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Endothelin-1 Induced Phosphorylation of ERK1/2 in Bovine Corneal Endothelial Cells

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ENDOTHELIN-1 INDUCED PHOSPHORYLATION OF ERK1/2 IN BOVINE CORNEAL ENDOTHELIAL CELLS

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ENDOTHELIN-1 INDUCED PHOSPHORYLATION OF ERK 1/2 IN BOVINE CORNEAL ENDOTHELIAL CELLS

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Finally, I dedicate the thesis to my parents Mr. and Mrs. Kalavathi Venkatrajam Bethi, my sisters, my friend Anvesh Reddy and to Dr. Crawford who supported and encouraged me the most during my challenging times here at Western Kentucky University.

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Directed by: Kenneth Crawford, Jacobshagen Sigrid and Claire Rinehart

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The purpose of this study was to determine whether Endothelin-1 (ET-1) induced cellular responses in bovine corneal endothelial cells (BCECs) involves MAPK pathway by phosphorylating ERK1/2 protein kinase and to find out the phosphorylation patterns of ERK1/2 in confluent and sub-confluent cells. BCECs were isolated from bovine corneas and cultured in medium supplemented with 10% serum. Confluent (contact inhibited) and sub-confluent (actively growing cells) serum starved cells grown in T-75 flasks were treated with 10nM Endothelin-1. The control cells were left untreated. Total cellular protein was isolated using RIPA buffer and quantified according to the Peterson modification of the Lowry method. The level of expression of phosphorylated ERK1/2 (pp44, pp42) proteins relative to overall ERK1/2 (p44, p42) was determined by western blotting technique. Densitometry analysis of immunoblots revealed differential phosphorylation patterns in confluent and sub-confluent cultures. The pERK1/2 levels were significantly increased at 15 min and 24 hrs after post incubation with ET-1, whereas following the initial rise levels declined to 6hrs of incubation with ET-1 in confluent cultures. In sub-confluent cultures pERK1/2 levels increased gradually to 6hrs of incubation with ET-1, returning to pre-incubation levels at 24hrs. In conclusion, ET-1 treatment was shown to induce phosphorylation of ERK1/2 in BCEC. ET-1 treatment in confluent and sub confluent BCEC exhibited time dependent phosphorylation of ERK1/2. ET-1 treatment affected the phosphorylation pattern distinctively in confluent and sub-

confluent BCEC. These observations led to the conclusion that ET-1 induced cellular events in BCEC may involve the MAPK cascade and that these ET-1 induced MAPK cascades may exhibit a negative feedback mechanism, suggested by a distinctive oscillations in pERK 1/2 levels. The contrasting effects of ET-1 in confluent and sub-confluent cells may suggest a density dependent phosphatase activity.

INTRODUCTION

The cornea:

The cornea is a transparent tissue that forms the anterior part of the eye, covering the pupil, the iris and the anterior chamber (1). It serves as the most important refractive tissue and provides 2/3 of eye's optical power. The refractive power of cornea in humans is 43 diopters. An important parameter of corneal health is its thickness. The thickness of the cornea in the periphery is 500μm-600μm, in the center 600μm-800μm and it has the diameter of 11.5 mm. The cornea does not have blood vessels (avascular), as its primary function is transparency. It absorbs the oxygen from air as it is exposed directly to the air and has no blood vessels. The oxygen in the atmosphere first dissolves in tear liquid and then it diffuses throughout the cornea to maintain it in healthy condition. Nutrients are supplied to the cornea inside from the aqueous humor, and outside from the tear film by diffusion. The cornea is a very sensitive tissue as it has many nerve endings.

Organization of corneal layers:

The human cornea is organized into 5 layers; anterior to posterior the corneal epithelium, Bowman's membrane, corneal stroma, Descemet's membrane, and corneal endothelium (2). This kind of organization is common in primates, but some carnivores like dog, cat and wolf have only a four layered cornea (3). The corneal epithelium is a multi-cellular layer consisting of cells that are easily regenerated and have fast growing ability. It is a non-keratinized, non-secretory, stratified squamous epithelium. It interacts with the tear film and forms a smooth refractive surface on the cornea. Bowman's membrane lies underneath the epithelium. It is a strengthen 8μm -14μm layer made up of

irregularly arranged type-I collagen fibers and serves as a Basement membrane for the epithelium. Carnivores do not exhibit this layer.

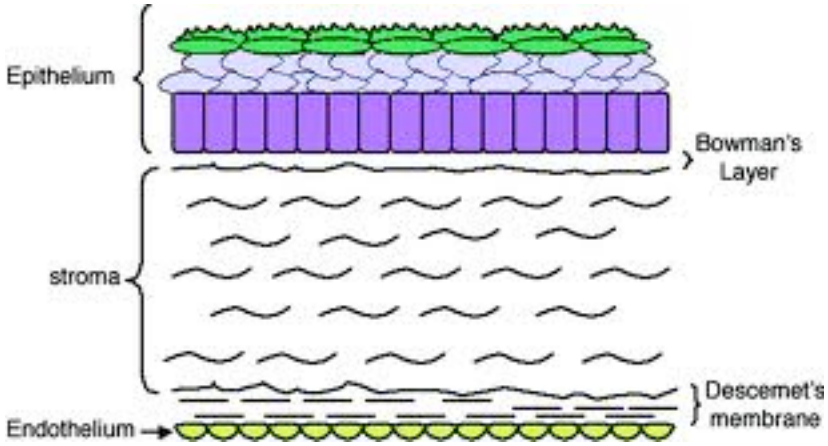

Figure 1: Organization of corneal layers (4)

The corneal stroma is located between Bowman's and Descemet's membrane and is made up of regularly arranged type-I collagen fibers and scattered keratocytes. It plays an important role in maintaining structural integrity and comprises 90% of the corneal thickness. Decscemet's membrane is found underneath the stroma. It is an acellular layer that contains type IV and type VII collagen fibers and acts as modified basement membrane for the corneal endothelium as it is continuously secreted by the corneal endothelial cells throughout life beginning in utero at the 8-week stage (1). Therefore the thickness of this membrane depends upon the person's age. The innermost layer of the cornea is the corneal endothelium, which is a single cell layer (simple squamous epithelium), composed of mitochondrial rich cells (5).

Endothelium:

The corneal endothelium is a monolayer of flattened, hexagonal cells, located at the posterior of the cornea, forming a physical barrier or boundary between the stroma

and aqueous humor (6). The main role of the corneal endothelium is to maintain proper corneal hydration, thickness, and its transparency. The healthy condition of the cornea depends upon the cell density of the corneal endothelium. In young individuals, the corneal endothelium is comprised of about 400,000 cells of 4-6 μm thickness and a diameter of 20μm (7). The corneal endothelial cell density varies with the subject's age. At birth it is ranges from 3500-4000 cells/mm^2, in adults it ranges from 1400-2500 cells/mm^2. A cell density below 400-700 cells/mm^2 leads to edema and loss of visual acuity (1). Corneal endothelial cells are flattened hexagonal shaped cells, containing numerous mitochondria and prominent Golgi apparatus. Endothelial cell shape is maintained by the actin filaments formed in the periphery of the cells (8) and the cell-cell contacts are strengthened by the presence of adhesion junctions between cells, which creates the close contact between plasma membranes of neighboring cells. Focal like tight junctions, who serve as a semi permeable ("leaky") barrier, are formed between cells (9) are calcium sensitive (10) and thus subject to break down at calcium concentration below the threshold level.

Importance of corneal endothelium:

The main function of the corneal endothelium is to maintain the transparency of cornea by regulating its hydration. Most of the nutrition of the cornea is supplied from the aqueous humor by the leaky tight junctions in the endothelium, that allows the movement of fluids and nutrients to flow into the stroma but strictly avoids the bulk flow of the fluid (11). The inward flow of solutes and water in the cornea is normally equilibrated by the action of pump proteins located on the plasma membrane of endothelial cells. The net fluid balance is maintained by tight junctions and pump proteins, depends upon the

integrity of the corneal endothelial single cell layer, which in other words depends upon the cell density of the endothelium. A decrease in endothelial cell density disturbs the barrier function and leads to corneal edema, bullous keratopathy, corneal clouding, and loss of visual acuity (7).

In humans the corneal endothelial cells do not divide *in vivo* once they undergo differentiation (12, 13). Normal gradual cell loss of the corneal endothelium, caused by increased age does not usually affect endothelial function, but the accelerated cell loss caused by aging, trauma, corneal dystrophy or previous corneal transplantation could affect endothelial function severely. The major means of repairing the corneal endothelium is by cell enlargement and cell migration rather than by cell division as these cells do not divide *in vivo* or divide at a low rate that is not adequate to compensate for cell death. Cell migration and enlargement of corneal endothelial cells in response to corneal damage changes the cell from a regular hexagonal shape to a more irregular, rounded polymorphic form, that can stress the cell-cell junctions and affects the monolayer integrity and its barrier function (14, 15, 16). Even though the mitotic activity of human corneal endothelial cells (hCEC) *in vivo* is very limited, they retain some proliferative capacity. hCEC were successively cultured under *in vitro* conditions by providing appropriate stimulating growth factors to stimulate cell division (17). Inhibition of hCEC proliferation *in vivo* is mediated by several factors including cell-cell contacts (contact inhibition), lack of stimulation by autocrine or paracrine growth factors, and negative regulation by transforming growth factor-beta2 (TGF-β2). In addition, corneal endothelial cells exhibit intrinsic, age-related differences in relative proliferative capacity. Endothelial cells cultured from younger donors grow more rapidly and can be

subcultivated more times than cells cultured from older donors. With increasing age, most hCEC enter a replicative senescence-like state in which they become increasingly refractive to mitogenic stimulation (18, 19). Age-dependent alterations in the relative expression and activity of the cyclin-dependent kinase inhibitors (CKIs) p27KIP1, p16INK4a, and p21CIP1 appear to play an important role in decreasing sensitivity of hCEC to mitogens. Studies on hCEC cell cycle regulation revealed that these cells stay in G1 phase and become non-proliferative. The characteristics of G1 phase-arrested hCEC cells include low proliferative capacity, expression of G1 cyclin, and increased expression of CKIs (19).

