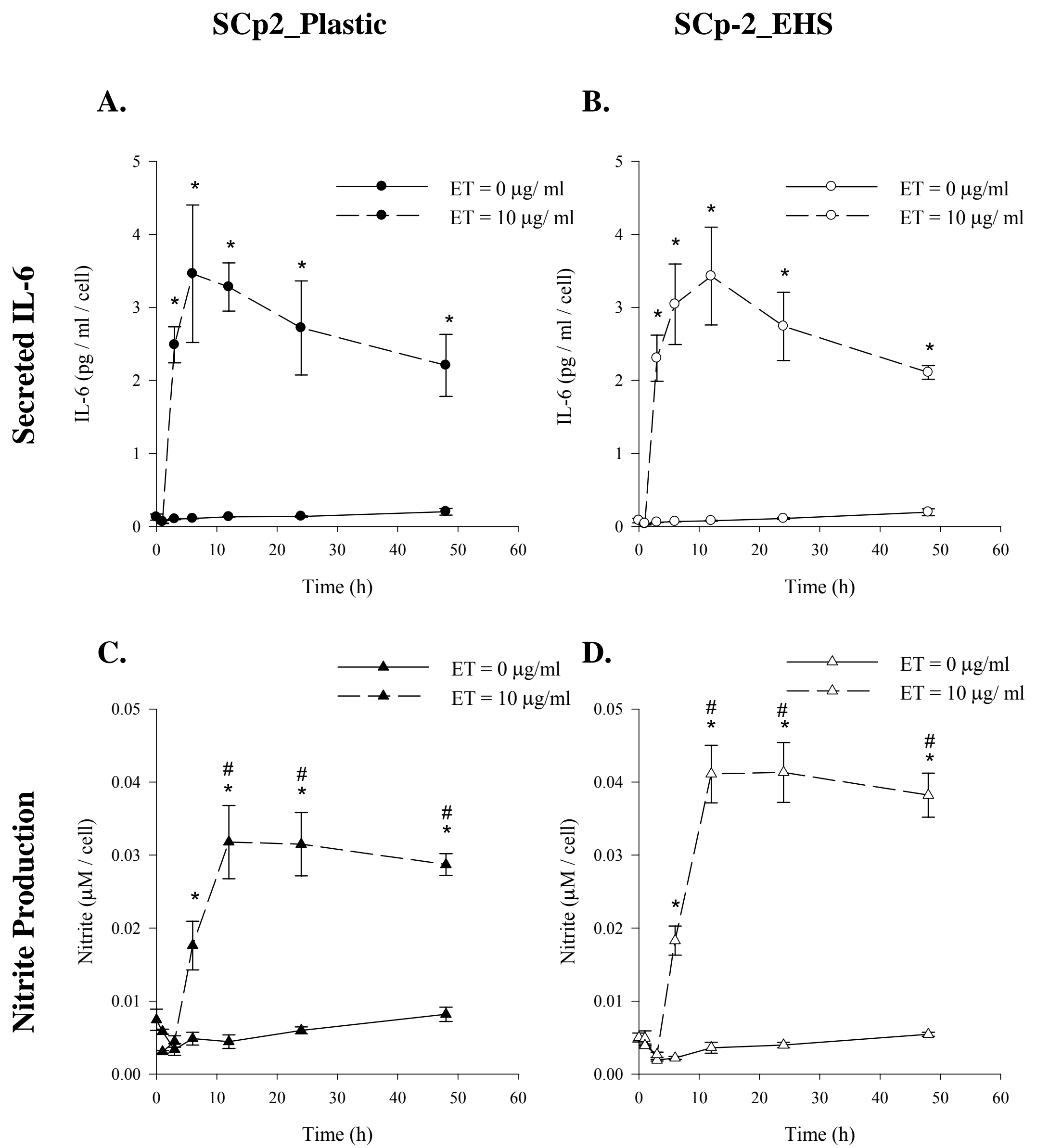


Figure 3.6

Figure 3.7: The effect of EHS addition on mRNA expression of IL-6 and iNOS in control and ET treated SCp2 cells. SCp2 cells were plated in 5% FBS-GM. At 50% confluence, the 5% FBS-GM was replaced with 0% FBS-DM alone or supplemented with EHS. After 24 h, ET (0 or 10 μg /ml) was applied in 1% FBS-DM for different time durations (0, 1, 3, 6, 12, 24, and 48 h) after which total RNA was extracted and quantified by RT-qPCR. The results are represented as the amount of IL-6 and iNOS mRNA expressed relative to that of GAPDH. The plots represent (A & B) IL-6 mRNA expression (circle) and (C& D) iNOS mRNA expression (triangle) in SCp2 cells cultured on plastic (closed symbol) or in the presence of EHS (open symbol) and treated with ET in 1% FBS-DM. Relative mRNA expression in control cells is depicted by a solid line, while that in ET-treated cells is depicted by a dashed line. The experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in triplicate qPCR reactions. Data represents the average for relative IL-6 or iNOS mRNA expression for duplicate samples ± SEM with (*) denoting significant differences between ET and non-ET treatment within each time point ($p<0.05$). There was no statistically significant difference between the respective IL-6 (A vs B) or iNOS (C vs D) mRNA expressions in response to EHS at all time points assayed.

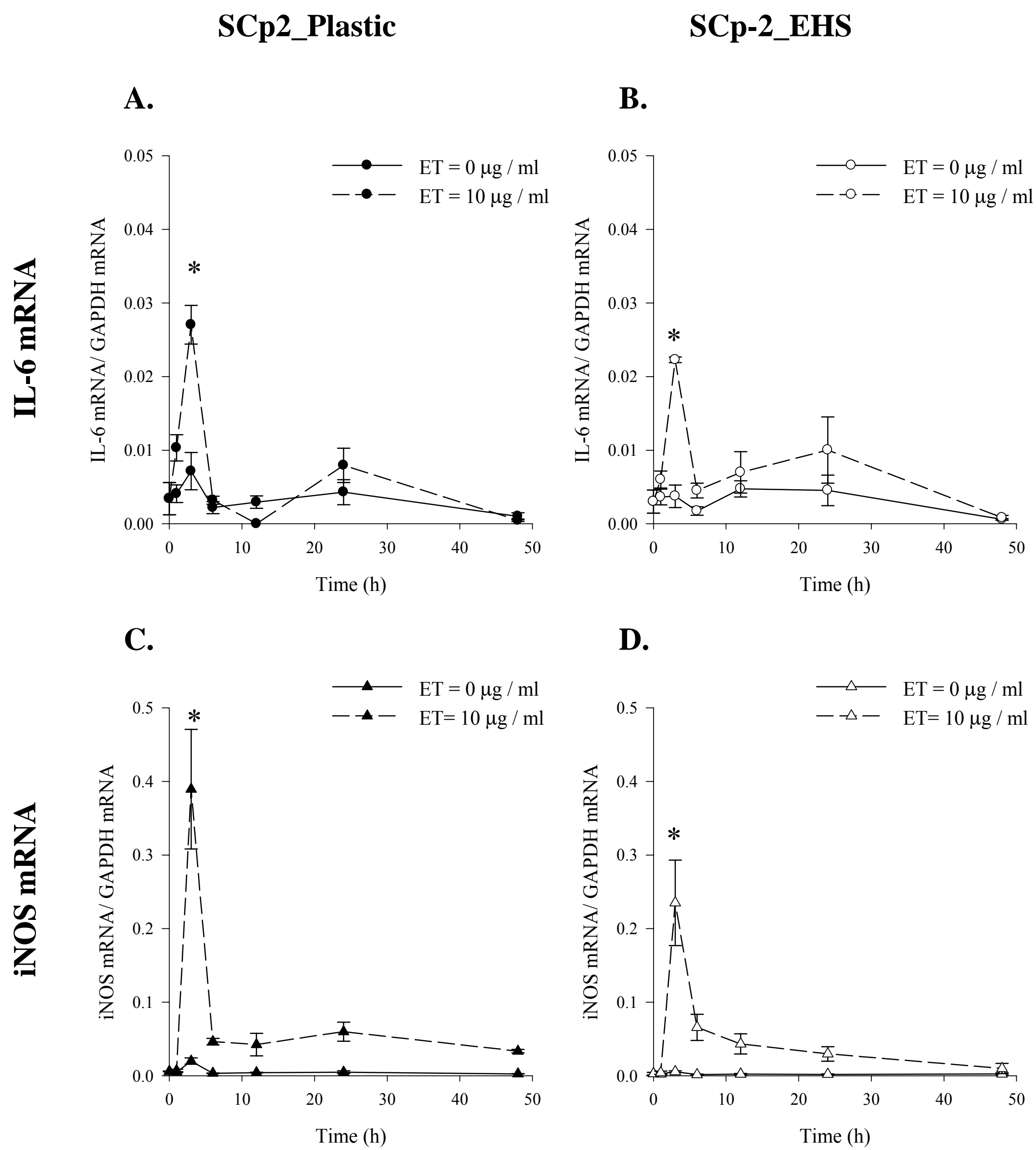


Figure 3.7

Figure 3.8: Effect of SCg6 monolayer on basal and ET-induced IL-6 secretion and NO production in SCp2 cells. SCg6 cells were plated at 4 x 10^4 cells / cm^2 as described in Fig. 3.1 B. On day 2 the 90% confluent SCg6 cells were fed with 5% FBS-GM with or without SCp2 cells at 4 x 10^4 cells / cm^2). On day 3 of the experiment all wells were washed with HBSS and fed 0% FBS DM. After 24 h incubation, ET (0 or 10 μg / ml) was applied in 1% FBS-DM. The medium was collected 24 h later and assayed for basal (open bar) and ET-induced (closed bar) (A) IL-6 secretion and (B) NO production in SCg6, SCp2, and SCg6/SCp2 coculture cells. Inset in (A) represents basal and ET-induced IL-6 secretion in SCg6, SCp2 and SCp2:SCg6 coculture normalized to cell number. The experiment was performed in duplicate samples / treatment. Each sample was assayed separately in duplicate analysis by both ELISA and Griess reaction assay. Data represents the average for IL-6 protein or nitrite concentrations for duplicate samples ± SEM. (*) denotes significant differences between treatment within each time point ($p<0.05$) and letters denote significant differences among cell types within treatment ($p<0.05$).

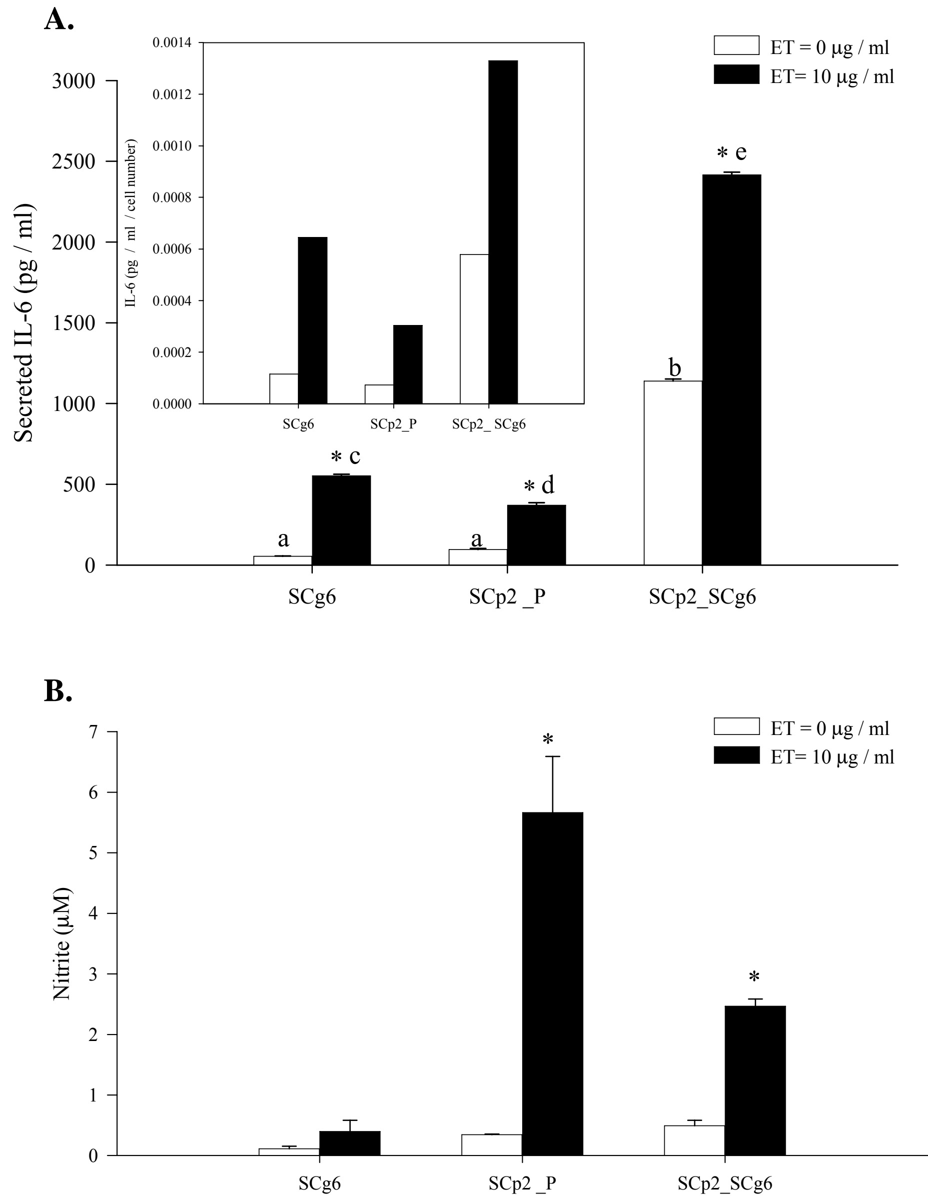


Figure 3.8

Figure 3.9: Effect of SCp2 cell plating density on IL-6 secretion and NO production in control or ET-treated cells cultured either on plastic or on a monolayer of SCg6 cells. SCg6 cells were plated at 4 x 10^4 cells / cm^2 as described in Fig. 3.1 B. On day 2 the 90% confluent SCg6 cells were fed with 5% FBS-GM with or without SCp2 cells at different cell densities (0, 1, 2, 4, and 8 x 10^4 cells / cm^2). On day 3 of the experiment all wells were washed with HBSS and fed 0% FBS DM. After 24 h incubation, endotoxin (0 or10 μg / ml) was applied in 1% FBS-DM. The medium was collected 24 h later and assayed for IL-6 secretion (A&B) and nitrite production (C&D) from SCp2 cells on (A, C) plastic or (B, D) SCg6 monolayer. IL-6 secretion or nitrite production in control cells are depicted by open bars, and those in ET-treated cells are depicted by closed bars. The experiment was performed in duplicate samples / treatment. Each sample was assayed separately in duplicate analysis by both ELISA and Griess reaction assay. Data represents the average for IL-6 protein or nitrite concentrations for duplicate samples ± SEM. (*) denotes significant differences between ET and non-ET treatment within each time point ($p<0.05$) and letters denote significant differences among SCp2 cell plating densities within treatment ($p<0.05$).

