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## Effect of Insulin and Vanadate on SRE-mediated c-fos Expression and JNK Activation

Francisco J. Berguido

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EFFECT OF INSULIN AND VANADATE ON SRE-MEDIATED *C-FOS*  
EXPRESSION AND JNK ACTIVATION

by

Francisco J. Berguido

A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Master of Science  
Department of Biological Sciences

Western Michigan University  
Kalamazoo, Michigan  
December 1997

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1997

## ACKNOWLEDGMENTS

First I would like to express my deepest gratitude to my family, especially my grandfather, Julio C. Berguido (deceased), the only father I ever knew; my grandmother, Blasina de Berguido, whose prayers and words of wisdom I keep deep in my heart; and my mother Maria Berguido, who taught me by example: perseverance and who always provides me with support, encouragement and love.

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Francisco J. Berguido

## EFFECT OF INSULIN AND VANADATE ON SRE-MEDIATED *C-FOS* EXPRESSION AND JNK ACTIVATION

Francisco J. Berguido, M.S.

Western Michigan University, 1997

Insulin mimetics are compounds that mimic the action of insulin in the body and are currently being studied as possible therapeutic agents against diabetes. Vanadium has been identified as an element with insulin-like properties with regard to metabolic processes such as lowering blood glucose levels. Few studies have implicated vanadium in mitogenic actions such as cell growth and differentiation. We studied the action of vanadate on the expression of the insulin-induced SRE, a cis-element of the well-characterized mitogenic gene *c-fos*. We measured this expression by means of a plasmid containing the reporter gene luciferase linked to the sequences from *c-fos* containing the SRE (SRE-Luc). We also examined activation of signal proteins that might be responsible for the insulin induction of SRE-Luc.

This study showed that insulin increased expression of SRE-Luc in a time-dependent fashion, vanadate had no effect on the expression of SRE-Luc. Use of inhibitors of the PI3K and MAPK pathways suggested that PI3K is necessary for insulin induction of SRE-Luc and PI3K maybe located somewhere between Ras and MEK. The use of inhibitors also helped determine that both, MAPK and PI3K pathway proteins may also be necessary for the insulin-induced expression of SRE-Luc. Additionally, we assessed JNK activity in the presence of insulin and vanadate. Our results indicate that insulin and not vanadate activate JNK, a close relative of MAPK.

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## CHAPTER I

### INTRODUCTION

#### Insulin, Insulin-Mimetics and Diabetes

Insulin action in the cell includes regulation of glucose and amino acid transport; activation of important enzymes in intermediary metabolism; modulation of protein, DNA and RNA synthesis; and regulation of cellular growth and differentiation (Saltiel, 1996; Jhun et al., 1995; Levy-Toledano et al., 1995). Any or all of these processes can be affected in disease states such as in insulin-dependent diabetes mellitus (IDDM) or non-insulin-dependent diabetes mellitus (NIDDM), conditions in which insulin is not produced or recognized adequately. In recent years, attention has been given to the use of elements and compounds with insulin-like effects to potentially treat IDDM and NIDDM. These compounds are commonly known as insulin mimetics (Weinstock et al., 1992; Balon et al., 1995; Esaki, 1990, Stapleton et al., 1997).

One of these mimetics is vanadium (vanadate), a transition element commonly found in the earth's crust. Vanadium has been shown to stimulate glucose transport in rat adipocytes and mouse skeletal muscle; and increase lipolysis, glucose oxidation and glycogen synthase in rat adipocytes (Goldfine et al., 1995). Its administration in oral doses has been found to reduce levels of blood glucose in diabetic animal models (Goldfine et al., 1995; Yale et al., 1995; Stapleton et al., 1993). Vanadium is also involved in regulation of DNA synthesis as well as in inducing expression of several genes that are usually increased in the presence of insulin (Weinstock et al., 1992; Chen and Chan, 1993; Imbert et al., 1994; Stapleton et al., 1993). Because of the

many documented studies about its insulin-like action, vanadium has recently been proposed for human clinical trials as a possible therapeutic agent for several forms of diabetes (Borman, 1994).

However, before concluding that this element completely mimics the effect of insulin intracellularly, it is relevant to determine the extent of the insulin mimetic action of vanadate in the cell. Within the cell, insulin induces metabolic actions such as lowering blood glucose levels, as well as mitogenic actions which are associated with cell growth and differentiation. Although vanadate mimics the metabolic action of insulin, its role in the insulin-like mitogenic effects has not been clearly identified (Yale et al., 1995). This aspect of insulin action should be mimicked by vanadate in order to consider this element a true insulin-like substance.

In order to determine the mechanism of action of vanadate as well as its effects as an insulin mimetic, it is first, necessary to understand the intracellular mechanism of insulin action.

### Insulin and Its Signal Transduction Mechanism

Intracellularly, insulin triggers a series of events, including transient protein phosphorylation and dephosphorylation, that are responsible for the insulin induced cellular effects. This cascade of events that triggers the regulation and modulation of mitogenic and metabolic effects of insulin in the cell is known as the insulin signal transduction cascade (Saltiel, 1996).

The first step in insulin signal transduction is the autophosphorylation of the insulin receptor (Saltiel, 1996; Jhun et al., 1995). The insulin receptor (IR) is a cell surface heterotetrameric protein complex with two alpha and two beta subunits (Saltiel, 1996; Zick, 1989). The alpha subunits contain the insulin binding domains, the beta subunits contain the tyrosine kinase activity (Saltiel, 1996; Lee and Pilch,

1994). Upon insulin binding to its receptor, autophosphorylation occurs via crossactivation of the beta subunits (Saltiel, 1996; Jhun et al., 1995). This autophosphorylation increases its kinase activity and results in the activation of downstream proteins (Saltiel, 1996). These activated proteins can be classified, based on their interactions, into two main groups or pathways: The IRS-1/PI3K and the Ras/MAPK pathways.

### Insulin Signal Transduction Cascade - The IRS-1/PI3K Pathway

One of the proteins that has been identified as a target of the activated IR (Biener et al., 1996), is the insulin receptor substrate 1 (IRS-1), a 185kDa protein with several tyrosine phosphorylation sites (Saltiel, 1996). This protein can interact with the src homology (SH) 2 domains of a membrane-bound kinase known as PI3K (Saltiel, 1996; Yamauchi et al., 1993) (Figure 1).

### PI3K and Its Signal Cascade

In mammals, there are several known classes of phosphatidylinositol 3-kinase (PI3K) (Ireton et al., 1996; Carpenter and Cantley, 1990). The best characterized for its effects in signal transduction, is a heterodimer composed of an 85kDa regulatory subunit (p85) and a 110kDa catalytic subunit (p110) (Wang et al., 1995), each of which exists in at least two isoforms ( $\alpha$  and  $\beta$ ) (Ireton et al., 1996; Inukai et al., 1997). p85 contains an SH2 domain, a break point region (bpr) homology domain inserted in the middle of two proline rich sequences and two SH2 domains flanking a p110-binding region (Wang et al., 1995).

PI3K phosphorylates phosphatidylinositol (PI), PI 4-phosphate and PI 4,5-bisphosphate at the D-3 hydroxyl position of the inositol ring. (Wang et al., 1995; Ireton et al., 1996; Karnitz et al., 1995; Carpenter and Cantley, 1990).