Cell cycle and its components:

The cell cycle or cell division is an essential mechanism by which all living cells reproduce new cells from previously existing cells. It involves a series of events that takes place in a cell leading to its division and duplication of its genetic content. The eukaryotic cell cycle is more complex when compared with the prokaryotic cell cycle as it involves well-timed coordinated processes. Although the duration of the cell cycle varies from one cell type to another, in typical rapidly dividing mammalian cells, it takes only 24 hours to complete. In eukaryotic cells, the cell cycle is divided into four discrete coordinated phases called Gap phase 1 (G1), synthesis phase (S), Gap phase 2 (G2), mitosis phase (M). Each phase of the cell cycle is unique for its processes. G1, S, G2 phases are combined into interphase, in which the cell grows, duplicating its DNA, synthesizing proteins and accumulating nutrients for mitosis. In a typical mammalian cell, 23 hours of the approximate 24-hour cell cycle is occupied by the interphase and the remaining 1-hour is occupied by M phase. M phase (mitosis) is proceeded by S phase and

is defined by chromosome segregation and cell division. It occupies less than 1 hour in mammalian cells. It involves two major events named nuclear division or karyokinesis and cytoplasm division or cytokinesis. During karyokinesis copied chromosomes are segregated and are distributed into a pair of daughter nuclei, where as in cytokinesis the cell itself divides into two daughter cells (20).

Cell cycle regulation is a complex process, which involves a complex network of regulatory proteins, known as the cell cycle control system, ensuring the proper cell cycle progression. Central components of the cell-cycle control systems are cyclins and cyclin dependent kinase (CDK) molecules. The cell cycle control system regulates cell cycle progression mainly at two checkpoints, the G1/S check point and the G2/M check point. At these check points, cellular events are monitored to decide whether or not to allow the cell to proceed to the next phase. The G1/S check point allows the cell to confirm that the environment is favorable for cell proliferation and whether its DNA is intact before entering into S phase. Cell proliferation through G1 phase depends upon extracellular conditions and signals. At the G2/M check point the cell confirms that any damaged DNA is repaired and DNA replication is complete, then it allows the cell progression through the G2 phase to enter into the M phase.

Cyclins and cyclin dependent kinase proteins play an important role in regulating the cell cycle. Cyclins are proteins that have no enzymatic activity and were named cyclins because they undergo a cycle of synthesis and degradation in each cell cycle. Cyclins bind with the cyclin dependent kinases to regulate kinase activity. Unlike cyclins, the concentration of CDKs are relatively constant during the cell cycle. Cyclins are classified into three classes depending upon the stage of cell cycle at which they bind

CDKs and function. They are G1/S cyclins, S cyclins and M cyclins. The cyclins that play an important role in G1-S transition are cyclin D and cyclin E. DNA synthesis in S-phase is regulated by cyclin A and M phase entry is regulated by the association of cyclin B with its respective CDK partner. Cyclin forms a complex with a particular CDK that drives the cell cycle from one phase to the next phase. Cyclin-CDK complexes phosphorylate substrates that are appropriate for the particular cell cycle phase. Activated cyclin-CDK complexes in earlier cell-cycle phase help in activating the cyclin-CDK complexes in later phase (21).

CDKs are a family of protein kinases. They are divided into 9 different classes. Among them, CDKs 1, 2, 3 and 4 are directly involved in cell cycle regulation. The CDKs that play an important role in G1 phase progression are CDK2, CDK4 and CDK6. The cell cycle fate is determined by CDKs (22). The levels of CDK molecules do not change throughout the cell cycle, but their activity varies within each phase of cell cycle (23). The activity variation of CDKs is due to the changes in the levels of regulatory cyclins. The activity of CDKs is regulated through phosphorylation and CKIs (24).

Upon mitogenic stimulation, the cellular levels of the CKIs p27Kip1, p21Cip1, and p16INK4a are decreased and the levels of the G1 phase regulatory protein, cyclin D is induced. Cyclin D forms a complex with CDK4 and activates its kinase activity. The activated Cyclin D/CDK4 complex prevents the binding of pRb to E2F transcription factor by hyperphosphorylating it. Thus the activated E2F transcription factor allows the cell to enter into S phase and also leads to the synthesis of cyclin E in late G1-phase. Generation of E2F activity is rate limiting for G1/S-phase progression, is sufficient to induce S-phase entry, and overcomes inhibition by G1-phase inhibitors (19). Cyclin E

binds CDK2 to form cyclin E/CDK2 complex, which forms auto regulatory circuit by maintaining the pRb in an autophosphorylation state. Cyclin A, which is synthesized in late G1-phase, associates with CDK 2 and forms CyclinA/CDK2 complex. This complex promotes the forward progression from S phase to G2 phase. Cyclin B, which is synthesized at the end of the S phase associates with CDK1 and forms cyclin B/CDK1 complex. This complex activates the molecular changes, which are needed to prepare the cell for M phase (7).

P27 is a member of the CIP/KIP family. P27 prevents the activity of Cyclin E/CDK2 or cyclin D/CDK4 complexes, which are required for G1 phase progression, by binding to them. CKIs inhibit the action of CDKs by binding with them physically or catalytically. CKIs also function as assembly factors for cyclin-CDK complexes (25).

Endothelin-1:

Endothelins are 21 amino acid peptides that possess vasoactive properties. Three isoforms of endothelins are known, namely, ET-1, ET-2, and ET-3. Endothelins contain two intra-chain disulfide bonds between cysteine residues.

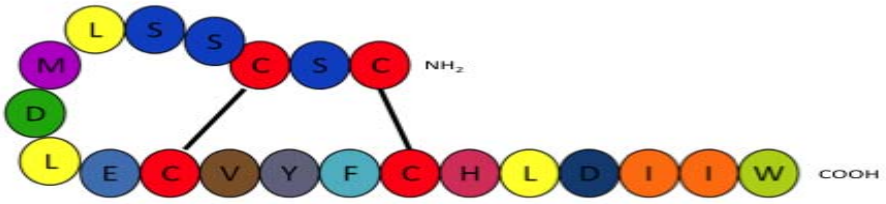

Figure 2: Primary structure of Endothelin-1

The C-domain and disulfide bonds are essential for endothelin activity. Endothelins are involved in numerous physiological and pathological conditions, including hypertension, cardiac failure, brain and myocardial infarctions, disseminated intravascular coagulation, Alzheimer's disease, and glaucoma (26-28). Among the three isoforms, ET-1 is the predominant one. It is a potent mitogen in many cells, including smooth muscle cells, fibroblasts, and astrocytes (29-31). The two key receptors of ET are ETA and ETB. ET-1 exhibits its mitogenic response by inducing ETA receptors in mesangial cells (32). Upon ET-1 binding, the high affinity receptors activate phospholipase C (PLC). Activated PLC activates the protein kinase C leading to the transcription of growth promoting genes like c-fos and c-myc (33), which are important for cell proliferation (34). ET-1 stimulates cell proliferation in U373MG astrocytoma cells by activating MAPK, PKC, and PI3K dependent pathways and it was suggested that there is no cross talk between these pathways (35). The binding of ET-1 to its receptor leads to the increased expression of growth promoting the proto-oncogenes, c-fos, c-jun and c-myc, which are important for cell proliferation through the activation of the MAP kinase (ERK) signal transduction pathway.

Mitogen Activated Protein Kinase (MAPK) signaling pathway:

Often cells adapt to rapidly changing environment, by recognizing and responding to various external stimuli. Membrane bound receptors and proteins detect these external stimuli and convert them into specific intracellular programs such as signaling cascades by the signal transduction process. The Mitogen-activated signaling cascade is a signal transduction pathway, which involves the stimulation of several cytoplasmic protein kinases collectively known as mitogen activated protein kinases (MAPKs) by the

activation of several membranal signaling molecules in response to external stimuli and elicits an appropriate response including cellular proliferation, differentiation, development, inflammatory responses and apoptosis in mammalian cells (36). In yeast, five MAPK modules have been identified which participates in regulation of mating, filamentation, cell wall remodeling, sporulation and high osmolarity responses (37, 38), In many other eukaryotes, the same number of MAPK cascades have been found, which appear to be linked to different signal transduction pathways. Some lead to the final activation of either p42/44 MAPK, which mainly involves in regulation of cell proliferation and differentiation, as well as c-Jun N-terminal kinase (JNK) and p38/ HOGMAPK, or stress-activated protein kinases (SAPKs), which function preferentially in stress responses like inflammation and apoptosis (39) and (40-42). The MAPK pathway is also involved in regulation of several developmental processes like eye development in *Drosophila melanogaster* (43), morphogenesis and spatial patterning in *Dictyostelium amoebae* (44, 45), T-cell development in mammals (46), and vulva induction in *Caenorhabditis elegans* (47). Depending upon the cell type, strength and duration of stimulation, external signals are transduced into specific cellular response (proliferation/ differentiation/ apoptosis) through one of the preferred MAPK cascade activation (48). MAPK cascades in all eukaryotes are conserved evolutionarily and play an important role in cytoplasmic activities as well as regulation of gene expression (49).