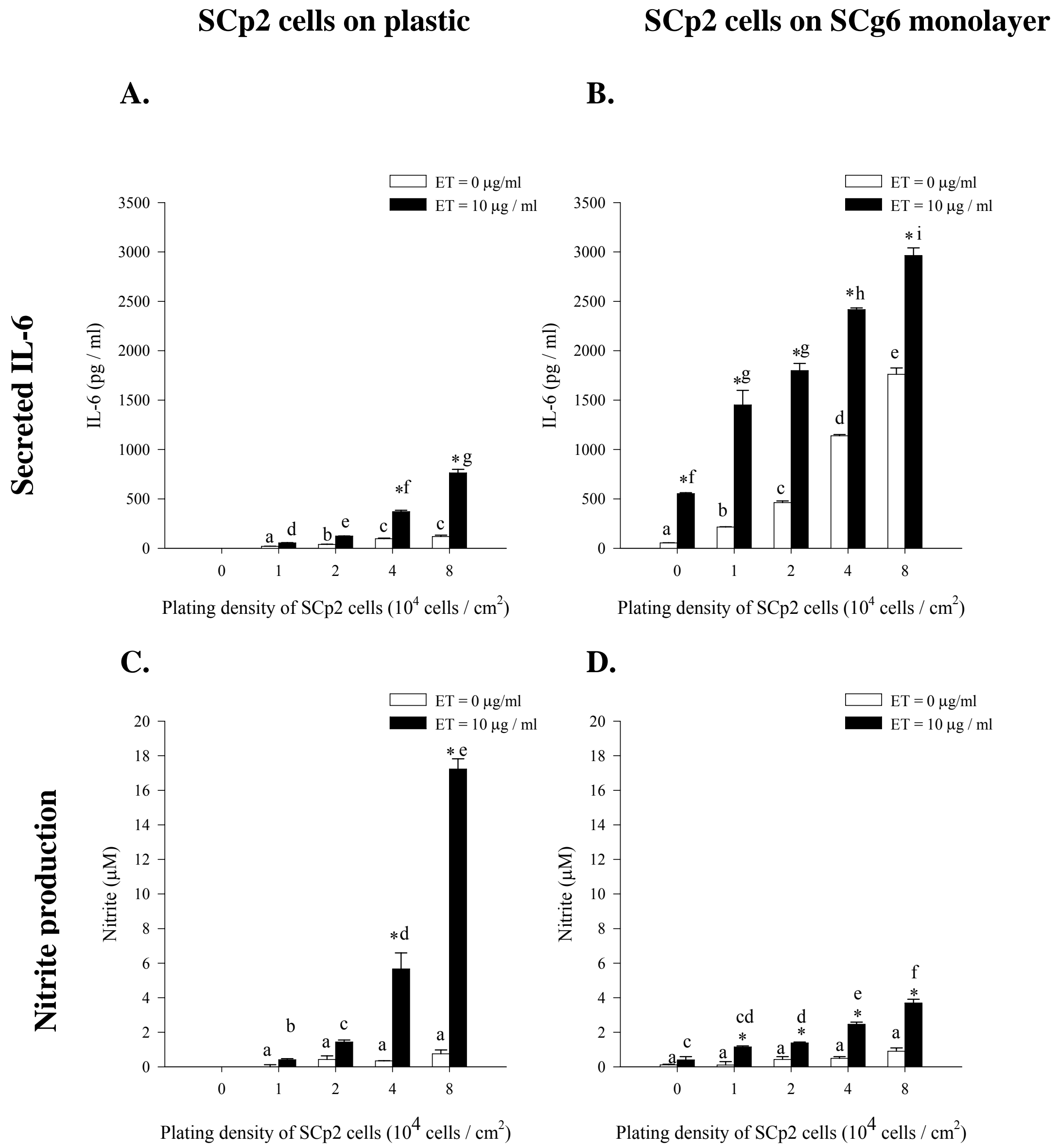


Figure 3.9

Gene &Genbank accession #	Primer Pairs 5'→3'	Size (bp)	Cycle #	Ref.
IL-6 NM_031168	F: 5'- GTTCTCTGGGAAATCGTGGA-3' R: 5'- GGAAATTGGGGTAGGAAGGA-3'	339	40	[38]
iNOS NM_010927	F: 5'-CCCTTCCGAAGTTTCTGGCAGCAGC -3' R: 5'- GGCTGTCAGAGCCTCGTGGCTTTGG -3'	497	40	NA
TNFα NM_013693	F: 5'-CCCCAAAGGGATGAGAAGTT-3' R: 5'-AGATAGCAAATCGGCTGACG-3'	319	40	[38]
GAPDH BC094037	F: 5'- ACCACAGTCCATGCCATCAC -3' R: 5'- TCCACCACCCTGTTGCTGTA -3'	452	30	[52]

Table 3.1: Nucleotide sequences of primer pairs used for real time PCR

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CHAPTER 4

PARTIAL PURIFICATION OF ANTI-INFLAMMATORY FRACTION OF LEBANESE *Centaurea ainetensis* FLOWER-HEADS USING BIOACTIVITY-GUIDED FRACTIONATION AND MOUSE MAMMARY EPITHELIAL CELLS AS A MODEL OF INFLAMMATION

ABSTRACT

Chronic inflammation not only leads to diseases such as inflammatory bowel diseases, rheumatoid arthritis, and fibrocystic inflammation of the mammary gland, but also is associated with increased risk of cancer in inflamed epithelial tissues. Therefore, the search for anti-inflammatory drugs is important not only in the fight against chronic inflammation but also for their recently suggested potential as an effective therapy against early stages of cancer. *Centaurea ainetensis* is a medicinal plant used in Lebanese traditional medicine to treat inflammatory diseases. Solid phase extraction (SPE) columns and reverse phase- high performance liquid chromatography (RP-HPLC) were used to fractionate the *C. ainetensis* crude methanol extracts for study of their inhibition of endotoxin (ET)-induced inflammation in non immune mammary secretory epithelial cells (SCp2) to screen for anti-inflammatory activity by inhibition of ET-induced IL-6 secretion. As a result, a partially purified fraction of *C. ainetensis* eluted in 60% methanol from SPE columns followed by methanol gradient elution on RP-HPLC

inhibited ET-induced IL-6 secretion with a drastic reduction of the cytotoxicity observed in the crude methanol extract. The partially purified fraction of *C. ainetensis* also reduced ET-induced nitric oxide production by SCp2 cells as assessed by Griess reaction and iNOS mRNA expression but not that of IL-6 mRNA expression assessed by RT-qPCR. Concentration and partial purification of anti-inflammatory bioactivity from crude extracts of *C. ainetensis* by SPE columns followed by RP-HPLC suggest feasibility of purification and characterization. Moreover, the use of non immune mammary epithelial cells as a model to investigate anti-inflammatory drugs presents a potential model to further investigate the link between chronic inflammation and cancers of epithelia.

INTRODUCTION

Inflammation is an innate immune response elicited in response to tissue damage or infection. Accumulating evidence suggests a functional relationship between long-term inflammation and increased risk of developing cancer especially in epithelial cells [1]. Thus, the search for anti-inflammatory drugs is important not only in the fight against chronic inflammation but also for their potential role as therapeutics against early stages of cancer [2-4].

Although immune cells such as neutrophils and macrophages are the major players during inflammation, non immune epithelial cells play an important role in initiating an inflammatory response by forming the first line of contact and defense against microbial infections and their response by secreting pro-inflammatory cytokines and chemokines to attract immune cells to the site of infection [5].

Bacterial endotoxin (ET) binds to toll-like receptor-4 (TLR-4) on the cell membrane of immune and epithelial cells (liver, intestinal), and induces, via activation of the nuclear factor kappa B (NFκB), a cadre of inflammatory respondents which include in addition to cytokines and chemokines, enzymes such as extracellular matrix (ECM) metalloproteases (MMPs), cycloxygenase-2 (Cox-2), and induced nitric oxide synthase (iNOS) responsible for the synthesis of nitric oxide (NO) [6-10]. Few studies have investigated the role of non immune cells during inflammation. Safieh-Garabedian et al (2004) showed that ET induced inflammation in mouse mammary cells was characterized by: inhibition of expression of the major milk protein β-casein, induction of NFκB, increasing levels of secreted cytokines such as interleukins 1 and 6 (IL-1, IL-6) and tumor necrosis factor alpha (TNFα), and activating MMPs (e.g., MMP-2 and MMP-9) [11].

Due to the intricate regulation of the inflammatory response by both immune and epithelial cells and because of the multiple roles that inflammatory molecules play in normal as well as inflamed tissues, search for effective and safe anti-inflammatory drugs has proven challenging because anti-inflammatory drugs that target one inflammatory respondent (e.g., Cox-2 inhibitors) might also induce serious adverse collateral side effects [12, 13] ultimately leading to withdrawal of the drug.

IL-6 is a pleiotropic cytokine produced by most immune cells as well as non immune cells such as endothelial, fibroblast and epithelial cells by acute up-regulation and secretion during the acute phase of inflammation; its persistence has been used as a marker of chronic inflammation (reviewed in [14-16]). In contrast, NO is a non-protein diffusible molecule produced through the conversion of arginine to citrulline by one of

the three forms of NOS; endothelial, neuronal or induced NOS (eNOS, nNOS and iNOS, respectively) [17, 18]. During inflammation, iNOS mRNA expression is upregulated to result in the production of high amounts (micromolar) of NO [19-21] to fight infection and modulate signaling pathways in immune as well as epithelial cells [22, 23].

Our previous studies have shown that ET induced IL-6 secretion and NO production in SCp2 mouse mammary secretory epithelial cells was regulated in part by NFκB (Chapter 2). Therefore, we suggest the use of ET-induced inflammation in SCp2 mouse mammary epithelial cells as a non immune cell model to screen for anti-inflammatory agents based on ability to inhibit ET-induced IL-6 secretion and/or NO production and identify possible mechanisms of action.

Because of their use in traditional folk medicine in several Mediterranean countries, the flower-heads of several *Centaurea* plant species have been studied for their therapeutic potential for treatment of inflammation and other diseases such as diabetes, cadiovascular diseases and microbial infections [24-26]. Moreover, of the 29 indigenous plants used in Lebanese traditional folk medicine and screened for anti-inflammatory and anti-cancer bioactivities, crude water and methanol extracts of *Centaurea ainetensis* flower-heads were the most effective in initial crude screening by reduction of ET-induced inflammation in mouse mammary cell lines (El- Jouni, WM., 2003, "Characterization of Potential Anti-Inflammatory Bio-Activities In Selected Indigenous Medicinal Plants Of Lebanon", MSc Thesis, The American University of Beirut, Beirut, Lebanon), in addition to their reported anti-bacterial [27], and anti-viral activities [28].

To study the mechanism of therapeutic action of *C. ainetensis* towards inflamed epithelial tissues, we describe here initial efforts to purify and characterize the anti-

inflammatory agents, and separate from cytotoxicity activites, using bio-guided fractionation based primarily on inhibition of ET-induced inflammation in SCp2 mouse mammary secretory epithelial cell cultures as an epithelial inflammation model system.

MATERIALS AND METHODS

Materials. Methanol (MeOH) (HPLC grade) and glacial acetic acid (HAc) (ACS reagent grade) were purchased from Fisher Scientific (Fairlawn, NJ). Bovine serum albumin (BSA), Dulbecco's modified eagle's medium/Ham F12 (DMEM/F12; 1:1), bovine insulin, hydrocortisone, ovine prolactin, and *Salmonella typhosa* endotoxin (ET; > 500000 EU (endotoxin units)/ mg) were purchased from Sigma (St. Louis, MO). Heat inactivated fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), and gentamycin were purchased from Cambrex Bioscience (Walkersville, MD). BD Falcon cell culture flasks (75 cm^2), 96-well plates and 6-well plates were purchased from BD Biosciences (Bedford, MA). Complete Protease Inhibitor Cocktail Tablets (product # 11697498001) were purchased from Roche Diagnostics (Penzberg, Germany), and tetramethyl benzidine (TMB) was purchased from BioFX Laboratories (Owings Mills, MD). All procedures were done at room temperature unless specified otherwise.

Plant collection and crude methanol extract preparation. *Centaurea ainetensis* flower heads were collected near Aineta (hence the species name ainetensis), a village in the semi-dry region of the anti-Lebanon mountain chain (1700-1800 m above sea level), during the peak flowering season (May until June) of 2003. The collected flower heads were air-dried at room temperature for 2 weeks, in the absence of direct sunlight. The dried plant flower

heads were ground and stored dry at -20°C until further extraction. Extraction of bioactivities involved soaking the finely crushed dry plant flower heads in 100% HPLC grade MeOH (10ml MeOH / g of dried material) for 16 h at room temperature, followed by filtering through three layers of cheese cloth. The MeOH filtrate was centrifuged at 274 x g for 10 min at 4°C. The supernatant was filtered through a 0.2 µm Acrodisc syringe filter (HT Tuffryn membrane; Gelman Laboratory, Ann Arbor, MI). The crude methanol filtrate (100 % crude methanol extract) was stored in a light-protected glass bottle at -20°C until further use.

Solid phase extraction and fractionation. Crude MeOH extracts of *Centaurea ainetensis* were fractionated by solid phase extraction (SPE) on SPE High Capacity Extract-Clean C18 (octadecylsilyl) reverse phase columns (packed bed weight = 1000mg, silica surface area = 537 m^2 / g) (Alltech Associates, Deerfield, IL) pre-activated by washing with 5ml of 100% MeOH followed by 5ml of water. Unless stated otherwise, SPE fractionation involved dilution of crude 100% MeOH extraction samples to 25% MeOH in water before loading (6 ml) onto an SPE column, washing with 6.0 ml 25% MeOH, and elution with 10 ml 60% MeOH followed by 8.0 ml 100% MeOH. Fractions of 1.0 ml were collected throughout the loading, wash, and step-wise elutions. The bioactive fraction eluted in the 60% MeOH eluate from the first SPE column (SPE1) was subsequently diluted to 25% MeOH and applied to a fresh SPE column (SPE2) with the same wash and elution steps as for the first. Maximum yield of anti-inflammatory bioactivity with minimal cytotoxicity was attained by a final loading of the bioactive fractions from the 60% MeOH eluate (diluted to 25% MeOH before loading) of SPE2

onto a third SPE column (SPE3), washing with an equal volume of 25% MeOH and final elution with 1.0 ml increments of 100% MeOH. The concentrated anti-inflammatory bioactivity was eluted in the second 1.0 ml 100% MeOH eluate after the wash (SPE3 bioactive fraction). Elution of fractionated materials and bioactivities (anti-inflammatory activity, cytotoxicity, and anti-proliferative activity) were monitored in each 1 ml fraction by the respective bioassays (described below) and by A_{280}.

Reverse phase- high performance liquid chromatography (RP-HPLC). In an effort to purify the bioactive agents of *Centaurea ainetensis*, the final SPE3 bioactive fraction was further purified by passage through a C18 reverse phase HPLC (Alltech Rocket column; Alltima C18- 3 μm, 7 mm x 53 mm; Alltech, Deerfield, IL) using a Beckman System Gold HPLC (Beckman Coulter, Inc., Fullerton, CA) equipped with a photodiode array (PDA) detector that provides for broad UV/Vis spectral (190nm-600nm) analysis of the eluate and collected fractions. For HPLC resolution, SPE bioactive fractions (in 100% MeOH) were diluted to 25% MeOH, 0.2% HAc in H_2O, centrifuged at 274 x g for 10 min at 4°C. The supernatant was filtered through a 0.45 μm syringe filter (PVDF Millex ®-HV Millipore, Billerica, MA) before loading 200-500 μl onto the HPLC column pre-equilibrated with 33% MeOH, 0.2% HAc in H_2O at a column temperature of 30°C. All elution gradients maintained constant 0.2% HAc. The loaded column was eluted at 1ml / min flow rate by programmed gradient of increasing MeOH concentration as follows: 33% to 40% over 3.5 min, 40% to 60% MeOH over 4min, isocratic elution at 60% MeOH for 5min, followed by
60% to 100% MeOH over 10 min to flush the column and a return to 33% of MeOH over 5min.

Eluted fractions (1ml) were collected every minute, dried *in vacuo* at room temperature (Speed Vac concentrator, Savant instruments, Inc., Farmingdale, NY), and either stored dry or resuspended in 50-125 µl deionised water and stored at -20°C for later use in biological screening assays.