# The Insulin Signal Cascades

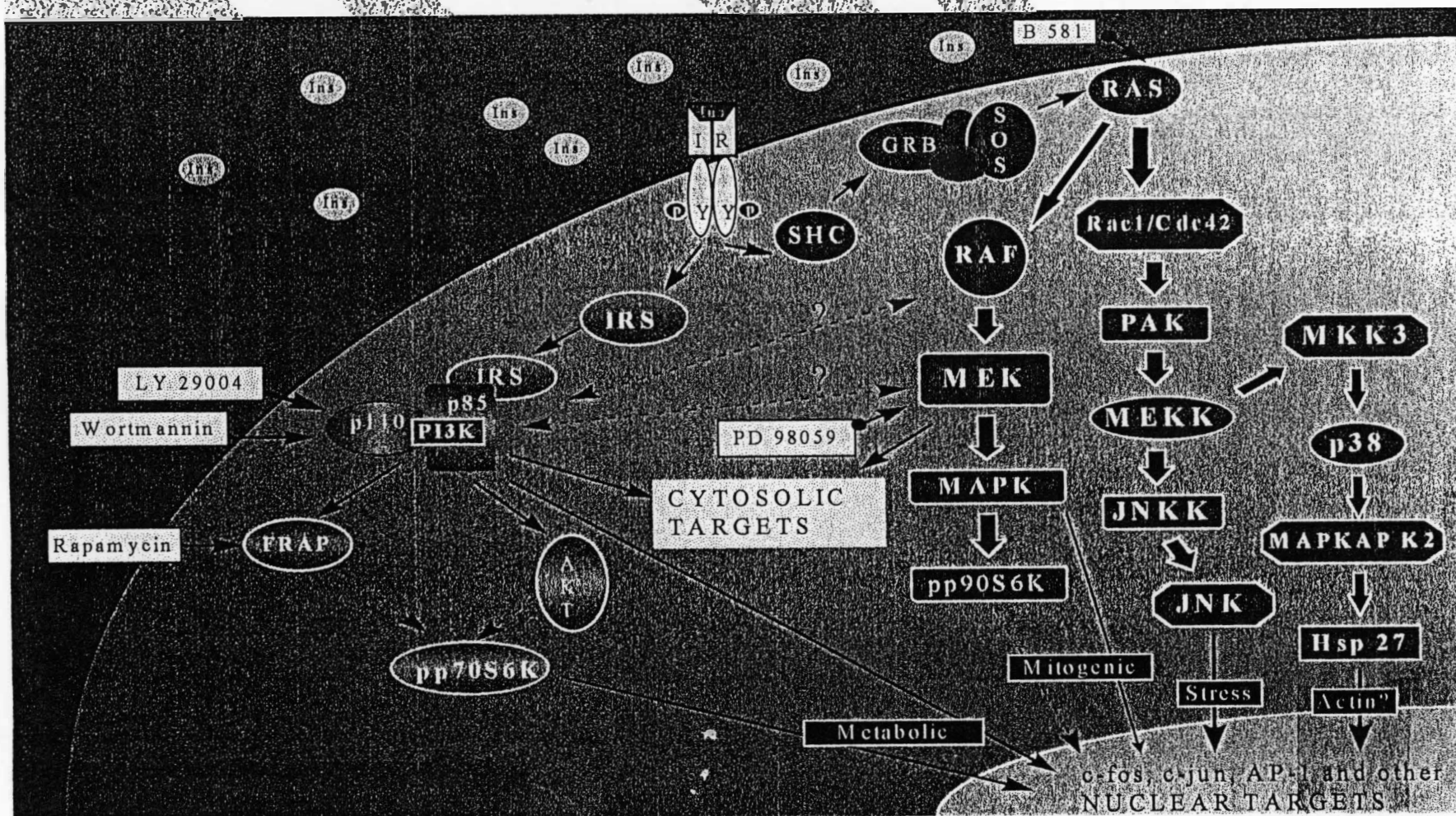


Figure.1 Proteins Known to Become Activated Upon Insulin Induction.

Several growth factors in addition to insulin, are capable of activating PI3K (Saltiel, 1996). PI3K activation by insulin and other growth factors has been correlated with membrane trafficking and ruffling, glucose and thymidine utilization and actin polymerization (Saltiel, 1996; Denton and Tavaré, 1995; et al., 1996; Suga et al., 1997; Harada et al., 1995; Lazar et al., 1995; Gabbay et al., 1996; Ireton et al., 1996; DePaolo et al., 1996; Hara et al., 1994). Other studies have also shown the involvement of PI3K in activation of gene expression associated with regulation of cell growth and mitogenesis (Hu et al., 1995; Karnitz et al., 1995; Hawes et al., 1996). Point mutations in the PI3K binding sites of the PDGF receptor, for example, impairs the ability of this receptor to mediate expression of several genes (Wang et al., 1995).

Two components of the PI3K cascade which were found downstream of PI3K have been identified as ribosomal S<sup>6</sup> protein kinase (p70S<sup>6</sup>Kinase) and PKB/c-Akt (Burgering et al., 1995; Monfar et al., 1995). In vitro assays show that Akt is capable of p70S<sup>6</sup>Kinase activation, suggesting that p70S<sup>6</sup>Kinase is downstream of Akt (Burgering et al., 1995).

Another protein implicated in the PI3K cascade is the FKBP-Rapamycin-Associated Protein (FRAP). FRAP (also known as RAFT in the rat system) is a 289kDa protein (Choi et al., 1996). This protein is characterized by a C-terminal kinase domain homologous to phosphatidylinositol kinases (Choi et al., 1996; Brown et al., 1995) (Figure 1).

FRAP, which is sensitive to the immunosuppressant rapamycin is required to bind p70S<sup>6</sup>Kinase for PI3K-mediated activation (Choi et al., 1996; Brown et al., 1995; Monfar et al., 1995; Mahalingam, et al., 1996) (Figure 1).

## Insulin Signal Transduction Cascade - The Ras/MAPK Pathway

Autophosphorylation of the insulin receptor results in the activation of a second group of proteins which forms the Ras/MAPK pathway. This cascade is initiated with the phosphorylation of members of the Shc family of proteins. Several members of the Shc family are phosphorylated via SH2 domains (Czech, 1995; Saltiel, 1996). Phosphorylation of Shc by IR results in activation of an adaptor protein: Grb2 (Saltiel, 1996). Grb2-Shc associates with a protein known as son of sevenless (SOS) on the SH3 domains of Grb2 (Su and Karin, 1996; Skolnik et al., 1993). Activated SOS acts as a guanine nucleotide exchange factor for a small G protein at the plasma membrane, Ras (Czech, 1995; Saltiel, 1996; Holt et al., 1996; Jhun et al., 1994) (Figure 1).

Ras is an inner leaflet plasma membrane associated guanine nucleotide binding protein that becomes activated upon several stimuli that are related to cell growth and differentiation (Jelinek et al., 1994; Avruch et al., 1994). Upon insulin presence, Ras activation results in the activation of a family of proteins: the MAPK's (Medema et al., 1991; Olefsky et al. 1993; Miller et al., 1996) (Figure.1).

### The MAPK's

Mitogen-Activated Protein Kinases (MAPK's) are a family of protein kinases that become activated by a proline-directed phosphorylation at specific serine/threonine residues (Su and Karin , 1996; Avruch et al., 1994).

Several studies with inhibitors of the MAPK pathway have determined that activation of the MAPK associated signal cascade is not sufficient for the regulation of glucose transport by insulin. However, activation of these proteins has been

directly associated with several aspects of cell growth and differentiation (Saltiel, 1996; Gabbay et al., 1996; Myers et al., 1994; Lazar et al., 1995).

There are several known members of the MAPK family which have been cloned. This family includes the extracellular signal regulated kinases (ERK's) 1, 2, 3, and 5, Jun-N-terminal Kinase/Stress-activated Protein Kinase (JNK/SAPK), Fos-regulating kinase (FRK) and p38/Mpk2 (Su and Karin, 1996; Childs and Mak, 1993).

During the insulin stimulation of the signal cascade, MAPK's are known to translocate to the nucleus and regulate gene expression by phosphorylating transcription factors such as Elk-1 which regulates the expression of c-fos (Saltiel, 1996; Medema et al., 1991).

The activating signals for ERK's 1 and 2 are specific extracellular stimuli associated with cell growth and differentiation, such as serum, cytokines and growth factors. These signals are different from the signals that activate the ERK-related protein kinases, JNK, p38 and FRK (Deng and Karin, 1994).

### ERK 1/2 Activation

In order to phosphorylate and therefore activate ERK1/2, Ras must first phosphorylate three different MAPKKK's: Raf-1 (Karin, 1995; Leever et al., 1994; Jelinek et al., 1994), c-Mos and MEKK1 (Davis, 1993; Su and Karin, 1996; Avruch et al., 1994). These MAPKKK's are protein kinases that phosphorylate the MAP kinase/ErK-activating Kinases, MEK1 and MEK2 (Karin, 1995; Dent et al., 1992; Jelinek et al., 1994; Blenis, 1993). MEK1/2 are dual specific (tyrosine and ser/thr) kinases that are directly responsible for the phosphorylation of ERK1/2 (Karin, 1995; Crews et al., 1992; Avruch et al., 1994) (Figure 1).