Each MAPK cascade is typically organized into no fewer than three sequentially phosphorylated protein kinases named as a MAPK kinase kinase (MAPKKK or MAP3K or MEKK), a MAPK kinase (MAPKK or MAP2K or MEK) and a MAP kinase (MAPK). These generic names were given to the enzymes that work at the same level in different

MAPK cascades. Isoforms of these enzymes working at the same level in different MAPK cascades were given distinctive names (Ex: ERK, JNK and Hog for the MAPK level). Current research on MAPK cascades supporting the presence of at least 14 MAPKKKs, 7 MAPKKs, and 12 MAPKs in mammalian cells (50).

A MAPKKK kinase (MAPKKKK, MAP4K or MKKKK), which is linked to the plasma membrane through association with a small GTPase, activates the MAPKKK (MAP3K). A serine/threonine MAPKK kinase (MAPKKK) activates the MAPKK (MAP2K) by phosphorylating the conserved serine and threonine residues on it, where as the dual specificity MAPK kinase (MAPKK/MEK) phosphorylates the tyrosine and threonine residues on MAPK leading to its catalytic activation. The protein kinases, which are activated by MAPK, are given a generic name MAPKAP kinases (MAPKAPK), for example, RSK (MAPKAP Kinase-1) and MAPKAP kinase-2 (see figure 3).

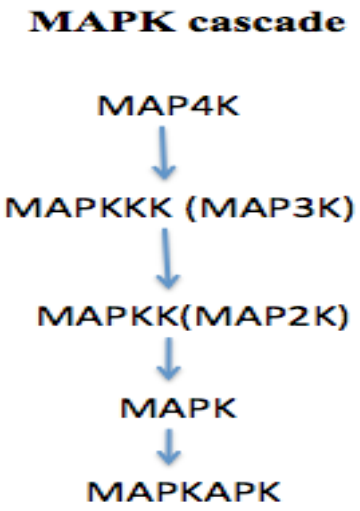

Figure 3: MAP kinase cascade

MAP4K and MAP3K:

PKC is membrane protein that serves as MAP4K in yeast as well as in several mammalian signaling cascades by phosphorylating MAP3K (Raf-1, MEKK, BCK1, Raf-B, c-mos) (51, 52). MAP3Ks participate in the activation of MAP2K. There are many different MAP3Ks, including Raf isoforms, Ste11 relatives MEKK1-MEKK4, mixed lineage kinases (MLKs), Tao proteins and Mos. From recent studies it is believed that each of these MAP3Ks activates a different group in the MAP2K family.

Raf-1 is a 70 to 75-kDa protein, that is one of the best-studied MAP3K found in the MAPK signaling pathway in response to mitogen stimulation. In Raf-1, the kinase domain is located on the carboxyl terminal side of the molecule and the remaining part is occupied by the regulatory domain. Upon mitogenic stimulation, Raf-1 undergoes transient activation within 2-3 min (53). Ras protein recruits the Raf-1 to the plasma membrane where it is activated by an unknown mechanism (54, 55). However, PKC and tyrosine kinases are believed to be Raf-1 activators. Among several reported proteins and peptides, the native forms of MAPKK are most preferred substrates for Raf-1. As Raf-1 itself is not solely responsible for complete activation of MAPKK, the involvement of complexation or additional factors is possible in MAPKK activation. I kappa B and NF kappa B activation by Raf-1 suggest that it might participate in more than one signaling pathway. Raf-1 was also demonstrated as a sufficient and a required molecule for activating a subset of early and late growth response genes.

MAP2K (MAPKK):

MEK is the generic term used to represent the genes or isoforms of a specific set of MAP2Ks (MAPKK) that works at the same level in the MAPK cascade. The MEKs

belong to the serine/threonine kinase family (56) and are composed of three highly homologous components named MEK-la (MKK-la and MAPKK), MEK-1b (MKK-lb, MEK-3), and MEK-2 (MKK-2). Several MAP3Ks can activate MEKs by dually phosphorylating serine and threonine residues within the activation loop of the catalytic domain. MAP3Ks can have large regulatory domains whereas MAP2K have smaller regulatory domains. MEKs show greater substrate specificity and selectively activate only native forms of MAPKs. These belong to a small family of dual specificity protein kinases along with downstream substrates of ERK1 and ERK2, and they are able to phosphorylate both regulatory threonine and tyrosine residues by itself. In spite of the fact that MEK-1a and MEK-2 are highly homologous, they exhibit some variations in phosphorylating different MAPKs. MEK-1b does not show MAPK phosphorylating activity and it does not undergo autophosphorylation. MEK-1 and -2, which phosphorylate ERKs, are linked to Ras mediated growth factor receptor via the Raf family of kinases (43).

MAPK:

Mitogen activating protein kinases (MAPK) pertain to a group of protein-serine/threonine kinases, which are potentially activated in all cell types by various mitogens such as growth factors, neurotransmitters, differentiating agents, and heat shock (57-60). Extensive research on the MAPK cascade revealed the presence of five different MAPKs in mammals namely, extracellular signal-regulated kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, p38 isoforms α, β, γ, and δ, ERKs 3 and 4, and ERK5 (61). The presence of six different MAPKs in yeast, suggest the possibility of having additional MAPK subfamilies in mammalian cells. Among the

five MAPKs, the most studied vertebrate MAPKs to date are the ERK1/2, JNKs and p38 kinases. JNKs and p38 kinases exhibit 40%-50% identity with ERK1/2. They are activated preferentially by pro-inflammatory cytokines and environmental stresses and are much less sensitive to growth signals (49), whereas the ERKs (ERK1/2) are strongly activated by growth factors and growth-promoting hormones. Dual specificity MAPKKs activate the MAPKs by dually phosphorylating conserved threonine and tyrosine residues within the activation loop (denoted T-X-Y) (62). The MAPKK, which can shuttle in and out of the nucleus carries MAPK and translocates it from cytoplasm to nucleus (62), where the activated MAPK is involved in stimulus-dependent gene regulation by modulating nuclear transcription factors (63, 64). MAPKs are localized to various subcellular structures, including endosomes, microtubules, endoplasmic reticulum and the actin cytoskeleton (62). MAPKs phosphorylate serine or threonine residues that are neighbors to proline so they are called proline-directed serine/threonine kinases. Primary preference of substrate for ERKs, JNKs and p38 MAPKs is having proline at the 1 position. The ERKs do have a second preference for proline at the 2^{nd} position (65-67). The activity of MAPKs are mitigated by dual specificity MAPK phosphatases, tyrosine phosphatase such as CD45 and by serine/threonine phosphatases, such as PP2A (68, 62).

ERK1 and ERK-2 (ERK1/2):

The extracellular signal-regulated kinases (ERKs) are widely characterized MAPKs. ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK) are readily detectable isoforms of MAPK (ERK) in mammals. Even though MAPK and ERK are often used interchangeably, ERK is the name for a specific group in the MAPK cascade. ERK1 and ERK2 are expressed in all tissues (69) and exhibit functional redundancy because of high

degree of similarity (83% amino acid identity). ERK 1/2 are strongly activated by growth factors and growth-promoting hormones. The activation of ERK1/2 involves the phosphorylation of threonine and tyrosine residues in a –TXY- motif. The phosphorylation of two residues occurs in a series, in which threonine phosphorylation (threonine 183) (70) is followed by tyrosine phosphorylation (tyrosine 185 in ERK-2). The main upstream molecule that phosphorylates ERK 1/2 is MEK, but it has also been reported that activation of ERK 1/2 is possible by p56 (71) and their association with the transcription factor Elk-1 (72). The activated ERK is translocated to the nucleus where it activates a wide variety of substrates. The ERK translocation into the nucleus (73) requires phosphorylation of both regulatory sites (74); whereas catalytic activity is not required. Nuclear retention, dimerization, phosphorylation and release from cytoplasmic anchors, have been shown to be important in nuclear accumulation of ERK1/2 (75).

The ERKS are proline-directed serine/threonine protein kinases. Because of the broad substrate recognition nature of ERK, it activates a number of proteins located in the cytoplasm and nucleus, which often act as regulatory molecules. The transcription factors and other nuclear proteins such as c-Jun, Elk-1, ATF-2, c-Myc, c-Myb, Ets2, NF-1L6, TAL-1, p53, and RNA serve as substrates for ERK, signifying ERK as an important regulator of nuclear transcriptional activity. Various membrane proteins (CD120a, Syk, and calnexin), cytoskeletal proteins (neurofilaments and paxillin) such as MAP-1, MAP-2, MAP-4, Tau, and others are also substrates for ERK1/2. ERK is also involved in feedback mechanisms by phosphorylating the upstream proteins of MAPK cascade such as the NGF receptor, the EGF receptor, PTP2C, SOS, Raf-1, and MEK. Another set of ERK1/2 substrates are downstream kinases in MAPK cascade such as RSKs, MSKs,

MNKs, several MAPKAP (MK) (76, 77) and protamine kinase (78). The above substrates activated by ERK1/2, along with other substrates seem to get involved in the control of cellular process that occur upon mitogenic stimulation.