Screening bioassays:

Cell culture model. SCp2 mouse mammary secretory epithelial cells, kindly provided by Dr. Pierre Desprez (Geraldine Brush Cancer Research Institute; San Francisco, CA), were maintained in 5% FBS growth medium (5% FBS-GM) composed of DMEM/F12 containing 5% FBS, insulin (5 µg/ ml) and Gentamicin (50 µg/ ml) in a 37°C humidified atmosphere with 5% CO_2. To insure predominance of secretory epithelial cell type in SCp2 cell cultures, SCp2 cells were passaged by selective trypsinization as previously described (chapter 2).

ET-induced inflammation in SCp2 mouse mammary secretory-epithelial cells. To screen for bioactivity in crude and purified methanol fractions of *C. ainetensis*, SCp2 cells were plated in 96-well plates at $3x10^4$ cells / cm^2 in 5% FBS-GM for cell attachment and proliferation to 70% confluence, generally after 48 h, before addition of crude methanol extract or purified fractions in 5% FBS-GM thirty minutes prior to induction of inflammation by addition of ET; (0 or 10 µg / ml). This concentration of ET used for routine induction of inflammation for screening was chosen from ET-dose / response studies as the concentration able to induce a maximal inflammatory response in SCp2 cells (defined by IL-6 secretion, see below) without cytotoxicity (measured by LDH

release). The medium was collected from each of triplicate wells per treatment at 24 h after ET application (post-ET) and used to assay for cytotoxicity and anti-inflammatory activity (by inhibition of ET-induced IL-6 secretion) as depicted in figure 4.2A.

For mechanistic studies of *C. ainetensis* bioactivity (depicted in Fig. 4.2 B), SCp2 cells were plated in 6-well plates at 4×10^4 cell / cm^2 in 5% FBS-GM for cell attachment and proliferation to achieve 50% confluence, generally after 24 h. Cells at 50% confluence were washed twice with HBSS before feeding with serum-free differentiation medium (0% FBS-DM) and incubating for 24 h before division into 6 treatment groups: control (no plant extract treatment), sham (MeOH or vehicle-treated (0.2% v/v)), and *C. ainetensis* SPE3 bioactive fraction-treated cells (0.2% v/v as µl SPE3 fraction / 0.1 ml of 1% FBS-DM per well), in the absence or presence of ET (10 µg/ ml) in 1% FBS DM (to enhance response to ET). The dose of SPE3 bioactive fraction used in this study was pre-determined from previous dose response analysis to inhibit ET-induced IL-6 secretion with minimal or no cytotoxicity (data not shown). The medium collected from all treatment cultures at 0, 1, 3, 6, 12, and 24 h post-ET for bioassay analysis was protected from non-specific protease action during handling and storage by immediate addition of Complete™ protease inhibitor solution (1 Complete tablet/ 2 ml deionized water) at 40 µl/ ml to collected medium before storing at -80°C for later analysis (North, 1969). The cells from each medium harvest were washed twice with HBSS and frozen at -80°C for subsequent total RNA extraction.

Immunoassay of Interleukin-6. To measure IL-6 secretion in response to ET in SCp2 cells, medium collected at various times post ET treatment was assayed by enzyme linked

immunosorbent assay (ELISA) for IL-6 (Duo set kit, R&D Systems Inc, Minneapolis, MN) according to the manufacturer's protocol. The IL-6 ELISA performed in Immunolon flat bottom 96 well plates (Thermo; Milford, MA) relied on rat anti-mouse IL-6 antibody, blocking with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 5% sucrose (Sigma, St. Louis, MO), mouse IL-6 standards dissolved in either 5% FBS-GM or 1% FBS-DM as for experimental samples, and detection with biotinylated goat anti-mouse antibody followed by streptavidin conjugated to horseradish-peroxidase (HRP). Addition of HRP chromophoric substrate TMB yielded color intensity (A_{690}) in proportion to the amount of IL-6 present in the sample. Samples were assayed in duplicate and data is represented as the average of duplicate samples $\pm$ standard error of mean (SEM).

Cell metabolic activity assay for cell number. The effect of crude plant extracts, SPE fractions, or HPLC eluate fractions on SCp2 metabolic activity relied on the Promega cell titer 96™ non-radioactive cell proliferation colorimetric assay (Promega Corp., Madison, WI) performed according to the manufacturer's recommendations. At 24 h after treatment with bioactive fraction treatment followed by ET addition 30 min later, MTS tetrazolium solution was added to cells in 5% FBS-GM at a final concentration of 317 µg / ml in each well. Cells were incubated at 37°C for 2 h, the conversion of MTS tetrazolium salt into a colored formazan product by living cells, quantified as A_{490}, was proportional to metabolically active cells. Relative cell metabolic activity is represented as percent of metabolic activity in control cells of triplicate samples $\pm$ SEM.

Cytotoxicity assay. The cytotoxicity of plant extracts, SPE fractions, or HPLC eluate fractions was monitored with the Promega Cytotox 96© non-radioactive cytotoxicity kit performed in 96 well plates and according to the manufacturer's instructions. The intensity of color (A_{490}) resulting from the conversion of tetrazolium salt to a red formazan dye is proportional to the amount of LDH released from dead cells into the culture medium. Each 96 well assay plate included two controls: a maximum LDH release control consisting of cells treated with the kit's proprietary cell lysis buffer for one hour before assay for cytotoxicity, and a spontaneous background LDH release control consisting of untreated control cells. The level of LDH released from cells treated with ET$\pm$ bioactive fractions was expressed as the (%) of the net maximum LDH release from lysed cells after substraction of the background spontaneous LDH release from control cells. Data is represented as the average of triplicate samples $\pm$ SEM. Cytotoxicity measured by LDH release was confirmed by microscopic examination of cells for morphological changes and visual scoring on a scale of (0 to 5), where 5 reflects a monolayer of confluent cuboidal shaped cells while 0 describes dead cells with 90-100% detached and floating in the medium.

Griess reaction assay of NO for NOS activity. The analysis of NO as a measure of NOS activity was accomplished by the Griess assay for nitrite (the spontaneous oxidation product of NO) using a Griess Reagent Kit [29] (Molecular Probes, Eugene, OR) involving addition of 20 µl of Griess Reagent (0.05% N-(1-napthyl) ethylenediamine dihydrochloride, 0.5% sulfanilic acid in 2.5% phosphoric acid) to 150 µl of nitrite-containing sample or standard and 130 µl deionized water, per the manufacturer's instructions. The color developed by azo dye formation in proportion to nitrite concentration (µM) in solution

was quantified by A_{550}. Assays of nitrite in cell culture medium used sodium nitrite ($NaNO_2$) standards diluted in appropriate medium from that experiment. Samples were assayed in duplicate and data is represented as the average concentration of NO_2^- (μM) of duplicate samples ± SEM.

RNA extraction, reverse-transcription and quantitative real time polymerase chain reaction analysis. Total RNA was scrape harvested from cells grown in 6-well plates using Qiagen RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Isolated RNA was quantified by A_{260}, and only RNA with ratio $A_{260} / A_{280} \geq 1.6$ was used for reverse transcription (RT) reactions. A defined amount of RNA (1μg) was treated with DNAse I (Promega, Madison, WI) before being reverse transcribed by incubation with random primer and avian myeloblastosis virus (AMV) reverse transcriptase at 42°C for 30 min in a 40 μl reaction using the Promega reverse transcription system (Promega, Madison, WI). Quantitative real time PCR (qPCR) was performed by adding 2 μl of undiluted RT product to 18 μl of Qiagen Hot start SyBR Green PCR master mix (Qiagen, Valencia, CA) containing 0.15 μM each of the sense (or forward; F) and anti-sense (or reverse; R) primers for each of IL-6 and iNOS target genes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene (Table 4.1). A no template control was included in each PCR plate. SybrGreen fluorescence was detected by MJ Research Opticon 2 real time detection system (BioRad, Hercules, CA), and amplified products were quantified relative to appropriate standard curve in autonomous qPCR assay reactions. Primer pairs were either adopted from the literature or designed using Primer_3 primer design software [30] and synthesized by Operon Biotechnologies Inc (Huntsville, AL). Target amplicons spanned an

intron to exclude amplification of genomic DNA. The standard curves for each of IL-6, iNOS and GAPDH genes of interest were generated by serial dilutions of the purified (QIAquick clean up kit; Qiagen, Valencia, CA (per manufacturer's instructions)) and sequenced RT-PCR product amplified from total RNA isolated from ET-treated SCp2 cells. Quantitative PCR conditions were, in sequence: a hot start at 94°C, 15min; followed by initial denaturation at 94°C, 5min, and subsequent annealing at 58°C, 30 sec. Each sample was analyzed in triplicate qPCR reactions with the average for each gene of interest normalized against the average for the reference GAPDH gene. The results of qPCR analysis are presented as the average for duplicate samples $\pm$ SEM.

Statistical analysis. . Significant differences between different groups were determined using Proc Mixed analysis of SAS 9.1 (SAS Institute Inc., Cary, NC). For each of the experiments studying the effect of ET alone or ET in the presence or absence of SPE3 bioactive fraction on IL-6 mRNA and iNOS mRNA expression or IL-6 secretion and NO production, the statistical model included time, treatment (ET, SPE3 bioactive fraction, or ET and SPE3 bioactive fraction), and time by treatment interactions. The effect of treatment within each time point was tested using the slice option by time. Results were expressed as mean $\pm$SEM, and significance was defined as $p<0.05$, unless noted otherwise.

RESULTS:

Crude methanol extracts of *Centaurea ainetensis* inhibit ET-induced IL-6 secretion in SCp2 cells. Pulverized dried flower heads of *C. ainetensis* were soaked for 16 h in

100% MeOH (10 ml / 1 g plant material) to yield a dark-green crude extract referred to as crude MeOH extract. The anti-inflammatory activity was analyzed by application of dilutions (0, 0.02, 0.08, 0.2, 0.4, 0.8, 1, 2, 5% [µl MeOH extract / 0.1 ml of 5% FBS-GM / well]) of the crude MeOH extract to ET-treated SCp2 mouse mammary cells (as described in materials and methods), and assaying for the inhibition of ET-induced IL-6 secretion. The effects of the MeOH extract on cell metabolic activity (Cell proliferation assay) and cytotoxicity (LDH release and visual scoring of cells) were tested to confirm that the reduced level of ET-induced IL-6 secretion is not due to cell damage by toxicity. Dilutions of *C. ainetensis* crude MeOH extracts to MeOH concentrations of less than 0.8% MeOH (v/v) reduced ET-induced IL-6 secretion in a dose dependent manner without inducing cytotoxicity. However, for samples assayed at MeOH greater than 0.8% (v/v) reduced IL-6 secretion was most likely due to decreased cell metabolic activity and/or increased cytotoxicity (Fig. 4.4). Vehicle (MeOH) concentrations higher than 1% (volume of MeOH/ volume of 5% FBS-GM) reduced ET-induced IL-6 secretion independent of the plant MeOH extract, most likely due to cytotoxicity (Fig 4.4 inset).

SPE fractionation of MeOH extracts of *C. ainetensis*. To purify and separate the anti-inflammatory activity, *C. ainetensis* crude MeOH extract was fractionated on a C18 SPE column (SPE1) as described in materials and methods. Crude MeOH extract (1.5 ml) of *C. ainetensis* was diluted to 25% MeOH with deionized water up to 6ml before loading on a pre-activated SPE column. This loaded SPE1 column was subsequently washed with 6ml of 25% MeOH and eluted with 10 ml of 60% MEOH followed by 8 ml of 100% MeOH (Fig. 4.3A). Fractions of 1 ml, collected throughout the load, wash, and 2-step

elution, were concentrated 4X *in vacuo*, and assayed for their bioactivity as described in materials and methods and Fig. 4.2A.

Anti-inflammatory activity of SPE1 fractions. Concentrated fractions of SPE1 were added to SCp2 cells at an optimized dose of 0.8% (fraction volume/ volume of 5% FBS-GM) per well thirty minutes before treatment with ET (10 μg / ml) (see Fig. 4.2A). At 24 h post-ET, the conditioned medium was collected and analyzed for induced IL-6 secretion by ELISA and for cytotoxicity by measure of LDH release, while the cells were fed with fresh medium (5% FBS-GM) in order to assay for their metabolic activity by cell proliferation assay per the manufacturer's instructions as previously described (materials and methods). Compared to the ET-treated control cells not treated with plant extracts, fractions from the load and wash steps of SPE1 (fractions 1 to 12) did not inhibit ET-induced IL-6 secretion (Fig. 4.5A) nor induce cytotoxicity (Fig. 4.5B). Fractions 14, 15 and 16 eluted with 60% MeOH, as well as fraction 24 eluted with 100% MeOH showed the most inhibition (~ 80-100%) of ET-induced IL-6 secretion (Fig. 4.5A), but also showed high LDH release and a low visual score (Fig. 4.5B) as well as 40-60% inhibition of metabolic cell activity (cell proliferation assay) (Fig. 4.5A), indicating cytotoxicity. Repeated extractions consistently yielded anti-inflammatory and cytotoxicity bioactivities in fractions 14 and 15 of SPE1, but the anti-inflammatory activity was inconsistently recovered in fraction 24 of SPE1, and therefore was not further analyzed.

A white precipitate often formed in the crude methanol extracts after dilution to 25% methanol and upon centrifugation at 278 x g for 10 min at 4°C. The supernatant and the re-dissolved precipitate (in 100% MeOH) were assayed for anti-inflammatory bioactivity. The

re-dissolved precipitate showed little or no anti-inflammatory activity assessed by little or no inhibition of ET-induced IL-6 secretion, whereas equivalent doses of the supernatant (25% MEOH extract) showed strong anti-inflammatory activity (data not shown).

Successive SPE purification reduced cytotoxicity of anti-inflammatory bioactive fractions. In an effort to separate the cytotoxicity from the anti-inflammatory activity, SPE 1 fractions 14 to 22 eluted with 60% MeOH were pooled, diluted to 25% methanol and reloaded on a fresh SPE column (SPE2). After applying the same load, wash, and 2-step elution as for SPE1 (Fig. 4.3B), the eluted fractions (1m each) were concentrated 4X and assayed for bioactivities (Fig 4.2A). Fractions 1 to 12 of SPE2 showed little or no effect on ET-induced IL-6 secretion and cytotoxicity. Fractions 14 and 15 consistently inhibited ET-induced IL-6 secretion by ~ 80-90% but showed less cytotoxicity, indicated by decreased LDH release and increased visual scores of cells, and little or no inhibition of cell metabolizing activity when compared to corresponding fractions of SPE1 (Fig. 4.6A & B).

Before further fractionation by RP-HPLC, the 60% MeOH eluates of SPE2 (fractions 14 to 22) were concentrated by pooling, diluting to 25% MEOH and reloading on a third SPE column (SPE3) (Fig. 4.3C). The loaded SPE3 column was washed with a volume of 25% MeOH equal to the loaded volume before eluting with 3 x 1ml aliquots of 100% MEOH. The second 1ml after 100% MeOH elution contained the highly concentrated anti-inflammatory activity and was referred to as SPE3 bioactive fraction.