### JNK/SAPK Activation

Ras is responsible, through Raf-1, for the activation of MEKK1. MEKK1 has been implicated in ERK1/2 activation, however, most of MEKK1 activation results in JNK phosphorylation (Minden et al., 1994). MEKK1 is a Ser/Thr kinase that activates JNKK1 (Karin, 1995; Minden et al., 1994; Lange-Carter 94, Cavigelli et al., 1995). JNKK1, also known as SEK1 or MKK4, is the kinase that phosphorylates and activates JNK (Derijard et al., 1995; Lin et al., 1995; Gerwins et al., 1997) (Figure 1).

JNK is activated mainly by stress related signals such as UV irradiation, osmotic stress, treatment with translational inhibitors, exposure to IL-1, ischemia and reperfusion (Su and Karin, 1996; Rosette and Karin, 1996; Adler et al., 1995; Morooka et al., 1995). However, JNK activation is also seen upon treatment with EGF and other hormones albeit to only about one third of the levels of ERK activation (Su and Karin, 1996).

### Insulin and Vanadate

As reviewed in the previous pages, the insulin signal cascade is a complex series of events that help target insulin action in the cell. Any insulin mimetic that could potentially be used in insulin replacement therapy must be tested with regard to its ability to similarly activate the proteins involved in the insulin signal transduction cascade.

Vanadate has been identified as an insulin mimetic in terms of metabolic processes such as glucose transport and protein synthesis. These insulin-like effects of vanadate maybe mediated through the activation of insulin signal cascade elements such as the insulin receptor  $\beta$ -subunit autophosphorylation and IRS-1 (Goldfine et al., 1995; Yale et al., 1995, Ruff et al., 1997).

In order to determine the insulin-like actions of vanadate in regulating gene expression through the insulin signal cascade, we proposed to study the insulin activation of a reporter construct that carries the serum response element (SRE) of c-fos. SRE is the cis-element used by insulin in mediating expression of this gene. If vanadate truly mimics insulin action, then it should, in a mechanistically similar fashion to insulin, increase expression of this reporter construct.

### Insulin in the Activation of c-fos

In early studies regarding activation of c-fos, it became apparent that insulin was one of many growth factors that produced a transient increase in the expression of this gene (Jhun et al., 1995; Thompson et al., 1994; Hattori et al., 1988). However, little was known regarding the cis-element responsible for the insulin induced activation of c-fos expression. Consequently, experiments were carried out in order to determine the exact sequences that respond to c-fos expression by insulin presence in the cell. It was found that mutation of four base pairs of a c-fos cis-element, later termed Serum Response Element (SRE), abolished expression of this gene by serum and insulin (O'Brien et al., 1996; Treisman, 1986). The Serum Response Element, which is located between -314 and -305 of the c-fos promoter was shown to be responsible in part for the insulin induction of c-fos (Treisman, 1986). Other studies have indicated that additional sequences present in the c-fos promoter are necessary in order to carry out a full response (Treisman, 1986; O'Brien et al., 1996). Yamauchi et al. demonstrated that an 80bp sequence surrounding the SRE region (-357 to -276) conferred a 14 fold increase in SRE expression when measured by means of the reporter gene luciferase (Yamauchi et al., 1993; de Wet et al., 1987). Another plasmid containing a shorter sequence of only the SRE region (SRE-Core) (-320 to -298) was also constructed and, upon insulin stimulation, produced only an

approximate 4-fold increase (Yamauchi et al., 1993). Yamauchi's studies demonstrate that while the SRE is capable of mediating an insulin induced increase in c-fos expression, the region surrounding the SRE is necessary for the full transcriptional response that is observed *in vivo*.

Even though, several groups have tried to determine which pathways are involved in SRE-mediated expression of c-fos by insulin, little can be concluded. These results seem to be cell specific, depending on the mitogenic or metabolic characteristics of the cells. (Olson and Pessin, 1994; Saltiel et al., 1994; Saltiel, 1996).

### Regulation of c-fos Expression: General Aspects

The expression of c-fos is now known to be regulated, at least in part, by its three known cis-elements: the cyclic AMP response element (CRE), the Serum Response Element (SRE) and the Sis-Inducible Enhancer (SIE) (Cavigelli et al., 1995). The first two are constitutively occupied in non-stimulated cells, while the third element is occupied only after specific stimulation (Cavigelli et al., 1995; Herrera, 1989) (Figure 2).

There are specific transcription factors that recognize each one of the cis-elements in the c-fos promoter. CRE is recognized by CREB and other similar transcription factors such as CREM and ATF-1 (Cavigelli et al., 1995; Mayer and Habener, 1993). CRE is responsible for the expression of c-fos when stimulated by neurotransmitters and hormones that use cAMP or  $\text{Ca}^{++}$  as second messengers. The induction of the gene at CRE is produced by activation of protein kinase A or calmodulin-dependant protein kinases (Karin, 1995; Sheng et al., 1994). The SIE is recognized by members of the transcription factor family, STAT (Cavigelli et al., 1995) (Figure 2). SRE is specifically recognized by a bi-protein complex formed by a

serum response factor (SRF) and a ternary complex factor (TCF) (Cavigelli et al., 1995; Treisman, 1994).

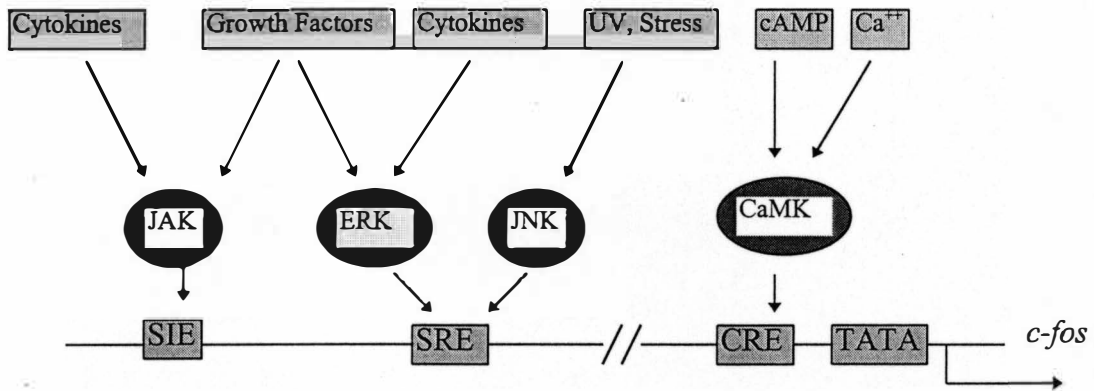


Figure 2. Regulation of *c-fos* Transcriptional Response by Diverse Extracellular Stimuli .

#### The *c-fos* Serum Response Element

The Serum Response Element (SRE) of the *c-fos* promoter contains a sequence comprised of a CArG box [CC (A/T)<sub>6</sub> GG] located near at least one DNA binding site (Gille et al., 1996; Latinkic et al., 1996). SRE initiates transcription of *c-Fos* upon binding of the Serum Response Factor (SRF) and the Ternary Complex Factor (TCF), forming a ternary complex (Treisman, 1994; Treisman, 1986) (Figure 3).

This large complex has been identified as the element that binds the extended sequences described previously in Yamauchi's studies (Yamauchi et al., 1993).

There are several models that describe the way in which the formation of the ternary complex occurs. Even though, some discrepancies exist regarding the series of events that are required for ternary complex formation, all these models agree that ternary complex occurs when: SRF becomes phosphorylated (Hill et al., 1995,

Franzoso et al., 1996), phosphorylated SRF binds to SRE (Cavigelli et al., 1995, Rivera, 1993), TCF becomes phosphorylated, phosphorylated TCF binds to SRF and its DNA binding site to complete the ternary complex (Cavigelli et al., 1995; Gille et al., 1992, Gille et al., 1995; Latinkic et al., 1996, Gille et al., 1996; Price et al., 1995).