ERK1/2 activity is stimulated in cultured cell lines by mitogenic stimulation of growth factor. Stimulating ERK1 activity results in enhanced cell proliferation, in eukaryotic cells, cell cycle events are regulated by ERK1 and ERK2 and are known to be an intracellular checkpoint for cellular mitogenesis. The ERK1/2 activation is required for G0 arrested fibroblast cells to enter into the cell cycle (79). This suggests the activation of ERK possibly controls the events that lead to the G0/G1 transition (80). ERK1/2 activation allows the cell to pass G1/S transition by stimulating early gene expression. ERK1 and ERK2 is activated biphasically in G1 phase, around the M-phase and involved in some activities in the G1 through S and G2/M phases. So it is suggested that MAPK may play some role in the cell cycle other than G0/G1 transition (81). It is also suggested that the function of microtubule organizing center (MTOC), which regulates the assembly of the cytosolic microtubules in interphase cells and the mitotic spindle of dividing cells, is possibily regulated by ERK1/2 (82). In Chinese hamster lung fibroblasts and ovary cells a biphasic activation of MAPK at G1 was correlated with the ability to enter S phase (83). This suggests MAPK involvement during G1/S phase transition. ERK1/2 plays an important role in cell cycle progression by decreasing the p27 levels through phosphorylation and degradation (37). Phosphorylated ERK1/2 seems to regulate cytoskeletal rearrangements and cellular morphology (84) by phosphorylation of cytoskeletal proteins. ERK1 and ERK2 have been suggested to participate in many cellular responses including proliferation, differentiation and meiosis, learning and

memory in nerve cells (65). These data suggests that the ERK cascade plays an important role in the control of cell cycle progression. In some conditions activated ERK1/2 does not lead to cellular proliferation. Rather it induces cellular differentiation that is often associated with interruption of proliferation. So it is also suggested that the ERK1/2 signaling pathway is one of the several other pathways that regulate cellular proliferation together. These pathways might work together or independently. As the ERK1/2 signaling pathway regulates cell proliferation, the inhibitors of ERK signaling pathway may be considered as potential anticancer agents.

Eukaryotic cell cycle control by MAPK pathway:

In eukaryotic cells progression through G1 phase involves the activity of G1 specific cyclin and G1 specific cyclin dependent kinases. In early G1 phase, the cyclin D family members are found associated with cdk4/cdk6. In late G1 phase, cyclin E is associated with cdk2. The activity of these complexes is potentially inhibited by CKIs. The MAPK cascade, in response to extracellular stimuli, participates in cell cycle regulation by modulating cyclin D1 expression and associated cdk activities (85). Cyclin D1 expression is positively regulated by Raf/MKK1/p42/p44 MAPK cascade in Chinese hamster fibroblast cell line CCL39. The ERK cascade is activated in response to growth factors thought to play an essential role in the modulation of cyclin D1 expression and in cell proliferation (86). Regulation of cyclin D1 expression in G1 phase involves the classic Ras/ERK pathway. AP-1 and ETS transcription factors, which are modulated by ERK, regulate cyclin D1 expression by binding to its defined elements. Activated Ras or MEK proteins induce the expression of cyclin D1 promoter genes. In addition to the regulation of cyclin D1 expression, the Raf/ MEK/ ERK pathway also post-translationally

regulates cyclinD-Cdk4/6 complex assembly. These activated complexes release the E2F factor by phosphorylating the pRb protein. This action is required for G1/S transition as well as cyclin E dependent kinase assembly and catalytic activity. The Cdk2 association with Cyclin E and Cyclin A also plays an important role in G1/S phase transition. The activity of cdk2 depends on it localization to the nucleus and is dependent on MAPK activity. Cyclin D –dependent kinases are the primary targets in the cell cycle for extracellular stimuli since there is little or no activity of these components in mitogen independent, normally proliferating cells. Regulation of these kinases at G1/S phase transition is one of the major functions of the MAPK pathway. Cdks are dephosphorylated and activated by Cdc25A phosphatases, which are activated by c-Raf-1 kinases. Ras/Raf signaling involves the stimulation of c-myc expression, which is a DNA binding protein involving transcriptional control of gene expression and cell proliferation. The c-Raf-1 protein regulates the Cdc25A expression via c-myc stimulation. Co-expression of Ras with Myc allows the generation of Cyclin E-dependent kinase activity and the induction of S phase (87). Over expression of c-myc protein inhibits the association of p27Kip1 with cyclin E-cdk2 leading to cell cycle progression through G1/S phase (88). The p27Kip1, which is driven out of cyclin E-cdk2, can be phosphorylated by ERK, and then this phosphorylated p27Kip1 is degraded by ubiquitin proteosome pathway. Thus the ERKs play a central role in the control of p27kip1.

The key molecular events modulated at G1/S transition by the ERK pathway include: 1) phosphorylation of cyclin D1-CDK4/6 which stimulates the release of the E2F leading to the synthesis of cyclin A and cyclin E mRNA; 2) Cyclin A and Cyclin E assembled to the CDK2 complex by the up regulation of CKI; 3) catalytically active

CDK2 complexes are released by the degradation of the CKI p27KIP1, that is bound tightly with these complexes.

The MAPK pathway also plays an important role at G2/M checkpoint, as through this pathway, the Mos protein activates and stabilizes M-phase-promoting factor (MPF)

The sustained activation of ERK1/2, cdk2 along with overexpression of cyclin D1, cyclin E and reduced levels of p15 and p27 as well as CKI activation are suggested consequences of the ERK pathway leading to pancreas regeneration (89). In Madin-Darby canine kidney epithelial cells (MDCK), contact inhibition of cell proliferation in the presence of serum occurs by cell density dependent regulation of ERK1/2 phosphorylation (90).

Upstream Events:

Upon growth factor stimulation, the activating signals are transmitted to the Raf/MEK/ ERK cascade by cell surface receptors such as tyrosine kinases (RTK) and G protein-coupled receptors (GPCR) through various isoforms of the small GTP-binding protein Ras. The binding of ligand to cell-surface receptor RTKs and GPCRs leads to the activation of Ras by associated SOS (son of sevenless), a Ras-activating guanine nucleotide exchange factor). SOS provokes the Ras to change GDP to GTP. Then this Ras-GTP leads to the activation of Raf isoforms and recruits them to the plasma membrane. The activated Raf in turn activates MEK1/ MEK2 then this activated MEK1/ MEK2 activates the ERK1/ ERK2. Activated ERKs modulate many nuclear transcription factors leading to the many cellular responses.

ET-1 acts as potent mitogen in many cell types. ET-1 also increases cytosolic Ca^{+2} levels and IP_3 levels upon binding to ETA receptor in BCEC (91). ET-1 has shown to

induce cell proliferation in BCEC through decreasing the p21 and p27 levels (92). The main purpose of the present research is to demonstrate the ET-1 effect on phosphorylation of ERK1/2 in the bovine corneal endothelial cell culture model system (See Figure 4). I hypothesized that ET-1 induced cellular events in BCEC involve the MAPK pathway and that ET-1 induces the phosphorylation of ERK1/2 in a time dependent manner.

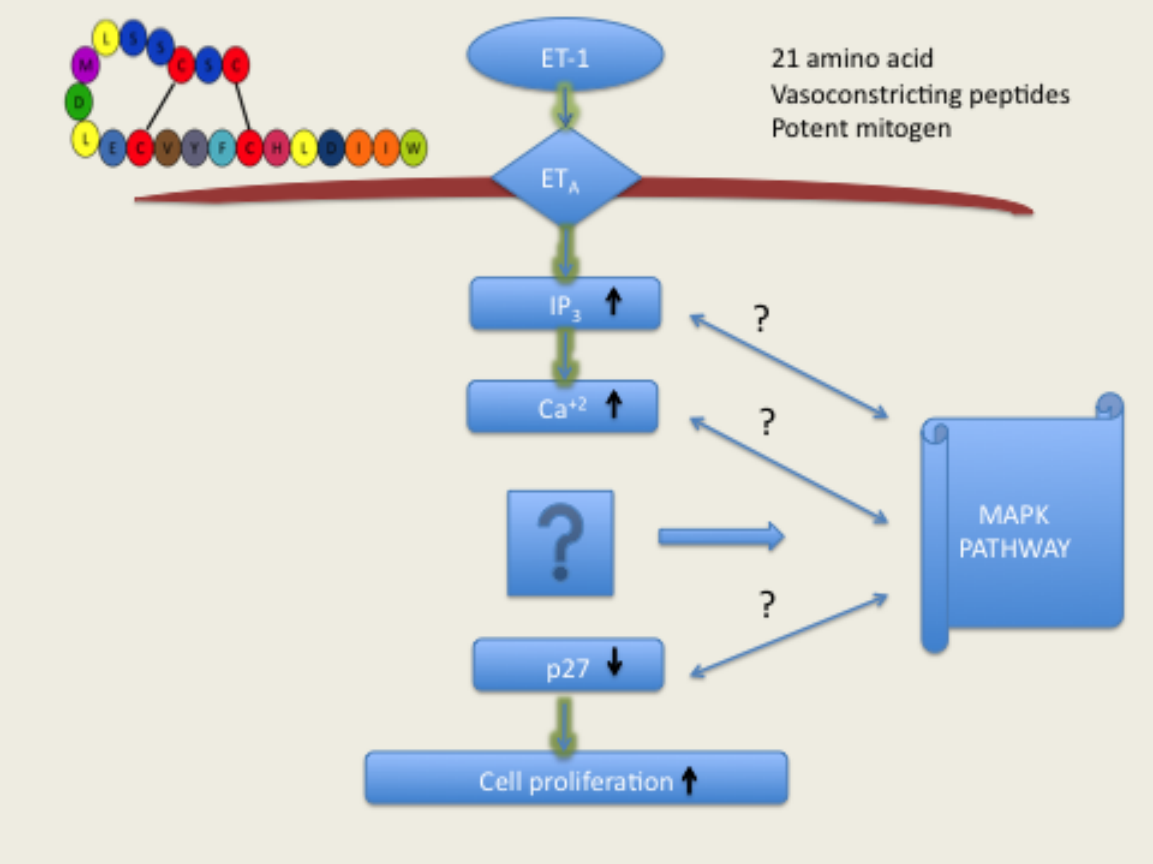

Figure 4: Schematic representation of ET-1 effect on Bovine corneal endothelial cells. ET-1 also induces cytosolic Ca^{+2} levels and IP_3 levels upon binding to ETA receptor in BCEC (90). ET-1 induces cell proliferation in BCEC through decreasing the p21 and p27 levels. The main purpose of the present research is to demonstrate ET-1 induced cellular events in BCEC involves the MAPK pathway.