Resolution of anti-inflammatory activity of *Centaurea ainetensis* by RP-HPLC. SPE3 bioactive fraction was diluted to 25% MeOH, 0.2% HAc, in water and loaded on a C18 reverse phase HPLC column (7 mm x 53 mm, 3 μm; Alltech) (Fig. 4.3D). The RP-HPLC

column was eluted with MEOH : H_2O programmed gradient containing 0.2% HAc at a flow rate of 1ml / min comprised of linear gradients from 33% to 40% MeOH over 3.5 ml, followed by 40% to 60% MeOH over 4.0 ml, isocratic elution at 60% MeOH over 5 ml, and a final linear gradient from 60% to 100 % MeOH over 5ml and isocratic elution at 100% MeOH over 10ml to flush the column before a decreasing linear gradient to 33% MeOH over 5 ml for column re-equilibration. The HPLC elution profile (HPLC1) was monitored by UV/VIS absorption using a photodiode array detector spanning wavelengths from 190 to 600 nm. Eluted 1 ml fractions of HPLC1 were dried *in vacuo* and redissolved in water for assay of anti-inflammatory bioactivity and cytotoxicity (Fig. 4.2A). Fractions 12 and 13 of HPLC1 contained most of the eluted anti-inflammatory activity, reducing ET-induced IL-6 secretion by ~25% and ~60%, respectively, relative to ET-treated solvent controls (Fig. 4.7 and 4.8A). The remaining HPLC1 fractions showed either no inhibition (fractions 1 to 11, 15, and 17-20) or inconsistent inhibition (fractions 14 and 16) of ET-induced IL-6 secretion (Fig. 4.7 and 4.8A). All HPLC1 fractions had minimal or no cytotoxicity on SCp2 cells as measured by LDH release and cell metabolic activity (data not shown). Fractions 11, 12, and 13 (F11, 12, and 13) of HPLC1 were separately diluted to 25% MeOH in 0.2% HAc and each was individually re-chromatographed on the same C-18 RP-HPLC as for HPLC1 (HPLC2). Activity of HPLC1-F12 eluted in fraction 12 of HPLC2 with ~30% maximum inhibition of IL-6 secretion and little activity in fraction 13 (Fig. 4.8C). The bioactivity of HPLC1-F13 eluted in fraction 13 of HPLC2 with ~55% inhibition of IL-6 secretion and little activity in fraction 12 (Fig. 4.8D). HPLC1-F12 eluted fractions showed no significant anti-inflammatory bioactivity. Each HPLC2 showed eluted activity in proportion to activity in the parent fraction from HPLC1. Each of the activities distributed between fractions 12 and 13

after re-chromatography and spectral analysis of either HPLC1 fractions 12 or 13 suggests multiple compounds responsible for the anti-inflammatory activity.

Liquid chromatography-Mass spectral analysis of HPLC2-F13. Eventhough The split anti-inflammatory bioactivity between fractions 12 (Fig. 4.8C) and 13 (Fig. 4.8D) of HPLC1 suggested multiple compounds, HPLC2 fraction 13 of HPLC1-F13 (Fig. 4.8D) was analyzed by liquid chromatography-mass spectral (LC-MS) analysis using a quadrapole and time of flight (Q-TOF2) electrospray MS (Mass Spectrometry & Proteomics Facility at the Ohio State Univeristy, Columbus, OH). LC-MS analysis results estimated ~20 compounds in the analyzed fraction without further information on their chemical structure or nature (Fig. 4.9). Further purification efforts were not possible due to limited availability of plant material.

The mechanism of anti-inflammatory activity of SPE3 bioactive fraction in ET-induced inflammation in SCp2 cells.. In an attempt to study the mechanism of SPE3 purified *C. ainetensis* anti-inflammatory activity in SCp2 mammary epithelial cells, we analysed the medium collected from the 6 treatment groups : control (no plant extract treatment), sham (MeOH or vehicle-treated), and *C. ainetensis* SPE3 bioactive fraction-treated cells (0.2% v/v), in the absence or presence of ET (10 μg/ ml) in 1% FBS DM as described in material and methods. ET ± MeOH induced both IL-6 secretion and NO production in SCp2 cells with an expected delay of ET-induced NO production (starting at 6 h post-ET) compared to an earlier ET-induced IL-6 secretion (starting at 3 h post-ET). Addition of SPE3 bioactive fraction reduced ET-induced IL-6 secretion and NO production by 50 % ($p<0.05$) (Fig. 4.10A) and ~40-50 % ($p<0.05$) (Fig. 4.10B), respectively. ET-induced IL-6 secretion was inhibited by the SPE3 bioactive fraction as early as 3 h post-ET (Fig. 4.10A), while ET-

induced NO production was inhibited at 6h post-ET (Fig. 4.10B). Sham (vehicle treated) and SPE3 treated cells in the absence of ET showed no significant induction of IL-6 secretion or NO production (Fig. 4.10). Despite the inhibition of ET-induced IL-6 secretion in SCp2 cells, further analysis of ET-induced IL-6 mRNA expression in the presence of SPE3 bioactive fraction showed no inhibitory activity of IL-6 at any of the time point assayed (Fig. 4.11A). In contrast, the SPE3 bioactive fraction reduced ET-induced iNOS mRNA expression by ~40 % ($p<0.05$) only at 3 h post-ET (Fig. 4.11B). Surprisingly, SCp2 cells treated with ET $\pm$ MeOH showed an ET-induced IL-6 mRNA expression as early as 1 h post-ET, that increased significantly ($p<0.05$) at 3 h and remained high at 6 h (Fig. 4.11A) contrary to our previous description of rapid decrease of ET-induced IL-6 mRNA expression at 6 h post-ET (chapter 2). In contrast, iNOS mRNA expression was not induced at 1 h post-ET in absence or presence of MeOH, showed a sharp and significant increase ($p<0.05$) at 3 h post-ET from near zero to peak at a maximum level then decreased sharply at 6 h post-ET (Fig. 4.11B) as expected from our previous studies (chapter 2). The high level of IL-6 mRNA expression in SCp2 cells at 1 h after addition of SPE3 bioactive fraction in the absence of ET (Fig. 4.11A) was inexpicable in view of the apparent increase, but with high error bar.

DISCUSSION

This study succeeded in partially purifying and concentrating the anti-inflammatory bioactivity from dried flower heads of *C. ainetensis* in a time and cost effective bioactivity-guided fractionation using ET-induced inflammation in SCp2 non immune mouse mammary epithelial cells. We further obtained preliminary insights into

the mechanism of action for the anti-inflammatory activity of the partially purified SPE3 bioactive fraction for key inflammatory respondents; IL-6 generation and NO production.

SPE-based fractionation proved to be a quick, easy, and a reproducible method to separate the bioactive fractions from non-bioactive components of plant crude extracts. The SPE fractionation described here also offers good throughput at modest cost for screening candidate plant samples or populations. Moreover, while regular chemical fractionation required several rounds of differential solvent extraction or component precipitation and re-dissolution, with pH monitoring and use of multiple solvents such as chloroform, petroleum ether, etc. [31, 32], SPE fractionation required only an SPE column pre-activation step with MeOH followed by water before loading the MeOH crude plant extract and subsequent washing and elution with appropriate mobile phases. The procedure was scalable by loading and eluting multiple SPE1 columns in parallel (or using large single columns) and pooling the corresponding bioactive fractions eluted with 60% MeOH. Importantly, by eliminating the several steps of precipitation and re-suspension that occurs during chemical purification, SPE purification likely reduced the loss of bioactive material during the purification process.

In order to monitor and compare the anti-inflammatory bioactivity yield in different plant MeOH extract preparations or bioactive SPE and RP-HPLC eluate fractions, we monitored absorbance of A_{280} at the different steps of plant extract fractionation. The absorption profile for A_{280} monitored fractionation appeared to correlate with the anti-inflammatory activity of SPE eluted fractions in 60% MeOH and was therefore used for routine monitoring of bioactivity in SPE fractionation protocol for different plant extract preparations. However, RP-HPLC eluted activity was not

coincided with the A_{280} absorption profile or any of other wavelengths monitored by the photodiode array (190-600nm). However, the A_{280} decreased with decreasing cytotoxicity of the anti-inflammatory bioactive SPE eluted fractions (data not shown).

Dilution of the crude MeOH extract of *C. ainetensis* to 25% MeOH before loading on SPE columns often caused formation of a white precipitate, which when re-dissolved in 100% MeOH showed little or no anti-inflammatory activity (data not shown). Further fractionation of the re-dissolved precipitate by HPLC and monitoring elution by A_{280} resulted in a major peak at 11.6 min elution but had no anti-inflammatory activity or cytotoxicity in SCp2 cells (data not shown).

The anti-inflammatory bioactivity of crude MeOH extracts and SPE3 100% MeOH elutates from *C. ainetensis* retained anti-inflammatory bioactivity after 2 years of storage at -20°C; in contrast to HPLC purified fractions dried *in vacuo* and resolubilized in water (data not shown).

Reverse phase HPLC fractions from the isocratic 60% MeOH elution step of the gradient elution program recovered the anti-inflammatory activity in fraction 13 and to a lesser extent in fraction 12 of HPLC1 (Fig. 4.7 and 4.8A). Further HPLC fractionation of the two bioactive fractions consistently recovered the anti-inflammatory bioactivity in F13 of HPLC2 profile of HPLC1-F13 (fig. 4.8D) and to a lesser extent in F12 of HPLC2 profile of HPLC1-F12 (Fig. 4.8C). The results of LC-MS analysis of HPLC2 F13 showed the existence of an estimated 20 compounds but was unable to define a specific chemical identity or composition as the bioactive agent without further purification; which was precluded by limited availability of plant material for the remainder of this study. It is worth noting that the bioactive fractions from solid phase extraction of *C.*

ainetensis MeOH crude extracts,, 60% MeOH SPE1 / SPE2 and the 100% MeOH SPE3 elution fractions, all had a bright yellow-orange color characteristic of flavonoids, previously described in extracts of other *Centaurea* species [25].

In efforts to gain insight into the mechanism of action of the anti-inflammatory activity of *C. ainetensis*, we investigated the effect of SPE3 bioactive fraction on ET-induced IL-6 and NO production as well as IL-6 and iNOS mRNA expression in SCp2 cells at different time points after ET $\pm$ SPE bioactive fraction (co-treatment). As expected from our previous studies (chapter 2), ET treatment in the absence of the SPE3 bioactive fraction induced IL-6 secretion at 3 h post-ET (Fig. 4.10A), and a later NO production at 6 h post-ET (Fig. 4.10B). SPE3 bioactive fraction applied to SCp2 cells in the presence of ET reduced both IL-6 secretion by ~50% (Fig. 4.10A) and NO production by 40% (Fig. 4.10B) relative to levels induced in response to ET alone. The time of onset for each inhibition was different depending on their respective time of induction post-ET; the inhibition of ET-induced IL-6 secretion started at 3 h after co-treatment with ET and SPE3 bioactive fraction, while the inhibition of ET-induced NO production was not observed until 6 h after co-treatment application. Further analysis of the effect of the anti-inflammatory bioactive fraction on the mRNA expression resulted in inhibition of ET-induced iNOS (Fig. 4.11B) but not IL-6 (Fig. 4.11A). Interestingly, the SPE3 anti-inflammatory bioactive fraction reduced IL-6 secretion but not IL-6 mRNA expression, consistent with our earlier findings of the involvement of different pathways in the regulation of ET-induced IL-6 and iNOS (chapter 2) mRNA expression in SCp2 mammary epithelial cells and suggestive of post-transcripitonal regulation of ET-induced IL-6 response.

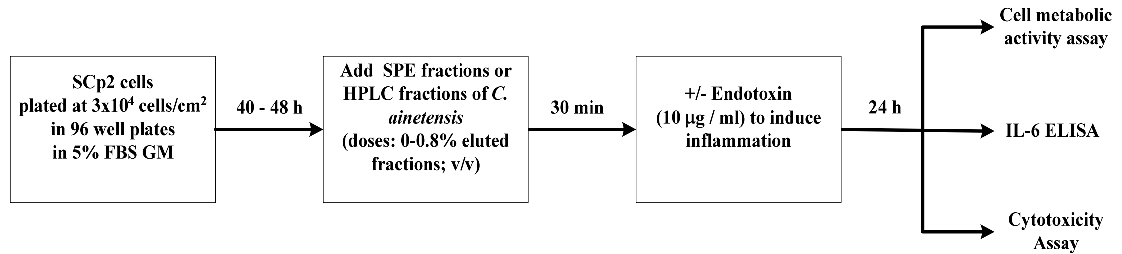
El-Jouni (2003) showed crude MeOH extracts of *C. ainetensis* to selectively inhibit MMP9 but not MMP2 protease activity in response to ET (El- Jouni, WM., 2003, "Characterization of Potential Anti-Inflammatory Bio-Activities In Selected Indigenous Medicinal Plants Of Lebanon", MSc Thesis, The American University of Beirut, Beirut, Lebanon). Taken together these findings suggest that *C. ainetensis* crude MeOH or SPE3 fractions likely selectively inhibit certain but not all pro-inflammatory markers induced by ET in SCp2 cells. In addition, other reports suggested a role for the mitogen activated protein kinase (MAPK) in MMP-2 activation (reviewed in [33]), iNOS mRNA expression [34, 35], and the translation of IL-6 and other cytokines [36]. Further definition of the signaling pathway through which they are acting and the regulatory factors involved (especially NFκB forms and regulators or MAPK) will help define the therapeutic potential for *C. ainetensis*.

Finally, we demonstrated that SCp2 mouse mammary secretory epithelial cell cultures can be scaled down to 96 well plate formats to be used for high-throughput screening of anti-inflammatory bioactivity, and can potentially allow determination of the mechanism of the anti-inflammatory activity of *C. ainetensis* in inflamed epithelial cells. Our preliminary mechanistic investigations suggest a selective inhibition of ET-induced iNOS but not IL-6 mRNA expression, however, further studies are required to identify the target signaling pathway of *C. ainetensis*, and depend on further purification and identification of the bioactive agent responsible for the anti-inflammatory activity of C. ainetensis in order to be definitive. In conclusion, the use of non immune mammary epithelial cells as a model to investigate anti-inflammatory activity of candidate drugs

presents a potential model to further investigate the link between chronic inflammation and cancers of epithelia.

Figure 4.1: ***Centaurea ainetensis*** **floral head (photo courtesy of Khaled Sleem)**

A. Bio-screening assays:



B. Mechanistic studies of SPE3 bioactive fraction:

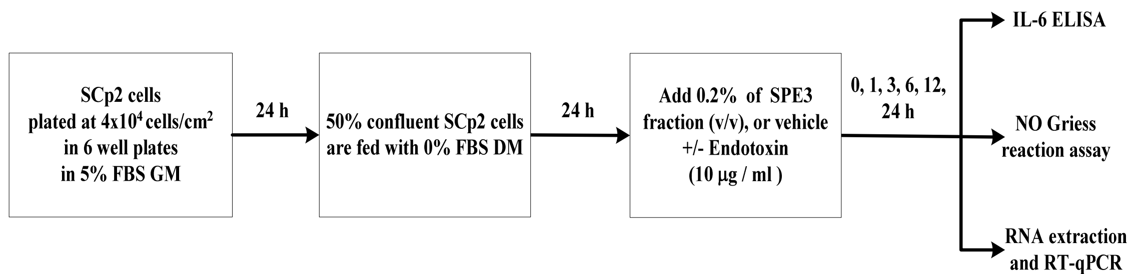


Figure 4.2: Schematic illustrations of. (A) Bioassay screening for anti-inflammatory, anti-proliferative and cytotoxic activities in SPE and/or HPLC eluted fractions. (B) Mechanistic assays for SPE3 bioactive fraction in ET-induced inflammation in SCp2 cells.