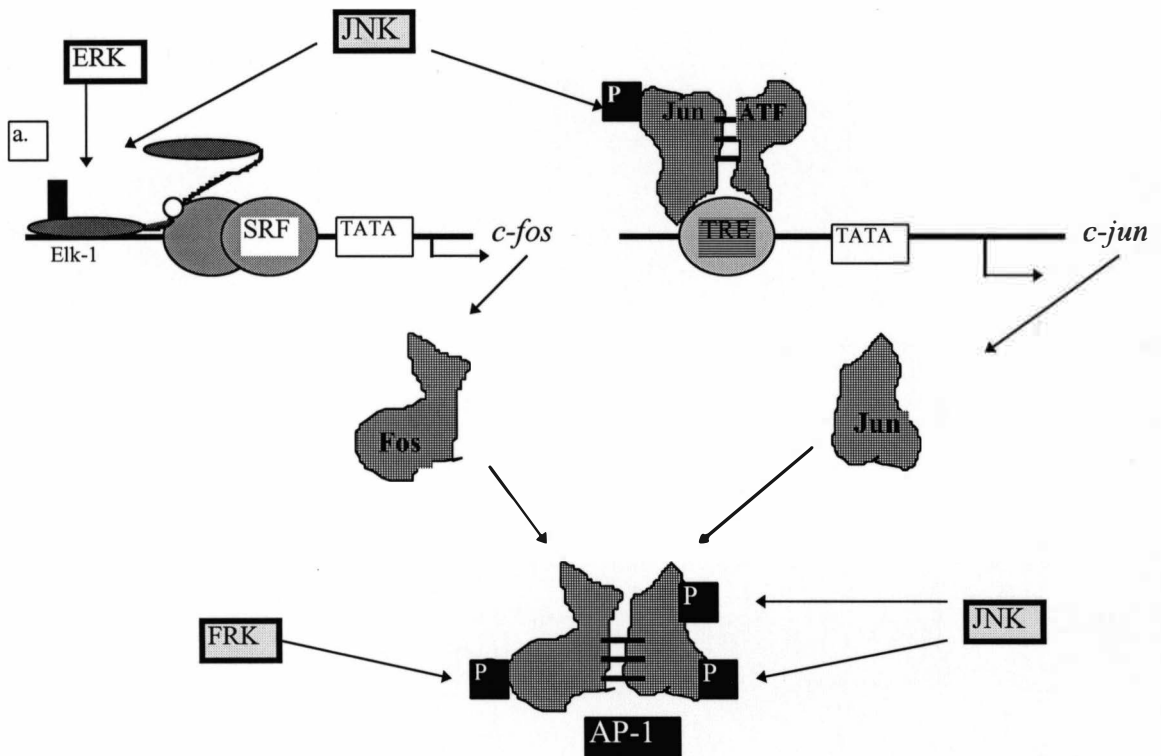


Figure 3. Activation of Fos and Jun by MAPK's. a. Representation of the ternary complex formation

SRF is rapidly phosphorylated upon mitogenic stimuli and/or stimuli associated with nuclear targets mediated by the Rho family of GTPases (Rivera, 1993; Hill et al., 1995a; Treisman, 1995). The major regulatory mechanisms in the formation of the ternary complex is therefore the phosphorylation of TCF (Cavigelli et al., 1995; Hill et al., 1994).

### Elk-1/TCF Phosphorylation

Ternary complex factor is a generic name used to describe a family of transcription factors that are required in the formation of the ternary complex in order to initiate transcription. TCF requires binding of SRF and SRE to form the ternary complex (Treisman, 1995).

Most of the TCF's are members of a family of transcription factors, which include Elk-1, SAP-1, and NET-1/ERP/SAP-2 (Whitmarsh et al., 1995; Latinkic et al., 1996). The first TCF to be identified and also the best studied is Elk-1 (Cavigelli et al., 1995; Hipskind, 1991). Elk-1 has several phosphorylation sites by its c-terminal domain, these phosphorylation sites are conserved in many of the TCF's (Cavigelli et al., 1995; Gille et al., 1992; Karin, 1995; Treisman 1994, Price et al., 1995; Latinkic et al., 1996).

Several *in vitro* studies have shown that the proteins responsible for the phosphorylation of Elk-1 are ERK1 and ERK2 MAPK's. Purified, these MAPK's phosphorylate Elk-1 in the exact same phosphorylation sites where Elk-1 is phosphorylated upon extracellular mitogenic stimuli *in vivo* (Cavigelli et al., 1995; Gille et al., 1992, 1995; Marais, 1993; Zinck, 1993; Whitmarsh et al., 1995; Cano et al., 1995; Price et al., 1995) (Figure.3).

Another member of the MAPK family, the Jun-N-terminal Kinase (JNK) is also known to phosphorylate Elk-1 in order to induce SRE-mediated c-fos expression, but in response to different stimuli, for example UV irradiation (Cavigelli et al., 1995; Derijard et al., 1994; Whitmarsh et al., 1995) (Figure 3).

## Objectives of the Study

If we are to fully understand the action of vanadate in terms of mimicking insulin, we need to address the issue of whether or not vanadate can similarly stimulate the expression of a well-characterized mitogenic gene, c-fos, through a well-characterized insulin response element, the SRE. For that purpose, we obtained the SRE-Luc construct utilized by Yamauchi et al., in the study described above (Yamauchi et al., 1993).

We hoped to link activation of part of the insulin signal cascade to the insulin activation of this construct. We then planned to similarly test vanadate to not only activate the expression of the SRE-Luc construct but also to link this activation to the same signal proteins used by insulin. To do so, several inhibitors of the MAPK and PI3K signal cascades were used to block one signal cascade at a time and show that this pathway was utilized by insulin and/or vanadate in the activation of SRE-mediated c-fos gene expression in primary rat hepatocytes in culture.

Additionally, we wanted to determine the effect of insulin and vanadate on the activity of JNK, since the Jun-N-terminal Kinase (JNK) is implicated in the phosphorylation of Elk-1, a necessary step in ternary complex formation and activation of SRE-mediated c-fos expression.

## CHAPTER II

### MATERIALS AND METHODS

#### Materials

The SRE-Luc plasmid (Yamauchi et al., 1993) was a generous gift of Dr. Jeffrey Pessin, Department of Physiology and Biophysics, The University of Iowa, College of Medicine, Iowa City, Iowa.

Male Sprague-Dawley rats were obtained from Harlen Sprague-Dawley (Kalamazoo, MI). Waymouth's MB 752/1 medium, Lipofectin, restriction enzymes and gentamicin were purchased from Gibco BRL (Grand Island, NY). DNase-free RNase and Collagenase D were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Phenol was purchased from Amresco (Solon, OH). Insulin was a gift from Eli Lilly Corp. (Indianapolis, IN). Rat tail collagen (type IV) was obtained from Collaborative Research Incorporated (Bedford, MA). SAPK/JNK and Luciferase Assay Kits were purchased from New England Biolabs (Beverly, MA) and Promega (Madison, WI), respectively. Hyperfilm was purchased from Amersham Life Science (Arlington Heights, IL). Nucleobond AX kit for plasmid purification was purchased from The Nest Group (Southboro, MA).

The MEK inhibitor PD 98059 was generously provided by Dr. Alan Saltiel, Parke-Davis Warner Lambert Pharmaceutical Research Division, Ann Arbor, MI. The PI-3 kinase inhibitor wortmannin was purchased from Sigma Chemical Corp. (St. Louis, MO). Farnesyltransferase inhibitor B581 and a PI-3 kinase inhibitor LY 29004 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The FRAP inhibitor Rapamycin was purchased from Calbiochem (San Diego, CA).



All other reagents were obtained from Sigma Chemical Corp. (St. Louis, MO).

### Extraction and Purification of Plasmid DNA

SRE-Luc transformed *E. coli* DH5- $\alpha$  bacterial cells were grown in 1L of LB medium (1L=10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl) overnight at 37 °C. The bacterial cells were harvested at 2000xg for 30 min at 4 °C. The pellet was lysed by the alkali method as described by Sambrook, Fritsch and Maniatis (Sambrook, Fritsch and Maniatis, 1989) by first, resuspending it in 10 ml of solution A (50 mM glucose, 25mM Tris.HCl, pH 8.0, 10mM EDTA, 5mg/ml lysozyme) and then, subsequently in 20 ml of solution B (0.2 N NaOH, 1% SDS) and 15 ml of solution C (3M potassium acetate and glacial acetic acid). The bacterial lysate was centrifuged at 15000xg for 30 min at 4 °C. Nucleic acids were precipitated by isopropanol and RNA degraded by incubation with DNase-free RNase (2000 U/ml activity). Further purification was obtained by phenol-chloroform extraction. The plasmid DNA was precipitated by adding ammonium acetate (final concentration 2M) and 100% ethanol. Plasmid was visualized by 1% agarose gel electrophoresis. Final purification of the plasmid was carried out by an ion-exchange chromatography column using the Nucleobond AX kit (The Nest Group, Southboro, MA). The concentration of the plasmid was determined by UV-Vis spectrophotometry (absorbances 260nm and 280nm).