MATERIALS AND METHODS

Isolation and Culture of Bovine Corneal Endothelial Cells:

Bovine eyes were obtained from Kirby and Poe slaughterhouse, Alvaton, KY. Excess tissues (muscle, fat and fascia) around the globe were trimmed and the eye was then placed cornea side up in a shallow dish lined with sterile gauze. The eyeballs were covered with sterile gauze and soaked using 2X antibiotic solution (100X stock pen/strep/amphotericin; Invitrogen, Carlsbad, CA) for 30 min at room temperature. The cornea was then dissected with a 1-2 mm sclera rim and transferred to a sterile eyecup with the endothelium side up. The endothelium was incubated with Dispase (0.125g/ 5mL, Roche) dissolved in EBSS (Earle's Balanced Salt Solution) for 90 min at 37^0C After incubation with the Dispase enzyme, endothelial cells were gently scraped off into solution using a silicon surgical rubber spatula. Cells were then aspirated with a sterile Pasteur pipette and transferred to a 15 mL tube containing 5mL DMEM (Dulbecco's Modified Eagle Medium). The cells were pelleted by centrifugation at 600xg for 2 min. The supernatant was discarded and the pellet of cells was resuspended with 5mL of growth medium (DMEM). Cells were transferred to T-25 flasks and fed three times per week with growth medium (DMEM supplemented with 10% iron supplemented calf serum (HyClone) and 100µL gentamicin (antibiotic) and 100µL fungizone (antimycotic) for each 100mL of complete growth medium. Cultures were maintained in a CO_2 incubator at 37^0C and 5% CO_2 (figure 4). Trypsin, 0.05% dissolved in calcium free EBSS, was used to subculture cells. The second passage cells were used for all experiments.

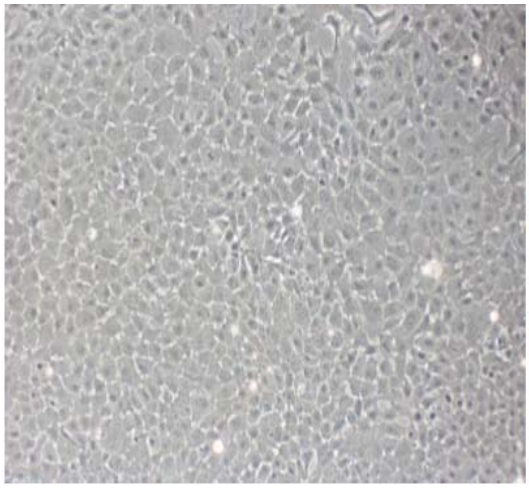

Figure 5: Cultured Bovine Corneal Endothelial Cells. Isolated BCECs were cultured in T-25 flasks at 37^0C in 5% CO2. The cells were fed 3X per week with complete DMEM.

Endothelin-1 Stock Preparation:

Endothelin-1 was obtained from Sigma Chemical, St. Louis, MO. The 20µM ET-1 stock was prepared in dilute acetic acid. One drop of glacial acetic acid was added to 15mL of nano-pure water, 100µL of the diluted acetic acid was then added to 10mL of nano-pure water and filter sterilized. One mL of dilute acid was added to 50µg of ET-1 and 25µL ET-1 aliquots were prepared and stored at -20^0C.

Antibody used:

P44/42 MAPK (ERK1/2) (catalog #9102), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (catalog #9101) and phospho-p44/42 MAPK blocking peptide (catalog #1150) were purchased from Cell Signaling Technology. The source of p44/42 MAPK (ERK1/2) antibody is rabbit and it detects the endogenous levels of total p44/42 MAPK protein in H, M, R, Mk, Pg, Sc, Hm, B, Z. The source of the Phospho-p44/p42 MAPK antibody is rabbit and it detects the endogenous levels of p44 and p42 MAP kinases when phosphorylated either individually or dually at Thr 202 and Tyr204 of ERK1 and Thr185

and Tyr187 of ERK2 in H, M, R, Mk, Pg, Hm, B, Dm, Z, Ce. It does not cross react with non-phosphorylated ERK1/2. The phospho-p44/42 MAPK blocking peptide was used to specifically block rabbit phospho-p44/42 MAPK antibody (Thr202/Tyr204). It was used to determine the specificity of phospho-p44/42 MAPK antibody.

ET-1 treatment in confluent cells:

BCECs were grown to 100% confluence in T-75 flasks and serum starved in DMEM for 24 hr to induce quiescence. Different serum starved confluent BCEC flasks were treated with 10nM ET-1 and incubated for 0.25hr, 2hr, 6hr, and 24hrs.Control cells were left untreated in serum free DMEM. Protein samples were collected from both control and ET-1 treated cells.

ET-1 treatment in sub-confluent cells:

BCECs were grown to 80%-85% confluence in T-75 flasks and serum starved in DMEM for 24 hr to induce quiescence. The different serum starved BCEC flasks were treated with 10nM ET-1 and incubated for 0.25hr, 6hr, and 24hrs.Control cells were left untreated in serum free DMEM. Protein samples were collected from both control and ET-1 treated cells.

Isolation of BCEC Protein:

After appropriate treatment, cells were rinsed twice with cold EBSS for 5 min. Then 2mL of ice cold RIPA buffer (25 mM Tris•HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor (1:500) and PMSF (1mM) was added to the T-75 cm^2 flask (0.6mL of buffer for T-25 cm^2 flasks) and the flask was incubated on ice for 5 min. Flasks were swirled occasionally on ice during incubation. Using a cell scraper, cells were scraped into a 15 mL blue cap tube. To increase yield, the

pellet was sonicated for 10 seconds with 20% amplitude. Samples were then kept at -20^0C over night.

Estimation of Protein Concentration:

Protein concentration was determined according to the Peterson modification of the Lowry protein determination assay (93). Samples and BSA standards (10-100µg) were pipetted into cuvettes. Volumes of all samples and standards were brought up to 0.5mL with dH_2O. 1mL of reagent A (equal volumes of copper-tartrate-carbonate solution, 10% SDS, 0.8N NaOH and dH_2O) was added to all tubes, vortexed immediately and left for 10 min at room temperature. Following the incubation with reagent A, 0.5mL of reagent B (one part of Folin-Ciocalteu Phenol Reagent to 5 parts of dH_2O) was added to all tubes and incubated for 25 min. Absorbance of standards and samples was read at 750nm wavelength. The protein concentration of samples was calculated from the standard curve of BSA standards.

SDS-PAGE and Western Blotting:

FiveX sample buffer was prepared by mixing 0.35g Tris base, 5mL glycerol, 1g SDS (gentle heating), pH to 6.8, and 5g bromophenol blue. Sample buffer was added in a 1:4 ratio (1 part sample buffer: 4 parts sample) to sample in a screw cap tube and the mixture was heated for 5 min. with 2-mercaptoethanol (2%) on a digital hot plate. An equal mass of sample (15µL) was loaded in each well of Any kD mini protean pre-cast gel (Bio-Rad) and the proteins were separated at constant voltage (150V for 1 hour). Five µL of protein molecular weight standards (Bio-Rad) were loaded in one well of the gel. After gel electrophoresis, the gel was removed from the gel cassette and the separated proteins were transferred onto PVDF membranes for immuno-staining. After

electrophoresis the gel was washed in 15mL transfer buffer on shaker at room temperature. The PVDF membrane and filter papers were wetted in transfer buffer and a blot sandwich was prepared in the following sequence from black side of the cassette to red side of the cassette: filter pad, filter paper, gel, PVDF membrane, filter paper and filter pad. Bubbles were removed by rolling a glass rod over the sandwich. The gel holder cassette was then closed and placed into the transfer tank filled with 1X transfer buffer (30.3 g trisbase, 144.1 g glycine, water to 1 lit for 10X). Proteins were transferred at a constant 100V for 2 hr 30 min at 4^0C. To detect the possible presence of non-transferred proteins, the gel was placed into a solution of Coomasie blue. The PVDF membrane was then placed into a dish containing approximately 20mL blocking solution (10 g non-fat dry milk, 20 mL 10X PBS, water to 200 mL) and incubated overnight at 4^oC. After the blocking step, the PVDF membrane was rinsed in 1X PBS for 15 min on shaker. The membrane was then incubated with an optimized dilution (1:1000 primary antibody ERK1/2 or pERK1/2 in blocking solution with 0.1% Tween-20) for 2 hr at room temperature or overnight at 4^0C, with shaking. The membrane was then rinsed five times in 1x PBS with 0.1% Tween-20 (30-40mL of PBS) at room temperature, with shaking. Next the membrane was incubated with an optimized dilution (1:1000 in blocking solution) of HRP-conjugated secondary antibody for 40 min at room temperature, with shaking, followed by another series of rinses and washes with PBS-T. The first three rinses in 1X PBS with 0.3% tween-20 for 5 min followed by next three rinses in 1x PBS with 0.1% tween-20 and a quick rinse in 1X PBS. A working solution (Super Signal West Femto Maximum Sensitivity Substrate) for detecting antigen-antibody complex was prepared by mixing equal parts of the stable peroxide solution and luminol/enhancer

solution (Invitrogen). The working solution was applied at 0.1mL per cm^2 of membrane and the blot was incubated 5 min. The blot was then removed from the working solution and an electronic image was taken with Alpha Innotech FluorChem HD2 [San Leandro, CA]. Densitometry analysis for two proteins (ERK and p-ERK) was done using NSF J Image software. ERK protein was used as a loading control to normalize the target protein levels by confirming that an equal amount of sample was loaded in each well of the gel. pERK values were normalized with ERK values. One-way Analysis of Variance (ANOVA) was performed on pERK/ERK densities to determine the difference between means of three or more groups. A Bonferroni multiple range test was performed on pERK/ERK values for pair-wise comparison. A p value less than 0.05 was considered statistically significant.