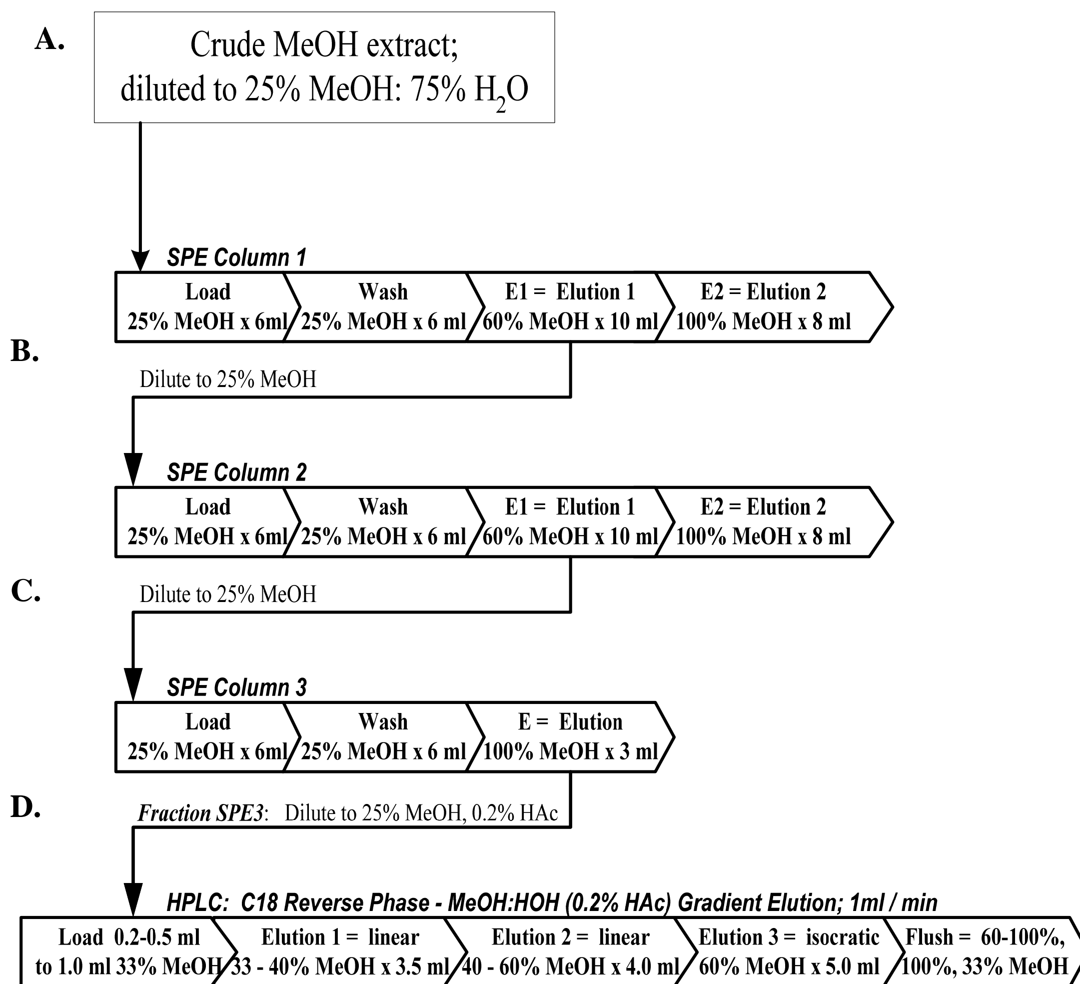


Figure 4.3: Fractionation and purification scheme for anit-inflammatory activity of *C. ainetensis* by MeOH extraction, repeated solid phase extraction (SPE) chromatography and reverse-phase HPLC.

Figure. 4.4: Dose response curve of anti-inflammatory, anti-proliferative, and cytotoxic bioactivities of crude MeOH extract of *Centaurea ainetensis* in ET-treated SCp2 cells. Different dilutions of crude MeOH extract of *C. ainetensis* in MeOH were applied to SCp2 cells 30 min prior to addition of ET (10 μg / ml). Medium was collected 24 h after ET treatment and was assayed for ET-induced IL-6 secretion expressed as % IL-6 secreted in ET-treated control SCp2 cells (closed circles), cell function expressed as % of control cell metabolic activity (open triangles) and cytotoxicity expressed both by % maximum LDH release (cross) and arbitrary visual scores (crossed square) on the secondary y-axis, with a score of 5 describing confluent and cuboidal monolayer of cells. Inset. ET-induced IL-6 secretion in SCp2 cells 24 h after treatment with either MeOH alone (solid line) or in the presence of ET (10 μg / ml) (dash line) at different dilutions in 5% FBS GM. The results are presented as average of duplicate or triplicate treatments ± SD.

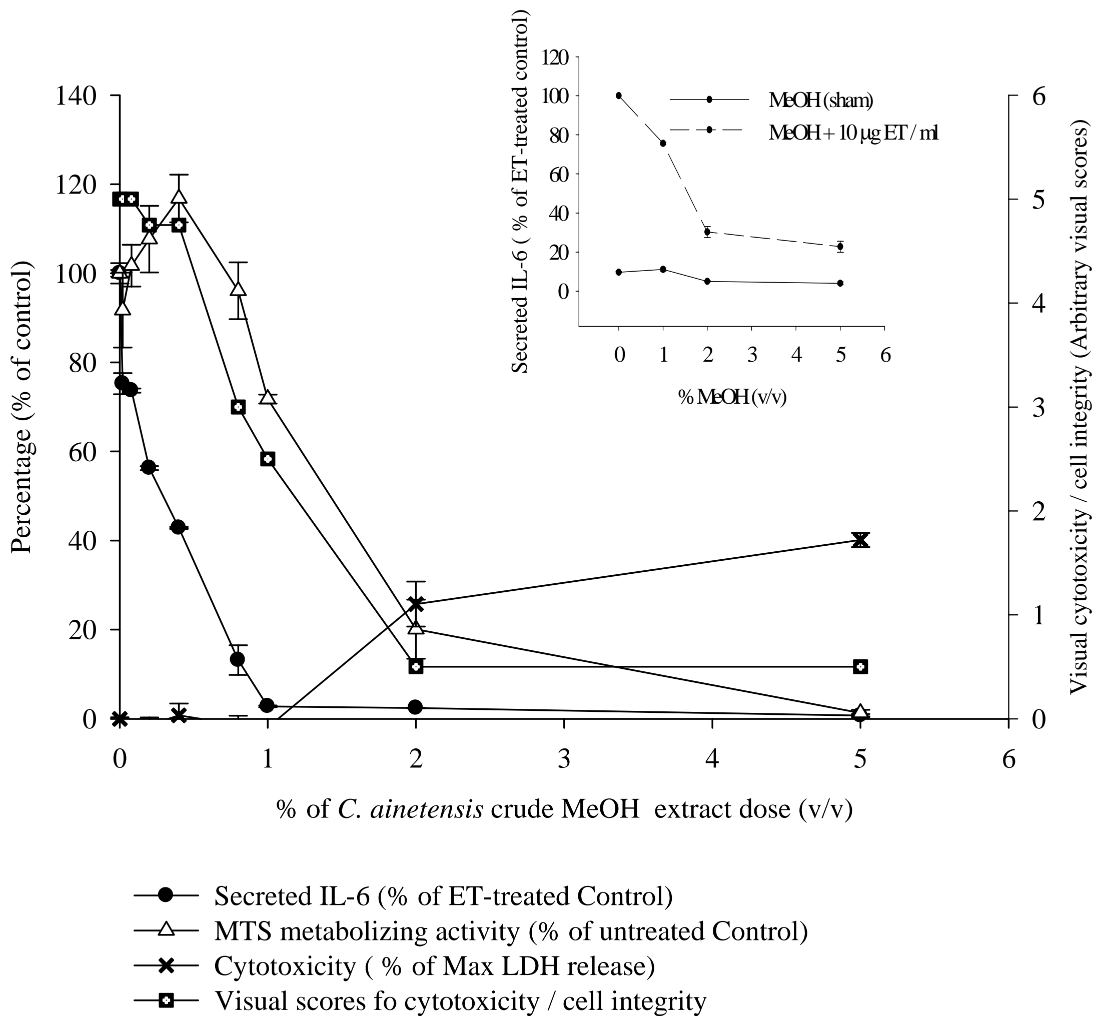


Figure 4.4

Figure 4.5: Bioactivity profile in SPE1 load, wash and elution fractions from *C. ainetensis* crude methanol extract. (A) Each of the 1 ml fractions collected from SPE1 during load (# 1-6) in 25% MeOH, wash (# 7-12) with 25% MeOH, elution 1 (# 13-22) with 60 % MeOH and elution 2 (# 23-30) with 100% MeOH were assayed at 0.8% (v/v)for anti-inflammatory activity (% ET-induced IL-6 secretion, closed circle) and proliferation (% Control, open triangle). (B) The same SPE fractions were assayed for their cytotoxic effects and results were represented as % maximum LDH release (cross, primary y-axis) and arbitrary visual scores of cell morphological appearance (crossed square, secondary y-axis) with a visual score of 5 describing a confluent and cuboidal monolayer of cells. The arrow diagram on the top of the figure represents the different steps of load, wash and 2-step elution of SPE1 column. Treatment with SPE1 fractions + ET was performed in triplicate samples for each SPE1 fraction. Each sample was assayed in singlets by cell metabolic acitivity assay and cytotoxicity assay and the results are presented as average of the triplicate samples. However, for IL-6 ELISA assays the medium from the triplicate samples was pooled for each fraction assayed and analysed induplicate ELISA assays and the results presented are the average of the duplicate ELISA assays for each fraction. The results shown in this figure are the bioactivities of SPE1 fractions from *C. ainetensis* crude MeOH extract representative of at least three different experiments with different crude MeOH extract preparations.

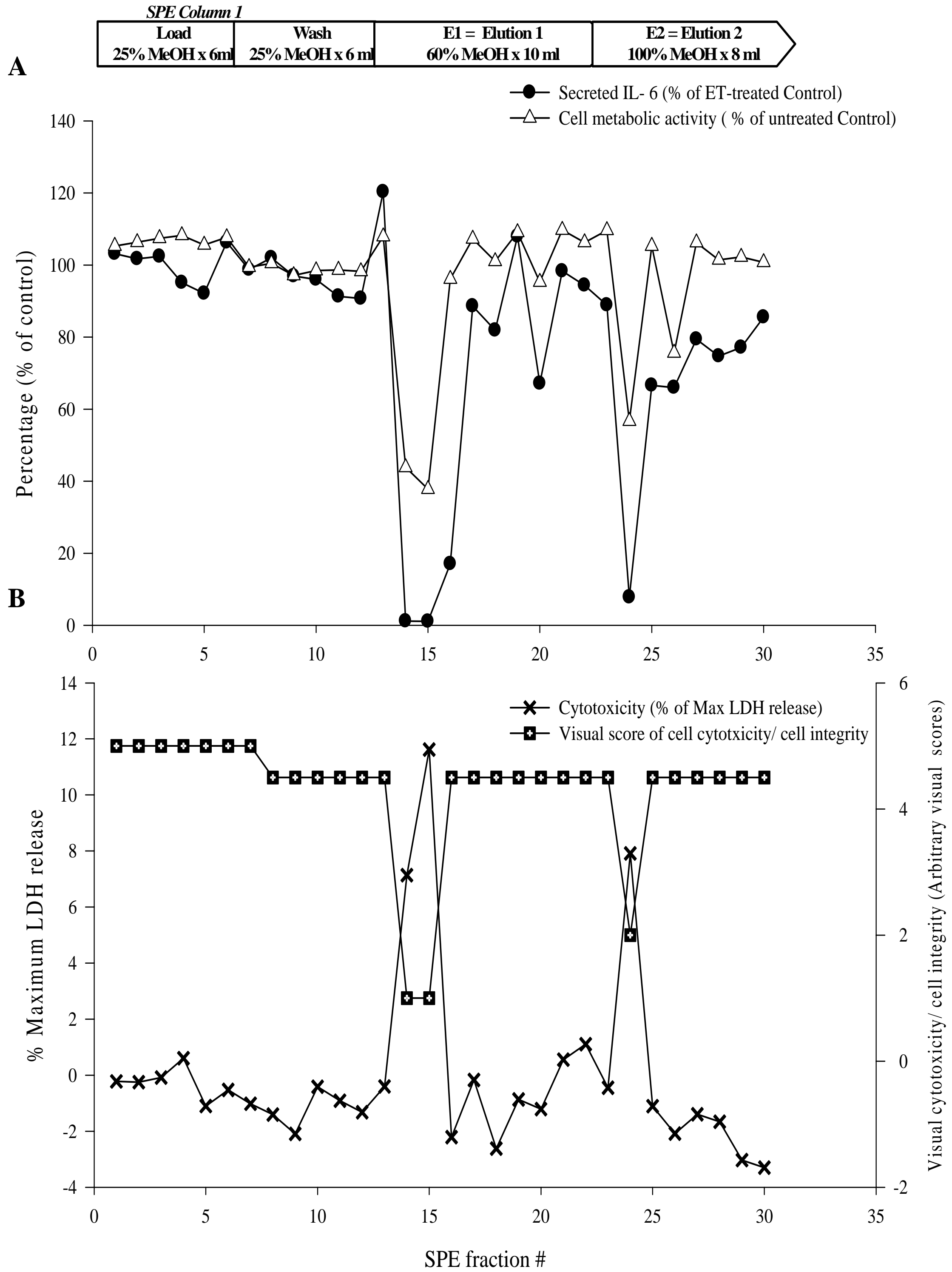


Figure 4.5

Figure 4.6: Bioactivity elution profile of SPE2 fractions. (A) SPE1 eluted fractions with 60% MeOH were diluted to 25% MeOH and reloaded on second SPE column (SPE2). Each of the 1 ml fractions collected from SPE2 during load (# 1-6) in 25% MeOH, wash (# 7-12) with 25% MeOH, elution 1 (# 13-22) with 60 % MeOH and elution 2 (# 23-30) with 100% MeOH were assayed at 0.8% (v/v)for anti-inflammatory activity (% ET-induced IL-6 secretion, closed circle) and proliferation (% Control, open triangle). (B) The same SPE fractions were assayed for their cytotoxic effects and results were represented as % maximum LDH release (cross, primary y-axis) and arbitrary visual scores of cell morphological appearance (crossed square, secondary y-axis) with a visual score of 5 describing a confluent and cuboidal monolayer of cells. The arrow diagram on the top of the figure represents the different steps of load, wash and 2-step elution of SPE2 column. Treatment with SPE2 fractions + ET was performed in triplicate samples for each SPE1 fraction. Each sample was assayed in singlets by cell metabolic acitivity assay and cytotoxicity assay and the results are presented as average of the triplicate samples. However, for IL-6 ELISA assays the medium from the triplicate samples was pooled for each fraction assayed and analysed in duplicate ELISA assays and the results presented are the average of the duplicate ELISA assays for each fraction. The results shown in this figure are the bioactivities of SPE2 fractions from *C. ainetensis* crude MeOH extract representative of three other different experiments with different crude MeOH extract preparations.