### SRE-Luc Restriction Digestion

Once the plasmid was isolated, a series of restriction digestions were carried out to confirm the DNA insert. The restriction enzymes BamHI (10 U/ $\mu$ l, Gibco BRL) and HindIII (10 U/ $\mu$ l, Gibco BRL) were used to cleave the plasmid in three

places. The fragments were compared to the original plasmid map (Figure 4) and subsequently, electrophoresed and separated by size (Figure 5).

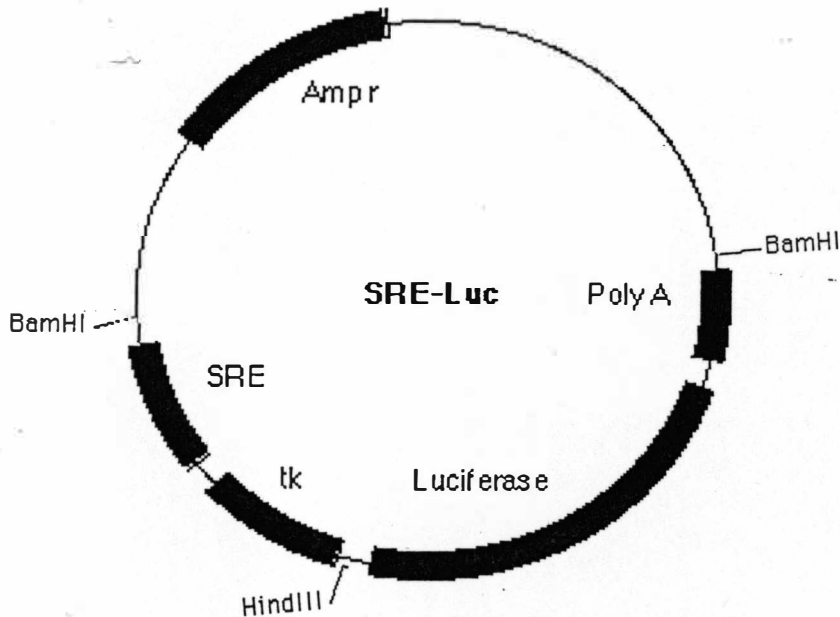


Figure 4. SRE-Luc Plasmid (5.5kb). Representation of plasmid containing a dyad of the SRE-c-fos, the tk promoter, and the luciferase gene.

#### Hepatocyte Isolation and Maintenance

Hepatocytes were isolated by the collagenase-hyaluronidase perfusion method as described by Stapleton et al. (Stapleton et al., 1993).

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Kalamazoo, MI) between 200 and 300 g were food-deprived 48h prior to hepatocyte isolation in order to minimize blood insulin levels. Rats were anesthetized with 50 mg/ml pentobarbital. These procedures conformed to NIH guidelines for animal care and use and were approved by IACUC (refer to appendix B). After the collagenase-

hyaluronidase perfusion, the liver was removed from the animal and passed through four layers of gauze for its dissociation into cells.

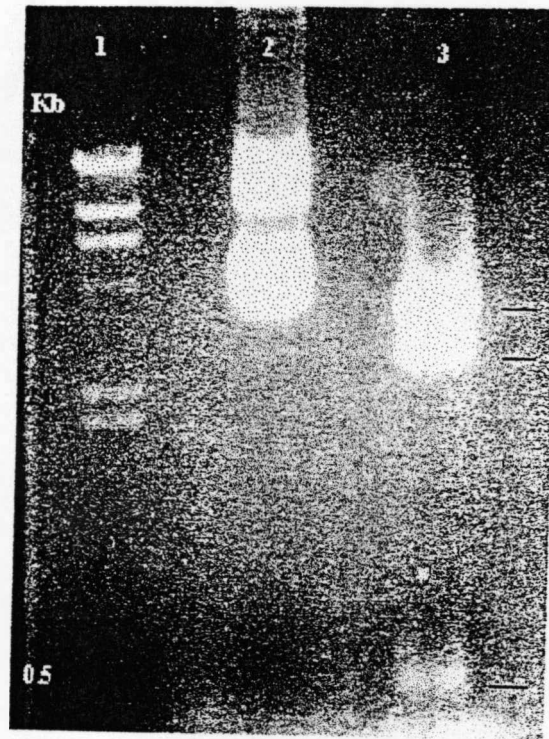


Figure 5. Restriction Digestion of the SRE-Luc Plasmid. The figure indicates the approximate size in Kb of the DNA fragments compared to a DNA ladder. Lane 1. represents the 1 Kb DNA ladder. Lane 2. shows the SRE-Luc plasmid prior to digestion. Lane 3. indicates the plasmid subjected to restriction digestion as indicated in Materials and Methods. The lines beside lane 3 indicate the position of the fragments.

The cells were washed twice with cold-serum free Waymouth's MB 752/1 medium (Gibco BRL, Grand Island, NY) plus 0.5% bovine serum albumin (BSA). This high protein content medium decreased cellular stress caused by the isolation procedure. The cells were then centrifuged for 3 min @ 4 °C and 50xg. The pellet was gently resuspended in medium and an aliquot of cells was counted with a hemocytometer. Cell viability was determined by trypan blue dye exclusion. Collagen coated 60mm tissue culture dishes were plated with  $3.0 \times 10^6$  cells/plate, if

cell were greater than 80% viable. The cells were incubated @ 37 °C in serum free Waymouth's MB 752/1 medium supplemented with gentamicin (10µg/ml) under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. After about 4 hours of incubation, a time considered adequate for the cells to have stabilized and adhered to the plates, the hepatocytes were washed and fresh medium without BSA applied. After cell stabilization and adhesion, it was necessary to removed BSA from the medium due to its random binding properties, which could alter the parameters of the experiment .

#### Cell Treatment for SRE-Luc Transfection

Approximately four hours after the last medium change, five micrograms of SRE-Luc plasmid per plate was transiently transfected using Lipofectin (Gibco BRL) (Rank et al., 1994). After a 12-hour transfection period, cells were simultaneously treated with either nothing (control), 500nM insulin, 500µM sodium orthovanadate and/or respective inhibitors. The concentration used for insulin, vanadate and each one of the inhibitors were based either on our previous studies or as a result of others documented in the literature (Sutherland et al., 1995, Cox et al., 1994; Vlahos et al., 1994). After incubation, the cells were harvested in lysate buffer provided in the luciferase assay kit (Promega). Luciferase activity was measured following the protocol in the Promega kit and activity was quantified in a liquid scintillation counter. Protein levels were measured using the Lowry method (Lowry et al., 1951).

#### Cell Treatment for Determination of JNK Activation

Preliminary studies carried out in our laboratory suggested that the optimal times for JNK activation upon insulin and sodium orthovanadate treatments, were 10 minutes and 1 hour respectively (data not published). After 10 min of insulin (500nM) and 1h of sodium orthovanadate (500µM) treatment, cells were washed

twice with ice-cold PBS. Cells were scraped and processed in the buffer provided by the JNK assay kit (New England Biolabs, Beverly, MA). The cell extracts were sonicated four times for five seconds each using level 5 on a 50 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) and centrifuged at 11000xg for five minutes @ 4 °C. The supernatant was used to carry out the JNK assays.

### JNK Assay and Western Analysis Detection

The protein JNK was assayed in cell extracts that were incubated overnight with c-Jun fusion protein beads provided in the JNK assay kit. After overnight incubation, the samples were microcentrifuged for two minutes @ 4 °C. The pellets were washed once with lysis buffer and once with kinase buffer, both of them provided in the JNK assay kit. ATP (100μM) was added to the samples in order to initiate the phosphorylation reaction. The reaction was terminated by 3X SDS sample buffer (1X=62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT, 0.1 bromophenol blue). The samples were then boiled for 5 min and microcentrifuged for 2 min in order to release the beads. The supernatant was electrophoresed in a 10% polyacrylamide gel and transferred to a PVDF membrane using a standard semi-dry electroblotting apparatus (NOVEX, San Diego, CA).