RESULTS

Determination of pERK1/2 antibody specificity to bovine pERK1/2 using blocking peptide:

To confirm the specificity of anti-pERK1/2 antibody to bovine pERK1/2, the polyclonal anti-pERK1/2 antibody neutralized with a blocking peptide against which anti-pERK1/2 was raised. Separated proteins were transferred onto the PVDF membrane and incubated with anti-pERK1/2 and HRP conjugated secondary antibody (Fig. 6, panel A), neutralized anti-p27 and HRP conjugated secondary antibody (Fig. 6, panel B). The blot (Panel A) incubated with anti-pERK1/2 alone gave conspicuous bands at ~ 44kDa and ~42kDa. The incubation of the immuno-blot with neutralized anti-pERK1/2 resulted in a complete loss of the bands at 44kDa and 42kDa

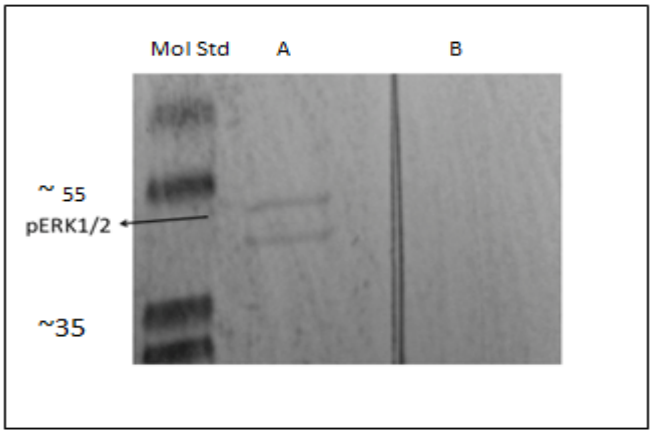

Figure 6: Specificity of anti-pERK1/2 for bovine pERK1/2. Panel A represents the result with no blocking peptide, panel B represent the result with the presence of blocking peptide. Equal amounts of protein were separated on Any kD precast mini-protean gels and separated proteins were transferred onto PVDF membrane. In panel A, the blot was immuno-stained with anti-pERK1/2 and secondary antibody. The blot, shown in panel B, was incubated with anti-pERK1/2 preincubated with pERK1/2 blocking peptide (1:2) against which pERK1/2 antibody was raised and secondary antibody.

Phospho-ERK1/2 Levels in serum starved confluent (contact inhibited) and sub-confluent cultures:

` To perform semi-quantitative analysis of the pERK1/2 protein expression in serum-starved cultures, protein was prepared from serum starved confluent and growing cultures of BCECs. Figure 7 shows the level of expression of pERK1/2 in confluent and growing cells. Densitometry analysis (Fig. 8) revealed the expression of pERK1/2 in growing cells is 74% higher than in confluent cells.

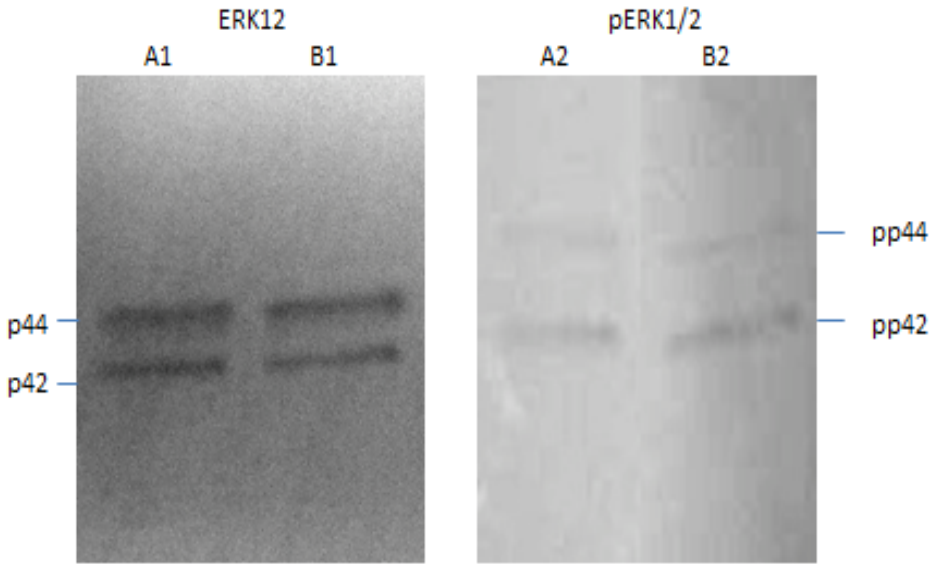

Figure 7: ERK1/2 and p-ERK1/2 levels in serum starved confluent and sub-confluent cultures of BCEC. Confluent cultures (A1, A2) and sub-confluent cultures (B1, B2) were serum starved in DMEM for 24 hrs then total cellular protein was harvested. Equal amounts of confluent and sub-confluent protein samples were loaded onto the Any kD precast mini protean gels for protein separation. Separated proteins were trasferred to PVDF membrane and immuno-staining was performed using ERK 1/2 (left) and p-ERK 1/2 (right) primary antibodies (1:1000).

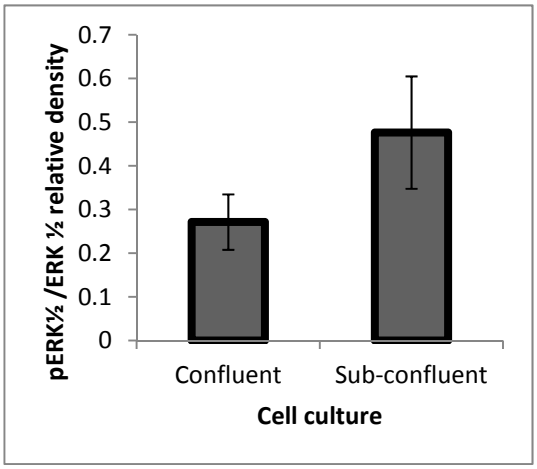

Figure 8: Cell culture Vs pERK ½ /ERK ½ relative density. Using J-image software, ERK1/2 and pERK1/2 levels were measured by densitometry. The pERK1/2 levels were normalized to ERK1/2 levels and compared between confluent cells and growing cells. Note: The values in the graph represent the average of two independent experiments.

Table-1: Differences in pERK1/2 levels between confluent and sub-confluent cells:

Protein	Confluent cells ((pERK ½ /ERK½)	Sub-confluent cells (pERK½ /ERK½)	Fold difference
pERK1/2	0.27	0.47	1.7X

Note: Results are the average of three independent experiments for confluent cultures and two independent experiments of sub-confluent cultures.pERK1/2 levels were normalized with ERK1/2.

Effect of ET-1 on ERK1/2 phosphorylation in confluent cells:

ET-1 is suggested to be a potent mitogen in many cell types and it was suggested

that ET-1 increases the BCEC cell proliferation and cell migration. It was hypothesized that ET-1 induced cellular responses involves the MAPK pathway by phosphorylation of ERK1/2. To determine whether ET-1 treated cells exhibit enhanced phosphorylation of ERK1/2, serum-starved cells were treated with 10 nM ET-1 for 24 hrs. Protein samples were then prepared from untreated and ET-1 treated cells to analyze the phosphorylated ERK1/2 levels through western blotting. The densitometry analysis revealed that phosphorylation was higher in ET-1 treated cells than in untreated cells (Fig. 10).

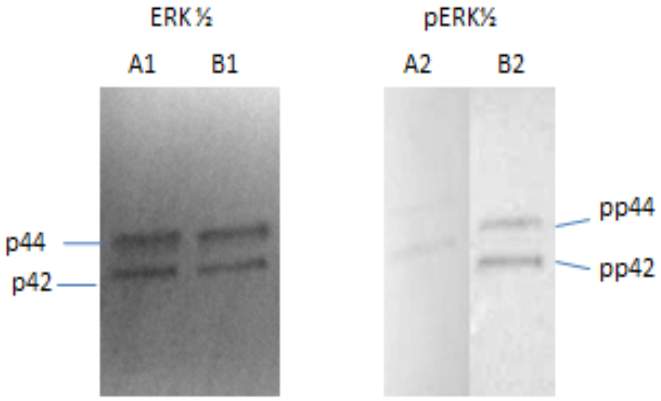

Figure 9: pERK1/2 levels in ET-1 treated cells. BCECs were grown to 100% confluence in T-75 flasks and serum starved for 24 hrs. Serum starved cells were treated with 10 nM ET-1 for 24 hrs. The control cells were left untreated. Total protein was isolated from both control (lane A_1, lane A_2) and ET-1 treated cells (lane B_1, lane B_2) and equal amounts of protein were loaded on Any kD mini-protean precast gel. The separated proteins were transferred onto PVDF membrane and immuno-staining was performed. Panel A1 and panel B1 were incubated with anti ERK1/2 (1:1000) and secondary antibody (1:1000). Panel A2 and panel B2 were incubated with anti pERK1/2 (1:1000) and secondary antibody (1:1000).