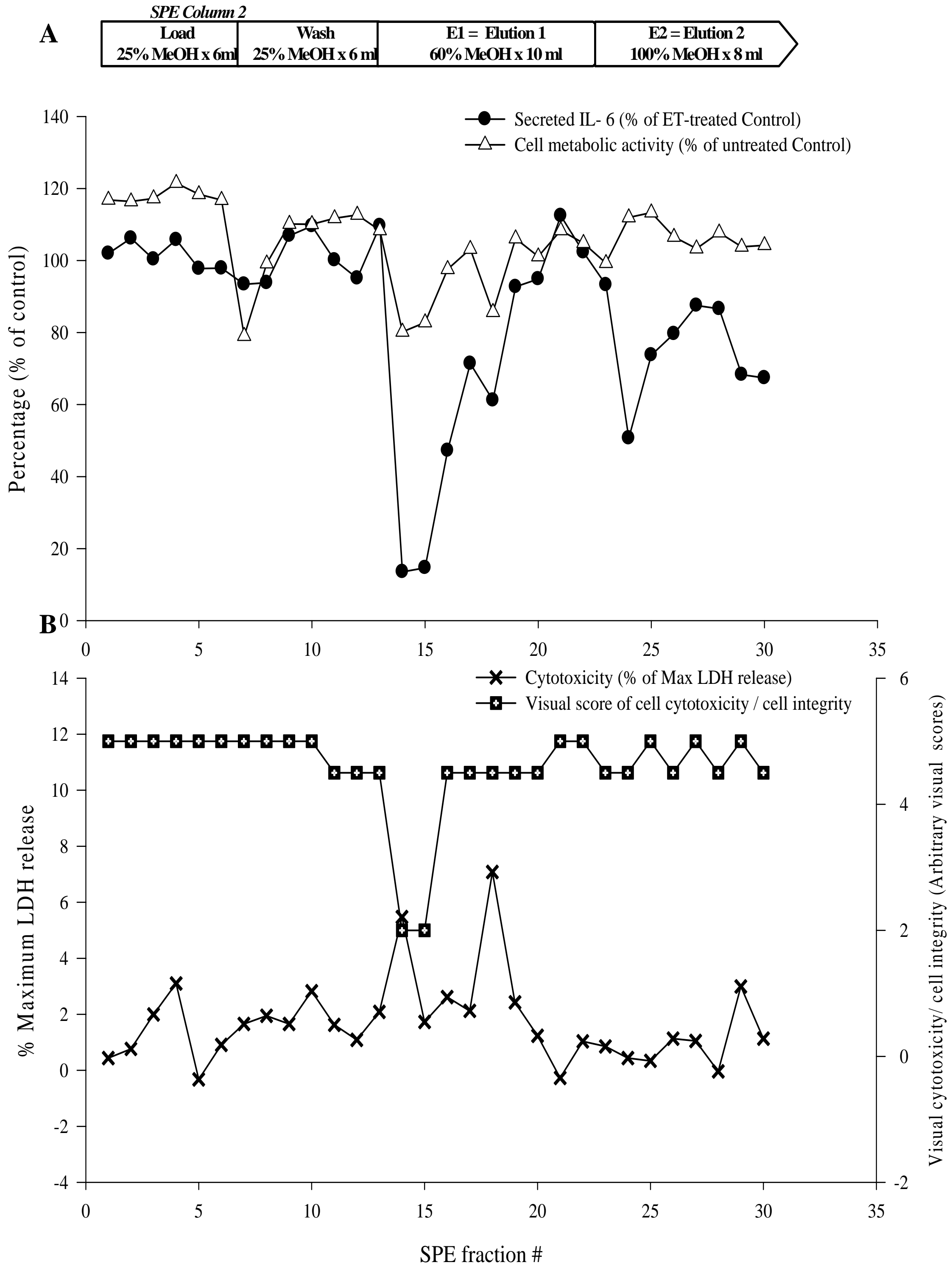


Figure 4.6

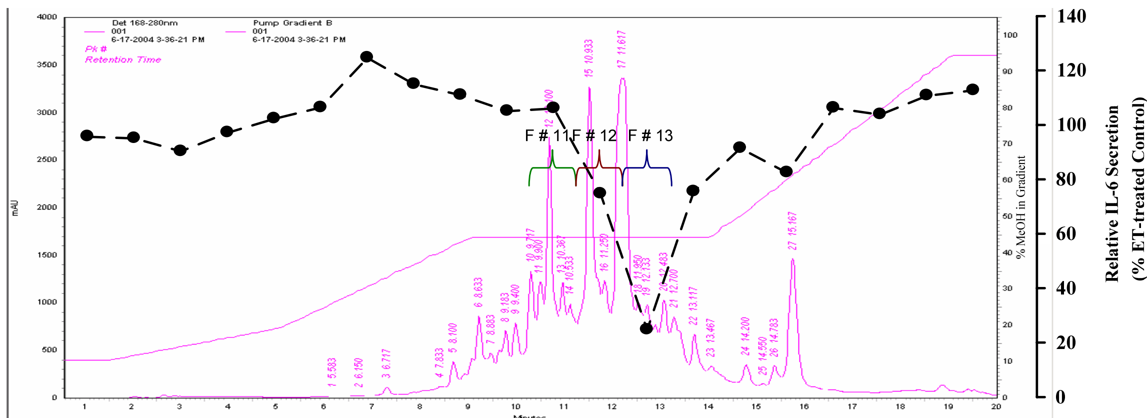


Figure 4.7: RP-HPLC1 A_{280} elution profile and corresponding anti-inflammatory activity of SPE3 bioactive fraction of *C. ainetensis* . The partially purified SPE3 bioactive fraction was diluted to 25% MeOH, 0.2% HAc, loaded on RP-HPLC column and 1 ml fractions were eluted at 1 ml / min, dried *in vacuo*, re-suspended in 125 µl water and assayed for their anti-infalmmatory activity by inhibition of ET-induced IL-6 secretion in SCp2 cells. The anti-inflammatory profile of collected fractions was depicted as % IL-6 secretion of the ET-treated control (closed circle, seconday offset right y-axis). The labeled HPLC fractions 11, 12 and 13 were diluted to 25% MeOH + 0.2 % HAc and re-chromatographed for further purification of anti-inflammatory activity. The elution gradient is superimposed on the A280 trace (solid pink line, secondary y-axis). Treatment with HPLC1 eluted fractions + ET was performed in triplicate samples for each HPLC1 eluate fraction. The medium from the triplicate samples was pooled for each fraction analysed in duplicate ELISA assays and the average of the duplicate ELISA assays is presented for each fraction.

Figure 4.8: A_{280} and anti-inflammatory activity elution profiles of HPLC collected fractions from partially purified SPE3 bioactive fraction of *C. ainetensis* and the re-chromatograms of fractions 11, 12, and 13. (A) The partially purified SPE3 bioactive fraction was diluted to 25% MeOH + 0.2% HAc, loaded on RP-HPLC column and 1 ml fractions were eluted at 1 ml / min, dried under vacuum, re-suspended in 125 μl water and assayed for their ability to inhibit ET-induced IL-6 secretion in treated SCp2 cells. The anti-inflammatory profile of collected fractions was depicted as % IL-6 secretion of the ET-treated control (closed circle, seconday offset right y-axis). (B) Fraction 11 (C) fractin 12 and (D) fraction 13 collected from (A) were each diluted to 25% MeOH + 0.2% HAc and re-loaded on RP-HPLC column. Then, 1 ml fractions were collected at 1 ml / min, dried under vacuum, re-suspended in water and assayed for their anti-inflammatory activity (closed circles, seconday offset right y-axis). The MeOH, 0.2% HAc gradient is included on the graph (pink solid straight lines, secondary y-axis). The results presented are average of duplicate IL-6 ELISA assay representative of at least two similar experiments with different crude MeOH extract preparation.

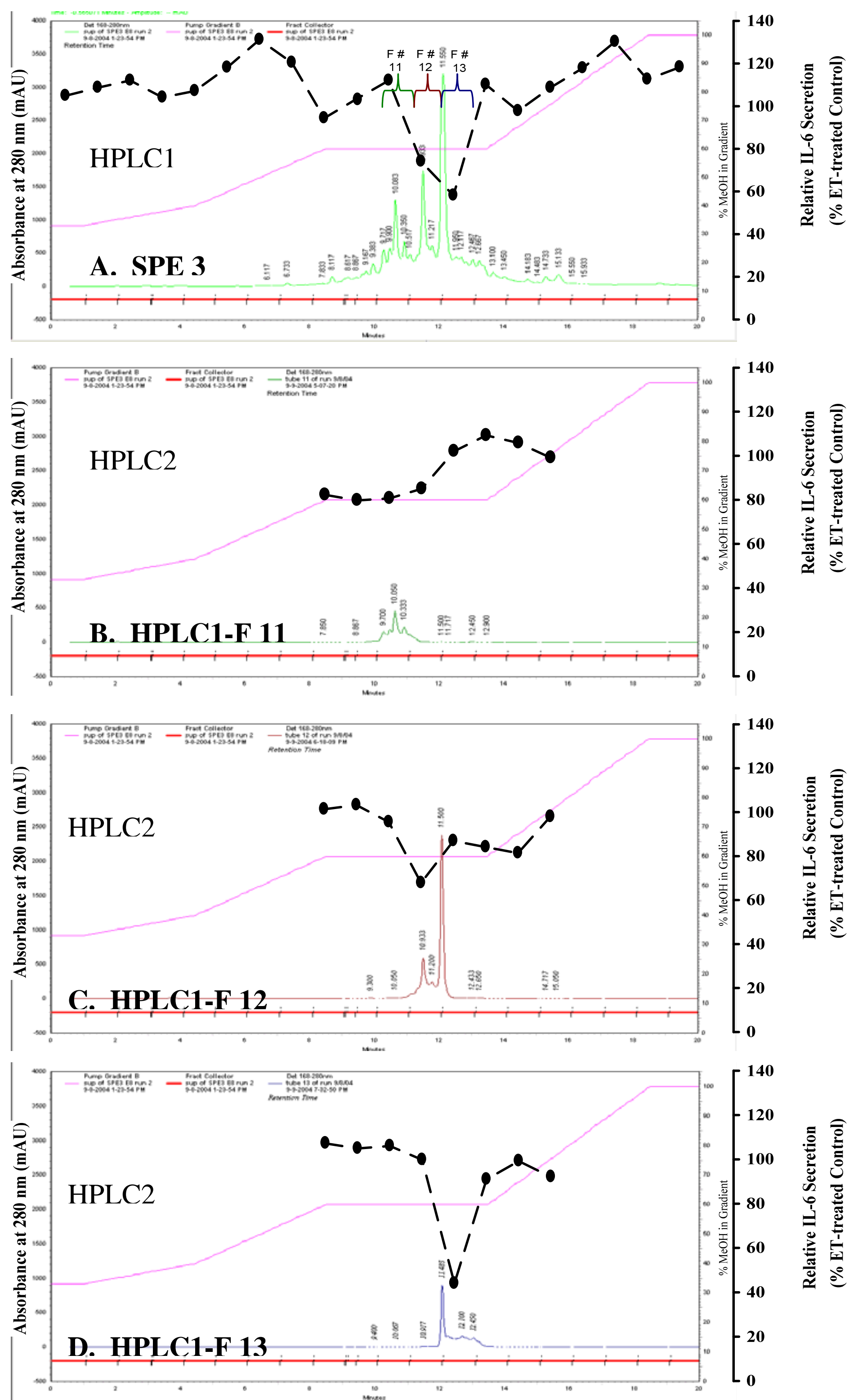


Figure 4.8

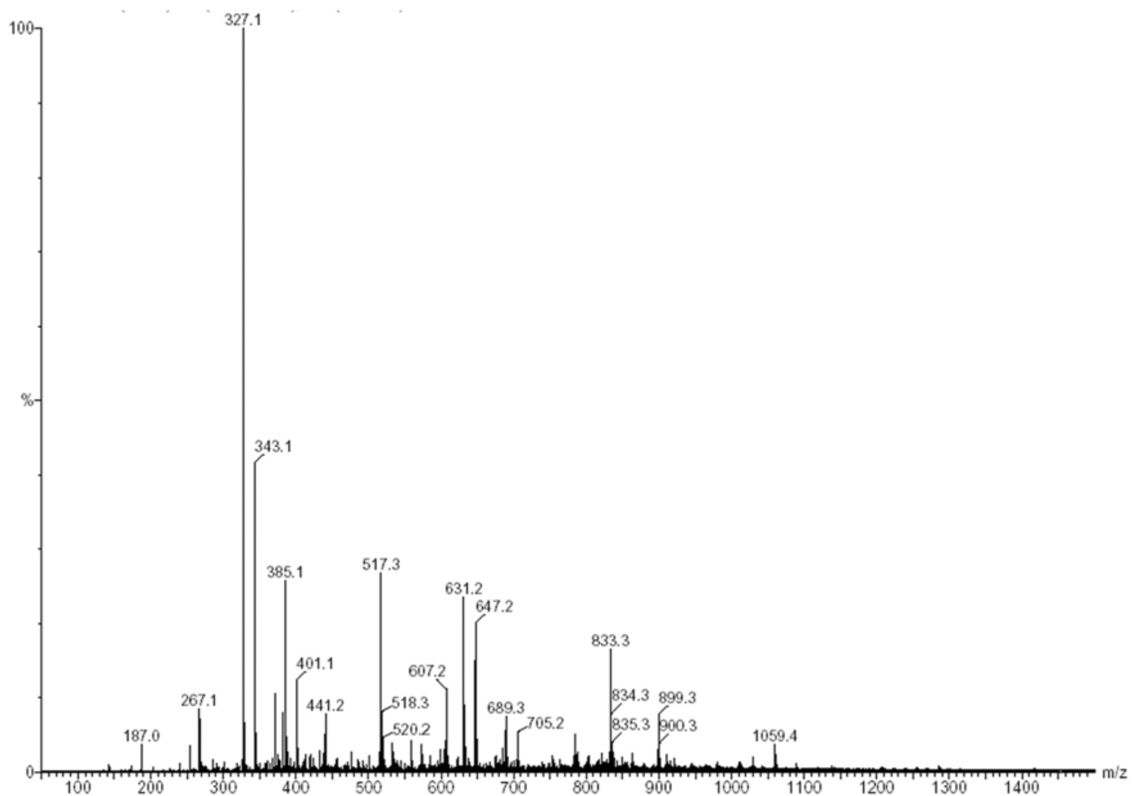
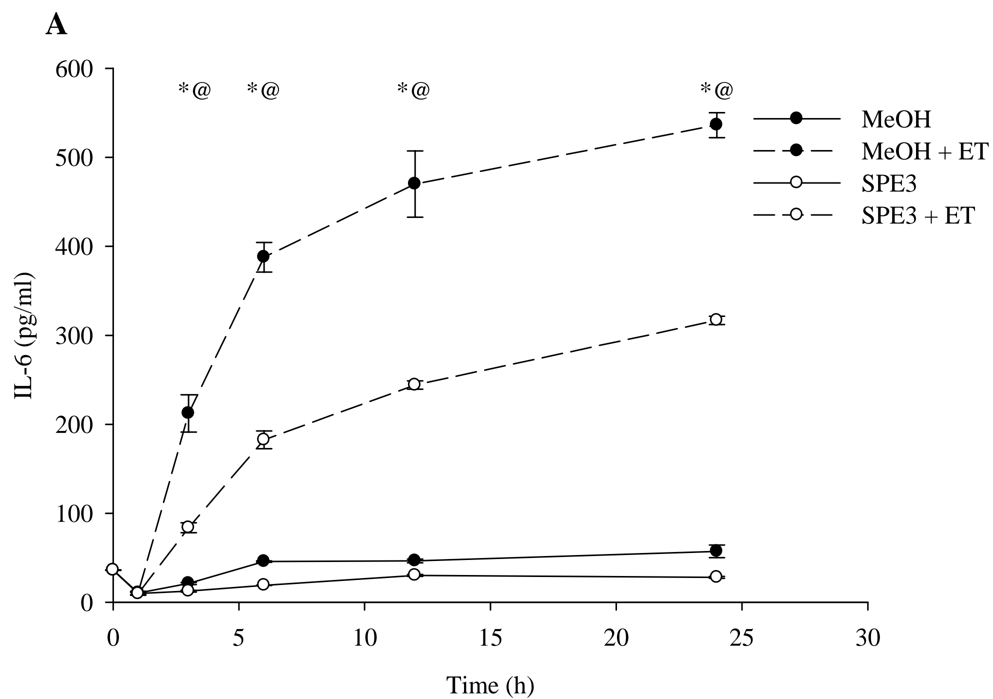


Figure 4.9: Mass spectrometry profile for fraction 13 from the re-chromatogram of figure 4.8 D. Fraction 13 of the HPLC chromatogram of SPE3 bioactive fraction of *C. ainetensis* (Fig. 4.8 A) was diluted to 25% MeOH + 0.2% HAc and re-loaded on RP-HPLC column. Then, 1 ml fractions were collected at 1 ml / min, dried under vacuum, re-suspended in water. Fraction # 13 of the re-chromatogram (Fig. 4.8 D) was analysed by LC- Mass spectrometry.