After transfer, the membrane was blocked for 1 to 3h with blocking buffer (1X Tris-Buffered Saline, 0.1% Tween-20 with 5% non fat dry milk). The membrane was then probed with phospho-specific c-Jun antibody (1:1000 dilution, provided in the JNK assay kit) in primary antibody dilution buffer (1X TBS, 0.05% Tween-20 with 5% BSA) overnight @ 4 °C. The membrane was washed several times with Tris-buffered saline plus Tween 20 (TBST 1X, 1L=2.42g of 20mM Tris Base; 8g of 137mM NaCl; 3.8ml of 1M HCl; and 1ml Tween 20) prior to incubation for one hour

with two antibodies: HRP-conjugated anti-rabbit secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000, to detect biotinylated markers).

Detection of the phosphorylated proteins was carried out by incubation in 1X LumiGlo solution of the Phosphotope-HRP system provided in the JNK assay kit. The membrane was exposed to Hyperfilm (Amersham Life Science, Arlington Heights, IL) and bands were quantified using an optical densitometer.

### Statistical Analysis

The results are expressed as the mean  $\pm$  S. E. Significance was evaluated by one-tailed Student's t-test and tested at  $p < 0.05$ .

## CHAPTER III

### RESULTS

#### Restriction Digestion of SRE-Luc Plasmid

The plasmid was amplified and isolated as described in Materials and Methods. According to the restriction map, the SRE-Luc plasmid contained two restriction sites for BamHI and one for HindIII, (Figure 4). After digestion with these restriction enzymes, the fragments were subjected to electrophoresis in a 1% agarose gel. Three DNA fragments were visualized by placing the gel under UV light after ethidium bromide staining (Figure 5). By comparing the sizes of the restriction fragments to a DNA ladder, the estimated sizes of the restriction fragments were 0.5, 2.0 and 3.0kb. The fragments corresponded to the plasmid portions that contained the tyrosine kinase promoter plus an SRE dyad (432bp), the luciferase gene (1811bp) and the remainder of the vector (2.8kb) (Yamauchi et al., 1993; Chen et al., 1987; de Wet et al., 1987). Therefore, the restriction map analysis confirmed the SRE-Luc plasmid reported by Yamauchi et al.

#### Effect of Insulin and Vanadate on SRE-Luc Expression

In order to determine the specificity of insulin induction of SRE-Luc in primary rat hepatocytes in culture, the cells were first transiently transfected with the plasmid, then incubated with insulin (500nM) for the following times: 5 min, 30 min, 1h, 3h, 6h, and 12h.

A significant insulin-induced increase in expression of the plasmid as measured by an increase in luciferase activity was not observed prior to 1h of incubation with insulin (Figure 6).

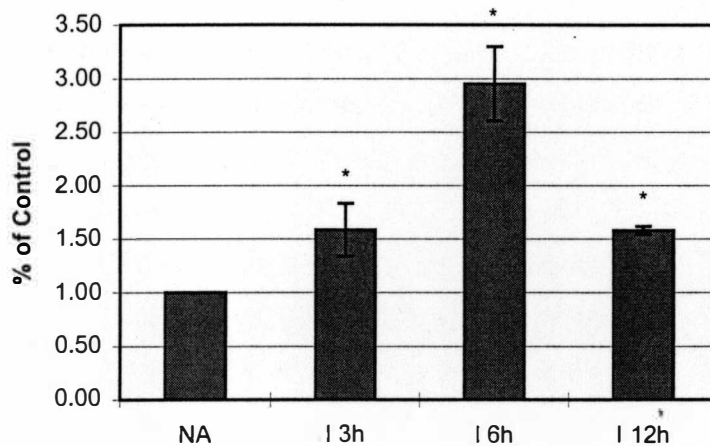


Figure 6. Effect of Insulin on SRE-Luc. Primary rat hepatocytes in culture were treated with nothing (control or NA) or insulin (500nM) for the SRE-Luc. Values represent the means $\pm$ S.E. Asterisk (\*) indicates significant stimulation ( $p < 0.05$ ). Minimum experiment per condition=3, except 1 12h where n=2.

As shown in Figure 6, the insulin-induced increase over control in SRE-Luc expression reached approximately 1.6 fold at 3h. Incubation of insulin for 6h resulted in approximately a 3 fold increase of SRE-Luc over non-stimulated control. A later incubation time of 12h showed a considerable decrease in the expression of the plasmid, suggesting that the insulin-induced expression of SRE-Luc was transient (Figure 6).

In order to determine whether or not vanadate could mimic the insulin induction of SRE-Luc in these cells, we incubated transfected cells with sodium vanadate (500 $\mu$ M) for 1h, 3h and 6h. As summarized in table 1, incubation of



hepatocytes with vanadate did not induce the expression of transiently transfected SRE-Luc to any significant extent for any of the time points analyzed.

Table 1

Effect of Vanadate on SRE-Luc in Primary Rat Hepatocytes

<i>Treatment</i>	<i>SRE-Luc Levels as % of Control</i>
<i>Control</i>	100
<i>V 1h</i>	1.07 $\pm$ 0.49
<i>V 3h</i>	1.11 $\pm$ 0.35
<i>V 6h</i>	0.98 $\pm$ 0.23

Primary rat hepatocytes in culture received no treatment (control) or were treated with vanadate (V) (500 $\mu$ M) for the times indicated. The control of each experiment was arbitrarily set to 100 and the change by treatment with vanadate was expressed compared to the control value. Data shown are the means $\pm$ S.E. \*=significantly different from control,  $p < 0.05$ .  $n = 4$ .

In conclusion, these studies suggest that activation of SRE-Luc expression in primary rat hepatocytes in culture by insulin occurs in a time-dependent fashion with a maximum increase observed at 6h of incubation. Surprisingly, vanadate, a known insulin mimetic, did not induce the activation of SRE-Luc expression at any of the time points considered. These results suggest that vanadate does not mimic insulin with regard to the regulation of gene expression through an SRE.

## Effect of Inhibitors of the Insulin Signal Transduction Pathways on Insulin-induced SRE-Luc Expression

In order to determine the role of insulin signal transduction proteins in the insulin activation of SRE-Luc expression, we used several inhibitors of the PI3K and MAPK pathways.

### Inhibitors of PI3K Activation: Wortmannin and LY 294002

Wortmannin (WT) is a fungal metabolite that inhibits the kinase activity of PI3K by covalently binding to the catalytic subunit of the enzyme (Ui et al., 1995; Norman et al., 1996). This inhibitor becomes unstable at room temperature after 2h of incubation at pH 7.0 (Norman et al., 1996). Studies utilizing the inhibitor for longer incubation times require re-addition of WT to the incubation medium every two hours (Shepherd et al., 1995).

As shown in Figure 7, wortmannin (100 $\mu$ M) completely inhibited the insulin-induced expression of SRE-Luc.

Since WT has been reported to inhibit the PI3K pathway in a non-specific fashion, a mechanistically different and more specific inhibitor of PI3K, LY 294002, was also used (Vlahos et al., 1994). LY 294002 inhibits PI3K by specifically interacting with the ATP-binding site of the enzyme (Vlahos et al., 1994). As shown in Figure 7, LY 294002 (50 $\mu$ M) also completely inhibited the insulin-induced SRE-Luc expression.

Since both, WT and LY 294002 were effective inhibitors of the insulin-induced SRE-Luc expression, these results suggest that activation of PI3K is a necessary step in the expression of this plasmid by insulin in primary rat hepatocytes.