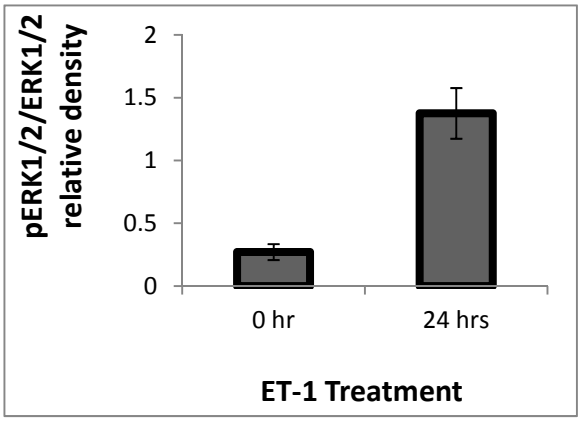

Figure 10: ET-1 treatment Vs pERK ½ /ERK ½ relative density. Using J-image software densitometry analysis was performed. ERK staining density was used to normalize pERK staining. The densitometry analysis revealed that the treatment with ET-1 at 10 nM concentration resulted in a 5.1 fold increase in pERK1/2 levels. Note: The values plotted in the graph are the average of three independent experiments.

Table-2: pERK1/2 in ET-1 treated cells:

Treatment	Without Serum	ET-1 treated for 24hrs	Fold increase
pERK½/ERK½	0.27	1.37	5.1X

Note: Results are the average of three independent experiments.

Effect of incubation time of ET-1 on pERK1/2 levels in confluent samples:

Considering mitogenic effects of ET-1, we hypothesized that ET-1 induces the phosphorylation ERK1/2 in a time dependent manner. To characterize the effect of incubation time of ET-1 on pERK1/2 levels in confluent cultures, different BCEC flasks treated with 10 nM ET-1 and incubated for 0.25hr, 2hr, 6hr, and 24hrs. Protein samples were collected from both control and ET-1 treated cells. Western blot analysis (fig. 11) revealed that ERK1/2 increased rapidly at 0.25 hr, and gradually declined at 2 hr and 6 hr

only to increase again at 24hr. The lowest level for pERK1/2 was observed at 6 hrs post incubation with ET-1 and the maximum level at 24hrs (fig. 12).

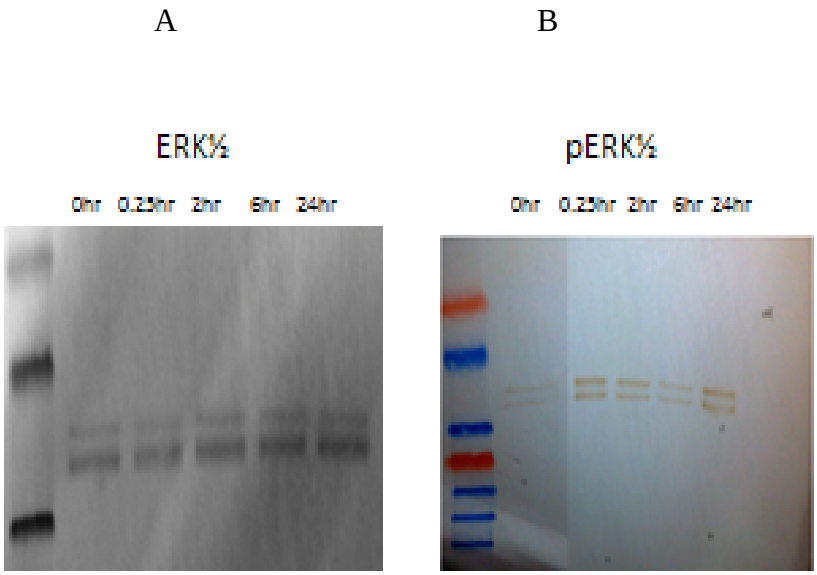

Figure 11: Effect of ET-1 on pERK1/2 levels in confluent BCECs. Confluent BCECs were serum starved in DMEM for 24 hr to induce quiescence. The serum starved cells were treated with 10nM ET-1. Control cells were left untreated in serum free DMEM and are considered as 0 hr. Protein samples were isolated using RIPA buffer at 0.25hr, 2 hr, 6 hr, 24 hr. Protein samples were separated on Any kD precast mini-protean gels and transferred onto a PVDF membrane. A: Immuno-staining was performed on PVDF membrane using antibodies against ERK1/2. B: Immuno-staining was performed on PVDF membrane using antibodies against pERK1/2.

A B

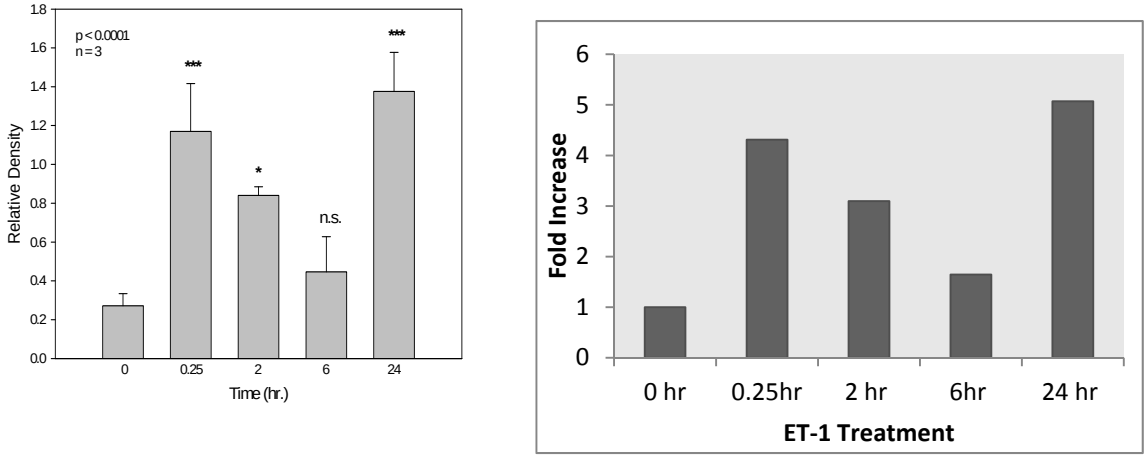

Figure 12: Densitometry studies of pERK1/2 levels in confluent BCEC. The density of bands was measured using J image software for ERK1/2 and pERK1/2. Panel A: The pERK1/2 levels were normalized with ERK1/2 and plotted against incubation time. In ANOVA analysis the P value is less than 0.0001, considered to be extremely significant. Multi range test revealed the probability of ET-1 treated cells for 0.25 hr and 24 hr is less than 0.001 compared to 0 hr. P value for ET-1 treated cells for 2 hrs is less than 0.005 and 6hrs is greater than 0.05 compared to 0 hr. B: Fold difference was plotted against incubation time. Note: The values plotted in the graph are the average of three independent experiments.

Table-3: Effect of incubation time of ET-1 on pERK1/2 **levels in confluent samples**

Treatment	**0 hr (Control)**	**0.25hr**	**2hr**	**6hr**	**24hr**
Density [pERK½/ERK ½]	**0.271**	**1.169**	**0.840**	**0.446**	**1.375**
Fold Increase [Treatment Density/control Density]	**1**	**4.311**	**3.098**	**1.644**	**5.069**

Note: Results are the average of three independent experiments. pERK 1/2 levels are

normalized with loading control, ERK1/2. The effect of ET-1 on pERK1/2 levels is represented as fold difference in pERK1/2 levels.

Effect of incubation time of ET-1 on pERK1/2 **levels in Sub-confluent samples:**

Considering the ET-1 induced differential phosphorylation in contact inhibited cells and sparse cells of mouse vascular endothelial cells (94) we hypothesized, the time dependent ERK1/2 phosphorylation manner is also different in confluent and sub-confluent cells. To determine the effect of incubation time of ET-1 on pERK1/2 levels in sub-confluent cultures (80%-85% confluence), we treated different BCEC flasks with ET-1 at 10 nM concentrations and incubated for 0.25 hr, 2 hr, 6hr, and 24 hrs.

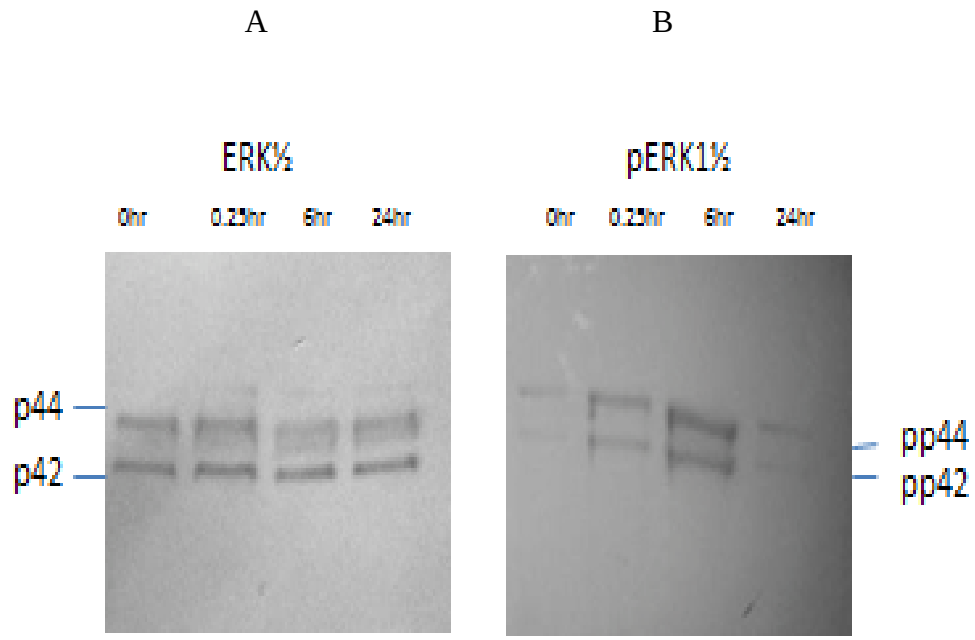

Figure 13. Effect of ET-1 on pERK1/2 levels in sub-confluent BCECs. Sub-Confluent BCECs were serum starved in DMEM for 24 hrs to induce quiescence. The serum starved cells were treated with 10nM ET-1. Control cells were left untreated in serum free DMEM and are considered as '0' hr. Protein samples were isolated using RIPA buffer at 0.25hr, 6 hr, 24 hr. Protein samples were separated precast mini protean gels and transferred onto a PVDF membrane. A: Immuno-staining was performed on PVDF membrane using antibodies against ERK1/2. B: Immuno-staining was performed on PVDF membrane using antibodies against pERK1/2.