Figure 4.10: Inhibition of ET-induced IL-6 secretion and NO production by the SPE3 purified anti-inflammatory fraction of *C. ainetensis*. Medium collected from SCp2 cells treated with 0 or 0.2% v/v SPE3 bioactive fraction of *C. ainetensis* (SPE3) in 100% MeOH (vehicle) along with ET treatment at 0 or 10 μg ET/ml for 1, 3, 6, 12, and 24 h as in Fig. 4.2B, were assayed for ET-induced (A) IL-6 secretion (circle) and (B) NO production (triangle). Control cells treated with vehicle alone (MeOH) in 1% FBS-DM only for 1, 3, or 6, 12, or 24 h (closed symbols, solid line); cells treated with MeOH with ET treatment (10 μg / ml 1% FBS-DM) for 1, 3, or 6, 12, or 24 h, (closed symbols, dash-line); cells treated with SPE3 (0.2% v/v) in 1% FBS-DM only for 1, 3, or 6, 12, or 24 h (open symbols, solid line); and cells treated with SPE3+ ET treatment for 1, 3, or 6, 12, or 24 h (open symbols, dash-line). This experiment was performed in duplicate samples / treatment / time point. Each sample was assayed separately in duplicate ELISA or Griess reaction analysis. Data represents the average for duplicate samples $\pm$ SEM. (*) denotes significant differences between ET treatment and non-ET treated control within each time point ($p<0.05$), and (@) denotes significant differences between SPE3+ET treatment and MeOH (vehicle) + ET treated control within each time point ($p< 0.05$).



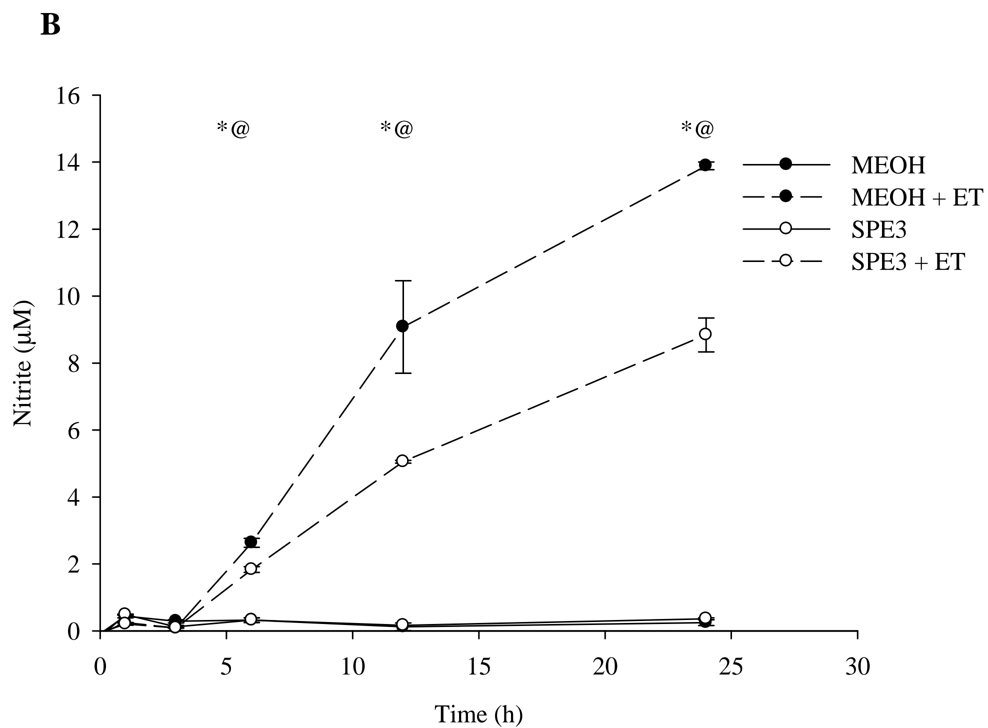
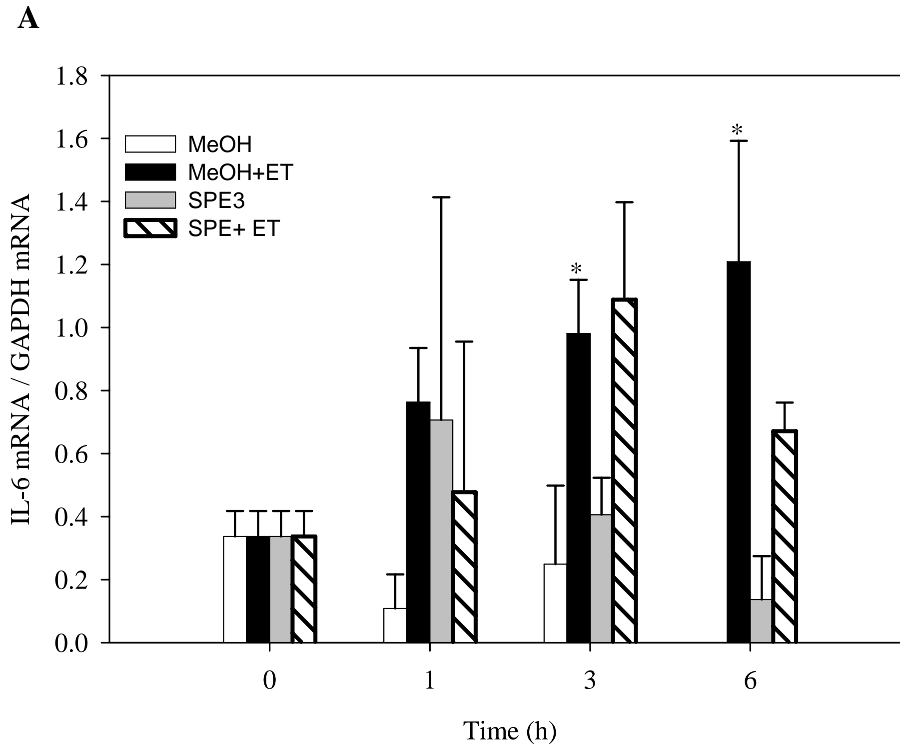


Figure 4.10

Figure 4.11: SPE3 partially purified anti-inflammatory fraction of *C. ainetensis* reduces ET-induced iNOS but not IL-6 mRNA Total RNA was extracted from SCp2 cells treated with 0 or 0.2% v/v SPE3 bioactive fraction of *C. ainetensis* (SPE3) in 100% MeOH (vehicle) along with ET treatment at 0 or 10 μg ET/ml for 0, 1, 3, and 6 h. Aliquots of 1μg total RNA from cells at each time point after ET application were reverse transcribed before amplification by real-time qPCR for ET induced (A) IL-6 and (B) iNOS mRNA. Amplified mRNA concentrations of IL-6 and iNOS were normalized to those of constitutive GAPDH mRNA. Amplified IL-6 and iNOS mRNA levels shown for: control cells treated with vehicle alone (MeOH) in 1% FBS-DM only for 1, 3, or 6 h (open bars); cells treated with MeOH with ET treatment (10 μg / ml 1% FBS-DM) for 1, 3, or 6 h, (closed bars); cells treated with SPE3 (0.2% v/v) in 1% FBS-DM only for 1, 3, or 6 h (grey bars); and cells treated with SPE3+ ET treatment for 1, 3, or 6 h (hashed bars). This experiment was performed in duplicate samples / treatment / time point. Each sample was analyzed in triplicate qPCR reactions. Data represents the average for duplicate samples $\pm$ SEM. (*) denotes significant differences between ET treatment and non-ET treated control within each time point ($p<0.05$), and (@) denotes significant differences between SPE3+ET treatment and MeOH (vehicle) + ET treated control within each time point ($p< 0.05$).

A



B

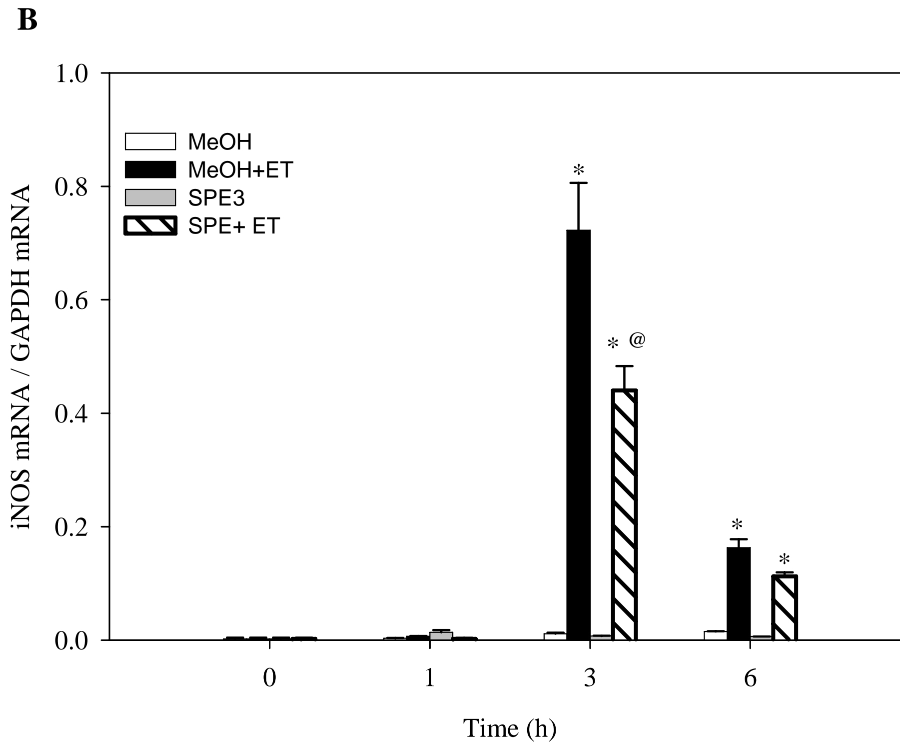


Figure 4.11

Gene and Genbank accession #	Primer Pairs 5'→3'	Size (bp)	Cycle #	Ref.
IL-6 NM_031168	F: 5'- GTTCTCTGGGAAATCGTGGA-3' R: 5'- GGAAATTGGGGTAGGAAGGA-3'	339	40	[30]
iNOS NM_010927	F: 5'-CCCTTCCGAAGTTTCTGGCAGCAGC -3' R: 5'- GGCTGTCAGAGCCTCGTGGCTTTGG -3'	497	40	NA
GAPDH BC094037	F: 5'- ACCACAGTCCATGCCATCAC -3' R: 5'- TCCACCACCCTGTTGCTGTA -3'	452	30	[37]

Table 4.1 Genbank accession numbers of genes and nucleotide sequences of primer pairs used for real time qPCR

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CHAPTER 5

CONCLUSIONS AND DISCUSSION

Inflammation is a coordinated innate immune response that defends the organism against infection or trauma and helps regain homeostasis. Inflammation involves the expression and secretion of a plethora of inflammatory markers such as cytokines (interleukin-6 (IL-6), IL-1, tumor necrosis factor alpha (TNFα)), inflammatory enzymes (induced nitric oxide synthase (iNOS), cyclooxygenase-2 (Cox-2)) and matrix remodeling enzymes (matrix metalloproteases (MMPs)). All of these inflammatory factors are orchestrated by a key inflammatory transcription factor nuclear factor kappa B (NFκB) [1], suggested to be the link between chronic inflammation and cancer and also a target for anti-inflammatory and/or anti-cancer therapies.

Failure to terminate an inflammatory response results in chronic inflammation and eventually might lead to cancer, especially in epithelia [2]. Although immune cells are the major players during inflammation, there is a growing interest in the role of epithelial cells in inflammation in part because epithelial cells form the first line of defense against microbial infections and therefore are highly prone to inflammation.

In contrast to immune cells, epithelial cells are anchorage dependent and rely on their extracellular matrix (ECM) and cell-cell interaction for proper cell organization,

differentiation and function [3]. The inflammatory respondents induced during inflammation are involved in several other signaling pathways that regulate normal physiological processes of the cell such as cell cycle (NFκB) [4], cell differentiation (IL-6) [5], and apoptosis (TNFα) [6]. Other inflammatory markers such as nitric oxide (NO) play dual roles as either anti-inflammation or pro-inflammation markers based on their level of expression and the microenvironment of the inflamed cell [7]. MMPs are involved in remodeling the tissue organization, and are upregulated during tissue remodeling associated with differentiation or inflammation [8-10].

The plethora of inflammatory markers induced during inflammation and their intricate regulation makes it difficult to develop a reliable model for epithelial cells to investigate the different aspects of inflammation under different conditions of growth, proliferation, and differentiation. Understanding inflammation in epithelial cells is essential to identify the pathways and key regulatory factors leading to chronic inflammation that is now linked to increased chances of cell transformation and cancer in epithelia and other tissues [2, 11].

Because NFκB was suggested to be the link between inflammation and cancer [12], and the key transcription factor regulating the different inflammatory respondents (Fig. 1.2), we proposed that activation of NFκB should induce the closely coordinated expression of the different inflammatory respondents IL-6, iNOS, Cox-2, and MMPs in response to an inflammatory stimulus (bacterial endotoxin (ET) also referred to as lipopolysaccharide (LPS)) (Fig. 1.2). The NFκB induced inflammatory respondents would in turn, via paracrine or autocrine pathways, modulate cell cycle and other cell

functions during inflammation. Therefore, we focused on IL-6 and NO as two markers of inflammation to understand their mode of regulation by NFκB.

This study showed that SCp2 cells respond to ET by secretion of IL-6 and a plethora of other cytokines and chemokines (such as RANTES, MCP-1, GCSF) and production of NO by induced nitric oxide synthase (iNOS) (Chapter 2). However, contrary to our suggested scenario of close co-regulation by NFκB (Chapter 1), our studies showed a difference in the temporal pattern of secretion of various inflammatory respondents that are expected to be closely regulated by NFκB. Cytokines mRNA such as IL-6 (Chapter 2) and TNFα (Chapter 3) were induced as early as 1 h after inducing inflammation by ET treatment (post-ET). IL-6 protein and granulocyte colony stimulating factor (GCSF) secretions were induced as early as 3 h post-ET, while NO was produced later starting at 6 h post-ET (Chapter 2), along with another chemokine RANTES (regulated on activation, normal T expressed and secreted) (Chapter 2).

Activation of NFkB is mainly regulated by inhibitory kappa B (IκB) kinase (IKK) complex that phosphorylates the inhibitor IκB labeling it for ubiquitin-dependent degradation to unmask the DNA binding site of NFκB free to translocate to the nucleus and induce gene transcription. Pharmacological inhibition of IKK resulted in inhibition of ET-induced IL-6 (chapter 2) and TNFα (data not shown) mRNA expression and IL-6 secretion but not that of iNOS mRNA expression or NO production (Chapter 2). This differential inhibition suggested that even though IL-6 and other inflammatory cytokines as well as iNOS are expected to be co-induced by NFκB in immune cells [13, 14], they showed a differential regulation in epithelial cells.

In addition, ET induced a transient increase in the activity of p65 at 1 h post-ET, while p50 was induced as early as 1 h post-ET and remained high for a longer period (up to 3h) after ET treatment (Chapter 2), suggesting that the regulation of NFκB might involve either different NFκB dimers or different temporal activation of NFκB. This regulation is critical in orchestrating the expression of specific inflammatory respondents.

Covert et al (2005) described two pathways for activation of NFκB depending on the inflammatory stimulus [15]. They showed that TNFα induces an oscillatory pattern of NFκB activation while ET induced a stable pattern of NFκB activation in mouse embryonic fibroblast cells (MEF), suggesting that ET induces NFκB activation via two parallel out of phase oscillatory pathways with a temporal delay. The sum of the out of phase oscillatory patterns resulted in a stabilized pattern of activation by ET [15]. Other reports by Werner et al (2005) and Hoffmann et al (2002) described a temporal control of IKK activity important in the selective gene expression by NFκB [16, 17]. Those studies in addition to the differential regulation of ET-induced IL-6 and iNOS mRNA by NFκB shown in this study confirm the importance of temporal regulation in the induction of inflammation.