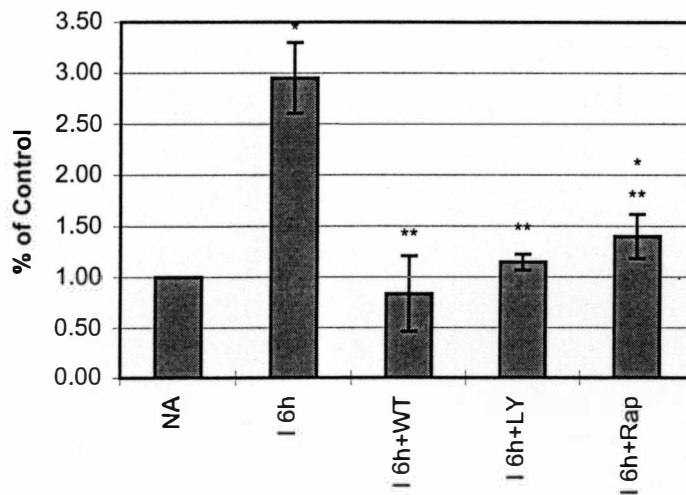


Figure 7. Effect of Insulin and Inhibitors of the PI3K Cascade on SRE-Luc Expression. Primary rat hepatocytes in culture were treated with nothing (control / NA) or insulin (500nM) plus the designated inhibitor of the PI3K cascade. Values represent the means±S.E. Asterisk (\*) indicates significant stimulation ( $p < 0.05$ ). Double asterisk (\*\*) indicates significant inhibition ( $p < 0.05$ ). Minimum experiment per condition= n= 3.

#### Rapamycin: Inhibition of FRAP Activation

FKBP-Rapamycin-Associated Protein (FRAP) is a downstream effector of PI3K that is, at least in part, responsible for the activation of p70S<sup>6</sup>Kinase (Choi et al., 1996; Shepherd et al., 1995; Brown et al., 1995; Monfar et al., 1995).

In order to determine the role of FRAP in the activation of SRE-Luc expression by insulin, the immunosuppressant rapamycin was used (Price et al., 1992; Choi et al., 1996). Rapamycin is an inhibitor of FRAP that interacts at a hydrophobic pocket within the protein and blocks its activation (Choi et al., 1996).

As shown in Figure 7, rapamycin (100nm) inhibited the insulin-induced SRE-Luc expression. The 3.0 fold insulin-induced increase in expression of SRE-Luc was

decreased to 1.40 fold upon rapamycin treatment (100nM). This represented a partial inhibition of the insulin-induced SRE-Luc expression.

These results suggest that the events responsible for the insulin induced-expression of SRE-Luc require, at least in part, an activated FRAP.

#### B581: Inhibition of Ras Activation

In order for Ras to obtain an adequate conformation necessary for its membrane interaction and activation, this protein must undergo a series of post-translational modifications. Ras post-translational modifications, which include proteolysis, methylation and farnesylation, are triggered by a consensus COOH-terminal CAAX motif (A=aliphatic residue) (Cox et al., 1994). In several cell types, B581 has been shown to act at the CAAX motif of farnesylated Ras causing inhibition of Ras activation (Gabbay et al., 1996; Cox et al., 1994).

As shown in Figure 8, B581 (50 $\mu$ M) partially inhibits the SRE-Luc induced expression by insulin. The 3.0 fold increase of SRE-Luc expression by insulin was slightly decreased to 2.5 fold upon B581 treatment.

#### Inhibition of MEK Activation by PD 98059

The MAP kinase/ErK-activating Kinases (MEK's) are downstream effectors of Ras that are implicated in the activation of ERK1/2 MAPK's (Karin, 1995; Crews et al., 1992; Avruch et al., 1994) (Figure.1). We utilized PD 98059 to determine the role of these dual specificity (tyr and ser/thr) kinases in the expression of insulin-induced SRE-Luc (Lazar et al., 1995). PD 98059 is a specific inhibitor of MEK that operates non-competitively with respect to ATP binding of MEK.

As shown in Figure 8, the 3.0 fold insulin-induced increase in expression of SRE-Luc was decreased to 1.40 fold upon PD 98059 treatment. These results suggest

that the signal responsible for the insulin-induced expression of SRE-Luc, requires an activated MEK.

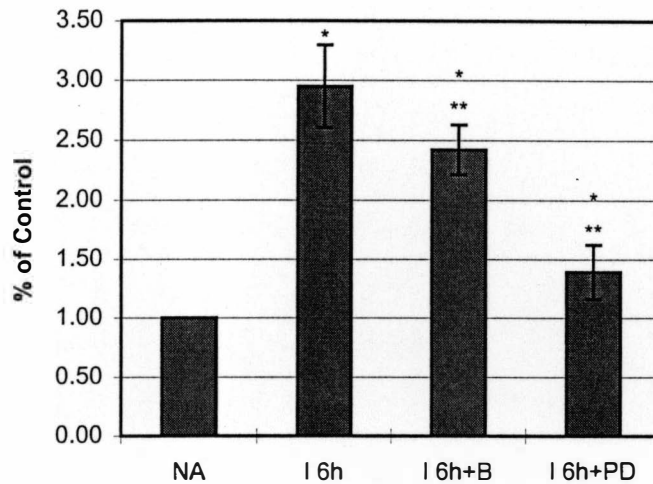


Figure 8. Effect of Insulin and Inhibitors of the MAPK Cascade on SRE-Luc Expression. Primary rat hepatocytes in culture were treated with nothing (control) or insulin (500nM) plus the designated inhibitor of the MAPK cascade. Values represent the means  $\pm$  S.E. Asterisk (\*) indicates significant stimulation ( $p < 0.05$ ). Double asterisk (\*\*) indicates significant inhibition.  $n = 4$ .

#### Effect of Insulin and Vanadate on JNK Activation

The Jun-N-terminal Kinase (JNK) and ERK1/2 are two members of the MAPK family that are implicated in the phosphorylation of ternary complex factors, such as Elk-1 (Cavigelli et al., 1995; Derijard et al., 1994; Whitmarsh et al., 1995) (Figure 3). Elk-1 phosphorylation is a necessary step in ternary complex formation and subsequent activation of SRE-mediated c-fos expression (Cavigelli et al., 1995; Gille et al., 1992, Gille et al., 1995). Cano et al. have suggested that Elk-1 activation requires both JNK and ERK MAPK's to elicit a complete nuclear response (Cano et al., 1995). Our laboratory have shown that insulin and vanadate are capable of activating ERK1/2 MAPK's in primary rat hepatocytes (data not published). These

data is in agreement with our data in where inhibition of MEK also causes inhibition of SRE-Luc expression. Since we have demonstrated that part of the signal used by insulin in inducing SRE-Luc may require Ras and most likely MEK activation, it seemed logical to evaluate next the effect of insulin and vanadate on JNK.

In order to determine the effect of insulin and vanadate on the activation of JNK, JNK assays were carried out as described in Materials and Methods.

Miller et al. reported peak JNK activation 15 min after insulin treatment in Rat 1 fibroblasts overexpressing human insulin receptors (Miller et al., 1996). We measured only a small increase of JNK activation 10 min after treatment with insulin. After 6h of incubation with insulin, a 1.40 fold increase over unstimulated control was observed (Table 2). This represented a statistically significant activation of JNK over no addition. However, it is unclear if this increase in JNK activation is significant in the mediation of the insulin-induced SRE-Luc expression.

In order to evaluate the effect of vanadate (500 $\mu$ M) on JNK phosphorylation, JNK activation was measured after 3h of incubation with the mimetic. As shown in Table 2, vanadate did not increase JNK activation to any significant extent.

To determine if JNK was possibly involved in mediating the insulin-induced SRE-Luc, as well as evaluating for cross-talk between the PI3K and MAPK cascades, WT and B581 were tested. As shown in Table 2, both B581 and WT, were effective inhibitors of JNK activation by insulin. These results suggest that insulin-induced JNK activity may require activation of Ras and PI3K.

Table 2

Effect of Vanadate, Insulin and Insulin Plus Inhibitors on JNK Activation  
in Primary Rat Hepatocytes

<i>Treatment</i>	<i>JNK Activation as % of Control</i>
<i>Control</i>	100
<i>V 3h</i>	1.28±0.2
<i>I 6h</i>	1.40±0.08*
<i>I6h+WT</i>	0.82±0.20**
<i>I6h+B</i>	0.83±0.16**

Primary rat hepatocytes in culture received no treatment (control) or were treated with vanadate (500μM), insulin (I) (500nM), wortmannin (WT) (100μM), LY 294002 (LY) (50μM), rapamycin (Rap) (100nM), B581 (50μM) or PD 98059 (PD) (20μM) as indicated. The control of each experiment was arbitrarily set to 100 and the change by treatment with vanadate, insulin or inhibitors was expressed compared to the control value. Data shown are the means±S.E. \*=significant activation, \*\*=significant inhibition. p<0.05. Minimum experiment per condition =n=3.