The maximum increase for pERK1/2 was observed at 6 hrs of post incubation with ET-1 and decrease in pERK1/2 was observed at 24hrs of post incubation with ET-1 (fig. 14). This contested markedly with the results with confluent cells (fig. 12).

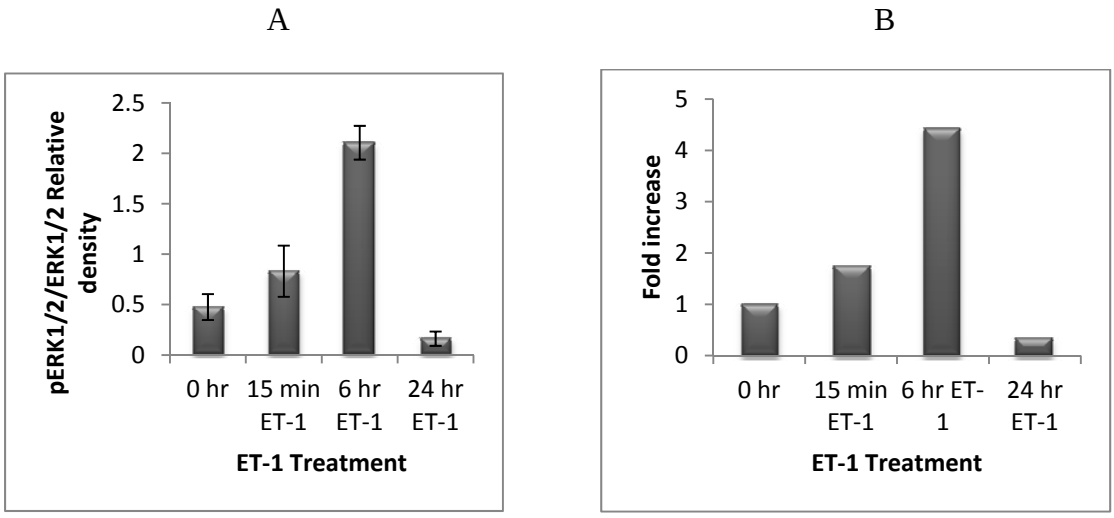

Figure 14. Densitometry studies of pERK1/2 levels in sub-confluent BCECs. The density of bands density was measured using J image software for ERK1/2 and pERK1/2. Panel A: The pERK1/2 levels were normalized with ERK1/2 and plotted against incubation time. Panel B: Fold difference was plotted against its time interval. The maximum increase in pERK1/2 levels in response to ET-1 was observed at 6 hrs following with ET-1.The decrease in pERK1/2 levels in response to ET-1 was observed at 24 hrs following with ET-1. Note: The values plotted in the graph are the average of two independent experiments.

Table-4: Effect of incubation time of ET-1 on pERK1/2 levels in sub-confluent samples

Treatment	Control (Without Serum)	0.25hr	6hr	24hr
Density [pERK/ERK]	0.476	0.831	2.105	0.162
Fold Increase [Treatment Density/Control Density]	1	1.746	4.421	0.340

Note: Results are the average of two independent experiments. pERK1/2 levels are normalized to ERK1/2. The effect of ET-1 on pERK1/2 levels is represented as the fold difference in pERK1/2 levels

DISCUSSION

The corneal endothelial monolayer, one of the important layers of the cornea, is essential for maintaining the thickness, transparency and hydration of cornea. It has been found that the endothelial cells are non-proliferative under *in vivo* conditions. Even though the adult corneal endothelial cells are non proliferative under *in vivo* conditions, they show cell proliferation under *in vitro* conditions when provided with sufficient nutrients and released from contact inhibition.

ET-1, a vasoconstring peptide, acts as potent mitogen in many cells, including smooth muscle cells, fibroblasts, and astrocytes (29-31). In our lab, it was shown that ET-1 elicits an elevation in cytosolic Ca^{+2} levels and inositol tri phosphate levels (IP_3) in Bovine Corneal Endothelial Cells (BCECs) through binding to ET-A receptor (91). In our lab, it was also shown that ET-1 exhibits mitogenic effects in BCECs by increasing cell proliferation through decreasing p21 and p27 levels (92). But the steps in between these two events are not known. In this research, I hypothesized that the MAPK pathway was involved in ET-1 induced cellular events in BCEC and it was confirmed with the induction of phosphorylated ERK 1/2 (pERK1/2) levels. There was an approximate 5-fold increase in the phosphorylation of ERK1/2 in response to ET-1 treatment compared to the control (Fig. 10). The ERK cascade, which is activated in response to growth factors, is thought to play an essential role in modulation of cyclin D1 expression and cell proliferation (86). The ERKs were also found to play an important role in controlling the p27 levels (37). These observations coupled with the results presented here support the hypothesis that the cellular proliferation occurs through p27 degradation in ET-1 treated

BCEC that is mediated by ERK1/2. However, further experiments are required to validate or not validate this hypothesis.

In this research, the time dependency phosphorylation of ERK1/2 in ET-1 treated confluent cells was analyzed (Fig. 12). A significant increase in ERK1/2 phosphorylation levels were found at 0.25 hr, 2 hr and 24 hr of incubation with ET-1 in confluent cells. After the rapid increase in phosphorylation levels at 0.25 hr, the phosphorylation of ERK1/2 declined at 2 hr and 6 hr only to increase again at 24hr. The biphasic response in phosphorylation may be considered as an oscillation in phosphorylation levels. These results may suggest the presence of a negative feedback mechanism in ET-1 induced MAPK pathway in BCEC confluent cells. ERK1/2 may negatively inhibit the levels of other upstream intermediates in the MAPK cascade. In the tyrosine receptor kinase linked MAPK cascade, it was shown that activated ERK1/2 inhibits Ras signaling through inhibiting phosphorylation of SOS (95). This inhibition creates a negative feedback in the MAPK cascade. It was also shown that negative feedback loop in association with intrinsic ultra sensitivity of the MAPK cascade can lead to a sustained oscillation in MAPK phosphorylation (96).

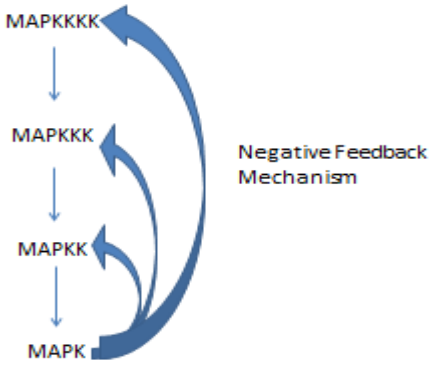

Figure 15. Assumed negative feedback mechanism in MAPK cascade

In this study, it was found that the ET-1 induced ERK1/2 phosphorylation pattern was different in confluent and sub-confluent cultures (Figs.12, 14). In confluent cultures, the higher phosphorylated ERK1/2 levels were found at 0.25hr and 24hr of incubation with ET-1 whereas the phosphorylation of ERK1/2 had declined at 2 hr and 6 hr. In actively growing cultures the pERK1/2 levels were increased from 0.25 hr to 6 hr then at 24 hr it was decreased. A difference in phosphatase activity may be considered as a reason for these fundamentally different response patterns. It has been shown that increases in both cytosolic and membrane-associated tyrosine phosphatase activities occur at high cell densities in osteoblast cells, in endothelial cells and in Swiss 3T3 fibroblasts (94). In mouse endothelial cells, it was found that confluent cells exhibited increased phosphatase activity compared to sparse cells. In mouse endothelial cells, cell density-dependent phosphatase activity contributes to a possible mechanism for maintaining a low level of pERK1/2. Cell density dependent regulation of ERK1/2 phosphorylation is shown to be responsible for contact inhibition of cell proliferation in the presence of serum in Madin-Darby canine kidney epithelial cells (90). It is suggested that in contact-inhibited BCECs the pERK½ levels declined after the initial risedue to higher phosphatase levels compared to actively growing cells. It was thought that decrease in pERK1/2 levels at 24 hrs in sub-confluent cells might be because they shifted from sub-confluent culture to confluent culture by that incubation time as they were 80%-85% confluent at the time of ET-1 treatment.

More research needs to be performed to identify the upstream molecules involved in the BCEC MAPK pathway, their roles in ET-1 induced cellular events and their regulation of ERK 1/2 phosphorylation. In conclusion, it was shown that the involvement

of MAPK pathway in ET-1 treated BCECs, time dependent phosphorylation of ERK 1/2, and differential phosphorylation in contact inhibited cells compared to sub-confluent cells. These findings provide a basis platform for further research in revealing BCEC MAPK cascade, their role in cell cycle regulation and contact inhibition.

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