The temporal patterns described in our study for IL-6 and iNOS differential regulation as well as in Covert et al (2005) for NFκB activation were in response to a single non-toxic application of ET, simulating an acute inflammatory response. However, it remains to be determined how multiple ET applications (simulating chronic inflammation) would affect the different temporal patterns of inflammatory respondents expression and secretion, as well as temporal pattern of NFκB activation. Understanding

of those temporal regulations is crucial for identifying the culprit in inflammation based cell transformation.

This study also showed the importance of the cellular microenvironment on the inflammatory response. ET treatment in the presence of serum was found to enhance the mRNA expression and secretion of inflammatory IL-6 and to decrease the half life of iNOS mRNA without significant effects on NO production (Chapter 3). On the other hand, serum alone in the absence of ET induced a transient peak of activation of the p65 form of NFκB at 1h post-ET (Chapter 2) without affecting that of p50 form of NFκB (Chapter 2). The addition of serum alone in the absence of ET also induced a transient peak in TNFα mRNA expression at 1 h after addition (Chapter 3).

Furthermore, SCp2 cells grown in the presence of EHS, a soluble extracellular matrix (ECM), or co-cultured with their myoepithelial counterpart SCg6 isolated from the same parent cell line (CID-9), modulated the magnitude and temporal regulation of the assayed inflammatory respondents (IL-6 and iNOS produced NO) (Chapter 3). Although EHS had no apparent effect on ET-induced IL-6 mRNA expression or secretion, and did not modulate the temporal pattern of iNOS mRNA expression, it showed a significantly higher level of NO production at 12 h -48 h post-ET (Fig. 3.6), likely due to ECM factors stabilizing NO in the medium.

Surprisingly, SCp2:SCg6 co-cultures manifested a spontaneous inflammation by inducing a dramatic increase in IL-6 secretion even in the absence of ET, and further increased the secretion of IL-6 upon treatment with ET (Chapter 3). In contrast, NO was not modulated in the non-ET treated co-cultures compared to the control individual cultures, and was induced only in response to ET treatment (Chapter 3). However, ET-

induced NO was only half of NO levels induced by ET in SCp2 cells grown on plastic (Chapter 3).

In addition, previous studies by Talhouk and coworkers demonstrated the activation of MMPs at 24h and 48h in response to ET-induced inflammation in mammary tissues [18] or in CID-9 cells [9], leading to altered ECM composition and subsequently altered cell differentiation and function in the epithelium. The activation of MMPs during involution of the mammary gland induces a cascade of responses similar to inflammatory responses [11, 19], leading to disruption of ECM and tissue structure and organization resulting in cell dedifferentiation (loss of β-casein expression) [9] and dysfunction. However, the activation of MMP during involution is well regulated and controlled by tissue inhibitor of metalloproteases (TIMPs) [10] to allow tissue regeneration in a following cycle of gestation and lactation. On the other hand, if the control of MMPs activity is lost during persistent inflammation, the increased catalytic activity of MMPS would alter ECM and cell-ECM interactions which are critical for tissue function and differentiation and shown in our study to be critical in modulating the inflammatory respondents (Chapter 3). As a result, uncontrolled high MMP activity would likely induce the tissue into an undifferentiated or disregulated disease state of which it would be difficult to regain its normal structure and function without external intervention (medical treatment). Such alteration may also be permanent as in scarring of stroma of the mammary gland tissue (cell death by apoptosis) and therefore loss of capability to produce milk [20, 21], or modulating cellular proliferation leading to cell transformation and tumorigenesis [22, 23].

These observations would allow further investigation of how modulation of the

cellular microenvironment would regulate the expression of inflammatory respondents during acute (single ET application) or chronic (repeated ET applications) inflammation, and help determine their temporal pattern of expression and their persistence in the cellular microenvironment in order to likely explain the link between chronic inflammation and cellular transformation.

One of the hallmarks of carcinogenesis is the loss of cell differentiation and uncontrolled cellular proliferation due to the cell becoming independent of its microenvironmental controls of proliferation (cell contact inhibition and other external cues that control cellular proliferation) and differentiation. The ability to induce SCp2 cells to differentiate (expression of milk proteins) in culture, and their responses to inflammatory stimulation by ET present a suitable model to investigate the changes in the cell microenvironment during inflammation and their effects on cell differentiation or dedifferentiation. For instance, the results described above (chapter 2 and 3) depict the responses of SCp2 cells to ET under differentiation conditions since all treatments (ET, and IKK inhibition) were performed in the presence of lactogenic hormones (insulin, prolactin and hydrocortisone) and reduced serum concentrations (0% or 1% FBS instead of 5% FBS). Therefore, modifying the growth conditions of SCp2 cells to mimic proliferating or differentiating cells allows us to study inflammation in cells at different stages of their development, and the different control points involved at each cell developmental state. Moreover, since NFkB plays a critical role in the inflammation, development, and involution of the mammary gland but not during lactation [19, 24], modulation of NFκB activity in inflamed SCp2 cells under differentiation or non-differentiation states might explain its role in inflammation dependent cellular

transformation.

The ratio of secretory epithelial to myoepithelial cells varies in ductular vs. alveolar mammary epithelia [25], with the ratio of secretory epithelial to myoepithelial cells low in ductal tissue and higher (>1) in the alveolus with dominance therein by the monolayer of secretory epithelial cells surrounded by a discontinuous layer of myoepithelial cells [25]. Therefore, by varying the ratio of SCp2 to SCg6 cells in SCp2:SCg6 cell co-cultures (chapter 3), we observed a spontaneous inflammatory response depicted by a dramatic increase in IL-6 secretion in the absence of the bacterial endotoxin stimulation that increased significantly with SCp2 cell plating density. In contrast, basal levels of NO production were not modulated in the co-culture and although ET treatment induced the production of significant levels of NO, the total amount of NO produced was ¼ of that produced in SCp2 cells cultured alone on plastic (Chapter 3), especially at the two highest SCp2 cell plating densities. Investigating the types of NFκB activated in these spontaneously inflamed co-cultures, in addition to investigating NFκB temporal pattern of activation in the presence as well as in the absence of ET and the subsequent modulation of the NFκB-dependent inflammatory respondents (cytokines, iNOS, Cox-2, MMPs and TIMPs) would provide further explanation of the role of NFκB in inflammation in general and in inflamed epithelial cells in particular, and subsequently in their transformation.

Understanding the inflammatory responses in epithelial cells in the absence of immune cells, and comparing their temporal regulation by NFκB or other transcription factors (e.g., activator protein-1 (AP-1)) in SCp2 mouse mammary secretory epithelial

cells would provide an epithelial cell model for investigating not only the role of epithelial cells in inflammation, but also to be used in co-culture with immune cells (e.g., macrophages) to investigate the role of the immune-epithelial cell interaction on inflammation in epithelial tissues.

In summary, the new corrected model of inflammation in the mammary epithelial cells would involve a set of early inflammatory respondents (IL-6 (Chapter 2) and TNFα (Chapter 3)), and late respondents iNOS, NO (Chapter 2), and MMPs [9, 10] induced in temporal sequences, rather than the initially proposed simultaneous regulation, that will ultimately lead to disruption of cell-substratum interactions and tissue function to eventually lead to disease (Fig. 5.1). All of these inflammatory markers are regulated by NFκB but not necessarily involving the same NFκB subunits, or NFκB activating machinery.

The difference in the regulation of two NFκB mediated inflammatory respondents (IL-6 and NO) described in this study in addition to the temporal regulation of NFκB for specific responses described by Hoffmann, et al (2002, 2003) [17, 26] and the oscillation pattern of NFκB activation by different inflammatory stimuli described by Covert et al (2005) [15] demonstrate the importance of timing in orchestrating the activation of inflammatory respondents during inflammation. The ability of SCp2 mammary secretory epithelial cells to respond to inflammatory stimulation as well as to changes in their environment provide a suitable model to further investigate the difference in temporal regulation of inflammatory respondents and their modulation by the cell state of differentiation.

The second aim of this study was to use the SCp2 mouse mammary secretory

epithelial cells as a model to screen for and identify anti-inflammatory bioactive plant products as potential anti-inflammatory and possibly anti-cancer therapies.

Contrary to immune cells that can grow in suspension, SCp2 cells as well as any other epithelial cell are anchorage dependent in order to grow and be used for screening experiments, resulting in a longer lag time before obtaining results when compared to immune cells. However, developing an epithelial system is an additional tool to allow investigation of anti-inflammatory drugs not only on immune cells, but also on epithelial cells and/or the co-culture of both to provide better understanding of the complex cellular inflammatory responses. Moreover, the availability of such a model provides a better in vitro model that might predict the possible side effects of prospective anti-inflammatory drugs and eliminate them early on in the process before moving to in vivo studies. Therefore, SCp2 epithelial non-immune cell model of inflammation was used in this study to screen for and identify mechanism of action of potential anti-inflammatory drugs in the Lebanese medicinal plant *Centaurea ainetensis*.

Despite the limitations of an anchorage dependent epithelial cell system, SCp2 cells were convenient for screening for bioactive compounds in medicinal plants (chapter 4). The preliminary investigation of the mechanism of action of SPE3 bioactive eluate of *C. ainetensis* in chapter 4 showed a differential inhibition of ET-induced iNOS mRNA expression but not that of IL-6 mRNA expression (Fig. 4.11), although both NO production and IL-6 secretion were significantly reduced in response to *C. ainetensis* SPE3 bioactive fraction and ET co-treatment (Fig. 4.10). This differential regulation is in contrast to the effect of the irreversible IKK inhibitor (Wedelolactone) that inhibited IL-6 expression but not iNOS. Thus, the anti-inflammatory activity of C. ainetensis might be

targeting a regulatory mechanism of NFκB other than the IKK complex.

Several studies have suggested a role for the mitogen activated protein kinase signaling pathway in the activation of NFκB as well as in the post-transcriptional regulation of cytokines which mRNA contain the adenine uracil (AU) rich element (ARE) in their 3'untranslated region such as TNFα and IL-6 [27]. Moreover, MAPK is known to be involved in the activation of the AP-1 [28, 29] also present in the promoter of inflammatory cytokines [30, 31] and essential for the expression of iNOS [32] and MMP-2 [8]. Other studies have suggested MAPK activated protein kinase-2 (MAPK-AP-K2 or MK2), a downstream target of p38 MAPK as a target for anti-inflammatory therapy after studies showing its role in enhancing the translation of specific inflammatory cytokines (reviewed in [33]). Furthermore, our purification scheme of the bioactive fraction of *Centaurea ainetensis* showed that the eluted bioactivity fraction had a bright orange color typical of flavonoids that were suggested to interfere in the MAPK pathway [34, 35] and inhibit NO prouction [36]. Further purification processes are required to completely purify and identify the identity of the anti-inflammatory reagent of *C. ainetensis* and determine whether they inhibit IL-6 secretion and NO production via modulating the MAPK pathway. Our preliminary results show the SCp2 cell model of inflammation to be a good model for such future investigations.

Efficient and cost-effective purification of bioactive agents from medicinal plants is essential for identifying candidate plant populations for purifying and characterizing the bioactivity, and for study of the mechanism of action. SPE chromatography coupled with reverse phase HPLC proved to be a powerful tool in reducing the labor intensive process of natural product extraction and purification, and minimizing the use of solvents

and variation in pH and temperatures during the fractionation procedures. The combined SPE-HPLC technique was effective, time efficient, and scalable to accommodate different amounts of starting material. However, due to limited availability of plant material, we were unable to completely purify and identify the chemical identity of the bioactive fraction of *C. ainetensis*.

In conclusion, the complexity of the signaling pathways of inflammation and the dual role of inflammatory respondents during inflammation as well as in normal physiological function in the cell makes it hard to find a selective anti-inflammatory drug that is completely safe and free of negative side effects (e.g., Cox-2 inhibitors). The biphasic regulation of NFκB, the modulation of the inflammatory response by the cell microenvironment in addition to cell-ECM and cell-cell interactions, all play a critical role in determining the temporal pattern of expression of inflammatory respondents and their level of secretion as well as their persistence in the microenvironment leading to disease state. Using SCp2 mammary secretory epithelial cells provide a suitable model to study the inflammatory response under the various conditions of cell growth and differentiation and would help identify the link between chronic inflammation and cancer.

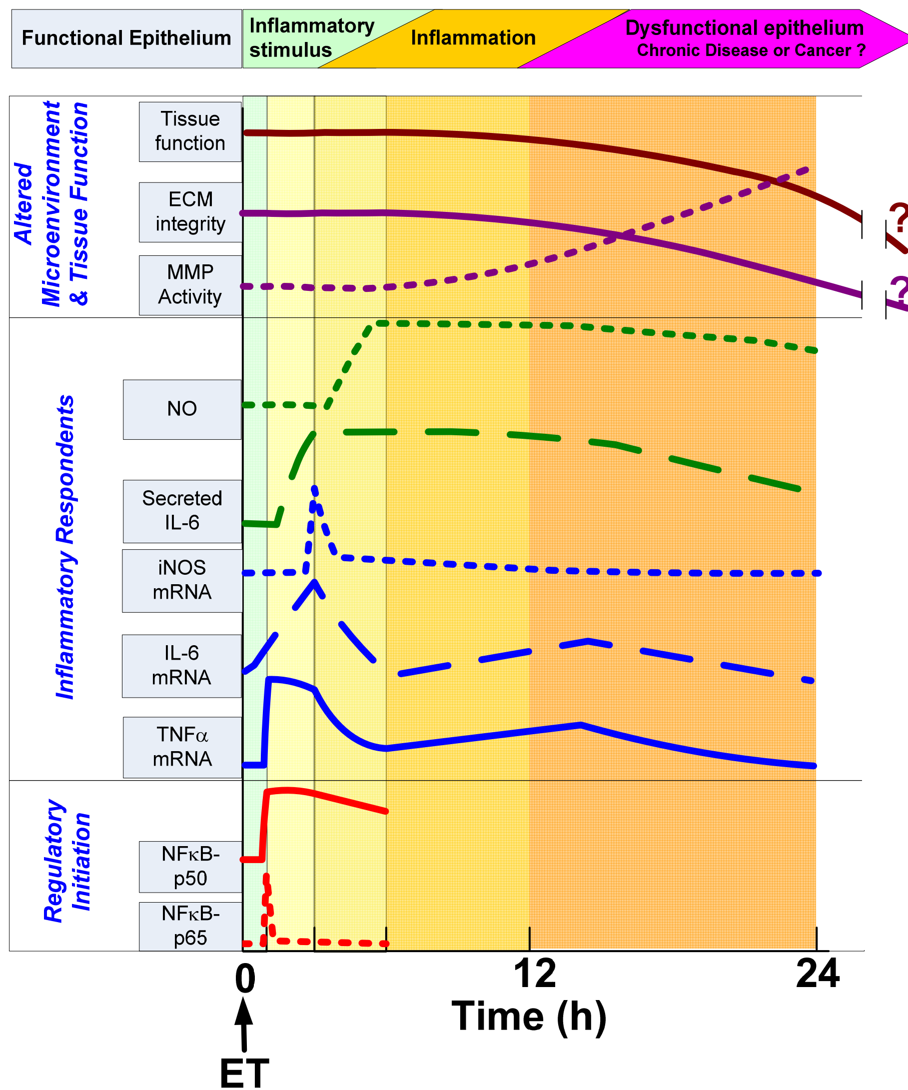

Figure 5.1: Scheme of temporal patterns and interactions for inflammatory responses and functional effects in epithelial cells and tissues, based on observed and proposed events in inflammation of mammary and other cells and tissues.

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