## CHAPTER IV

### DISCUSSION

The purpose of this study has been to determine the issue of whether or not vanadate can stimulate the expression of a well-characterized mitogenic gene, *c-fos*, using a signal transduction mechanism similar to insulin.

Vanadate has been shown to mimic several of insulin's actions (Posner, et al., 1994; Goldfine et al., 1995; Tamura et al., 1984). However, the intracellular aspects of vanadate action has not been clearly established. Therefore, before considering vanadate as a drug replacement therapeutic agent for diabetes, it is relevant to establish its effects on proteins associated with the insulin signal transduction cascades.

To accomplish our goal, we carried out experiments using a two-fold approach. First, transiently transfected primary rat hepatocytes in culture were treated with insulin and vanadate at various times in order to determine the effect of these compounds on SRE-mediated gene expression. Second, several inhibitors of the MAPK and PI3K signaling cascades were used in order to determine the possible signaling pathway(s) used by insulin and vanadate.

The SRE-Luc plasmid obtained from Dr. Jeffrey Pessin's Laboratory is an effective reporter of some of insulin's action in the cell (Yamauchi et al., 1993). The 5500bp-long construct contained a dyad (44bp) of symmetric SRE cis-elements that mediate the expression of the construct upon serum and insulin presence (Treisman, 1986; O'Brien et al., 1996). This dyad is surrounded by 59bps on each side, which are necessary for the full transcriptional response (Yamauchi et al., 1993). The SRE-



Luc dyad is linked to a thymidine kinase (tk) promoter (270bp) (Chen et al., 1987) and a luciferase gene (1811bp), as described by de Wet et al. (de Wet et al., 1987).

The SRE-Luc construct had been previously tested with regard to insulin induction in Chinese hamster ovary cells expressing high levels of the insulin receptor (CHO-IR) (Yamauchi et al., 1993). Yamauchi et al. reported a maximum increase of 14-fold of SRE-Luc observed between 4h to 6h of insulin treatment (Yamauchi et al., 1993). In our model, primary rat hepatocytes, SRE-Luc showed a 3-fold maximum increase between 3h and 6h of insulin treatment.

The higher plasmid expression levels observed in CHO-IR upon insulin induction represents amplified signals of multiple individual insulin receptor molecules enhancing the activation of the signal cascade proteins. These multiple insulin receptors are not present in the plasma membranes of the primary rat hepatocytes. Since primary rat hepatocytes closely resemble the liver and its actions as a primary target organ of insulin induction, the insulin-induced SRE-Luc expression levels observed in the hepatocytes better resemble the levels observed *in vivo*. Consequently, it was expected that the plasmid expression levels in CHO-IR and primary hepatocytes would differ. However, it was interesting to note that in both models, the incubation time for maximum SRE-Luc expression caused by insulin was very similar. In both, hepatocytes and CHO-IR, the maximum insulin-induced plasmid expression was observed between 4h and 6h. Therefore, in both models, insulin activates mechanisms of protein phosphorylation and gene expression within a similar time frame.

Since vanadate has been shown to activate the insulin signal cascades in response to metabolic effects, such as glucose transport and protein synthesis (Osamu et al., 1990; Balon et al., 1995; Goldfine et al., 1995; Yale et al., 1995), we decided instead to test the ability of vanadate to induce, in a fashion similar to insulin, the

expression of the SRE-driven reporter plasmid. Our results show that, even though, insulin (500nM) induced SRE-Luc expression, vanadate (500 $\mu$ M) did not induce expression of the plasmid to any significant extent. These results suggest that if vanadate induces the activation of the c-fos gene *in vivo*, it operates through a different mechanism than insulin. This has also been proposed by Chen and Chan who reported that vanadate and insulin induced c-fos expression in 3T3-L1 cells by stimulation of different cis-elements (Chen and Chan, 1993). Still others (Wang et al., 1994) suggest that differences in insulin and vanadate actions are due to differences in phosphorylation of signal molecules. However, irrespective of the possible mechanisms of action of insulin and vanadate, our results indicate that, in primary rat hepatocytes, vanadate does not induce gene expression through an SRE as insulin does. These results imply that vanadate does not trigger the same events as insulin.

Once we had determined that insulin induced the expression of the reporter plasmid through SRE, we were interested in determining the signals behind this activation.

In order to determine which proteins were activated by insulin-induced expression of SRE-Luc in our model, and consequently, the possible pathway(s) used in this process, we used several known inhibitors of the MAPK and PI3K signal cascade proteins. The level of SRE-Luc expression measured after treatment with insulin and the inhibitors helped characterize the role of the proteins in the signaling mechanism of insulin.

We found that two mechanistically different inhibitors of PI3K, WT and LY 294002, completely blocked the insulin-induced SRE-Luc expression in primary rat hepatocytes. Both of these inhibitors, in the presence of insulin, returned SRE-Luc

expression to no addition levels; which suggests that activation of PI3K is a necessary step in the expression of this plasmid by insulin in primary rat hepatocytes.

Similarly, treatment with the MEK inhibitor PD 98059, resulted in a significant inhibition of insulin-induced SRE-Luc expression, which suggests that the insulin-induced SRE-Luc expression in rat hepatocytes may require not only PI3K but also the activation of the MAPK pathway. Interestingly, we also found that inhibition of Ras activation by B581 resulted in inhibition of the insulin-induced plasmid expression. This suggests an interaction between the Ras/MAPK and PI3K signaling pathways. The involvement of phosphatidylinositol 3-kinase in the activation of Ras and similar proteins of the MAPK signaling cascade has been examined by several studies with results similar to ours (Hawes et al., 1996; Hu et al., 1995; Cross et al., 1994). For example, Hawes et al. established that wortmannin and LY 294002, inhibitors of PI3K, completely blocked MAPK activity (Hawes et al., 1996). These studies, however, differ with regard to the exact site of action of PI3K in the MAPK cascade. There are several possibilities which have been considered, however, these differences seem to be also related to cell specificity.

The data we collected suggests that PI3K is necessary for the insulin-induced expression of SRE-Luc. However, since the expression of SRE-Luc is only slightly reduced by inhibition of Ras and almost completely blocked by inhibition of MEK, it could be suggested that PI3K is located somewhere between Ras and MEK. This relationship has been considered previously by Suga et al., who has recently reported that insulin-activated PI3K is involved in the interaction between Ras and Raf-1 in 3T3-L1 cells (Suga et al., 1997). Some studies have also reported that in muscle cells, insulin and IGF-1 induced gene expression by activating proteins involved in the PI3K and MAPK cascades. Gene expression, in muscle cells, required PI3K activation which in turn, activated both the MAPK and PI3K pathways (Tansey et al.

1996, Cross et al., 1994). This relationship has been reported by several other studies that increasingly suggest crosstalk between the MAPK and PI3K cascades (Tansey et al., 1996; Band and Posner, 1997).

Even though, several studies have suggested interaction between PI3K and Ras/MAPK (Jhun et al. 1994a, Suga et al. 1997; Yamauchi et al. 1993; Cross et al., 1994; Welsh et al., 1994; Hu et al., 1995; Hawes et al., 1996; Standaert et al., 1995), several other studies have suggested that PI3K and Ras/MAPK pathways do not interact with each other. This difference in observations can be reconciled by considering that several of the studies that show no interaction between PI3K and Ras, have used different hormones which act on different target organs with different specificity (Harada et al., 1995; Yamamoto-Honda et al., 1995; Gabbay et al., 1996).

FRAP, a downstream effector of PI3K has been reported to participate in the activation of p70S<sup>6</sup>Kinase (Choi et al., 1996; Shepherd et al., 1995; Brown et al., 1995; Monfar et al., 1995). Addition of rapamycin, a FRAP inhibitor, partially blocked the insulin-induced expression of SRE-Luc. Since FRAP binding to p70S<sup>6</sup>Kinase is a required step for p70S<sup>6</sup>Kinase activation, these results suggest the involvement of FRAP, p70S<sup>6</sup>Kinase or both in the expression of SRE-Luc by insulin (Brown et al., 1995).

The proteins that participate in the activation of p70S<sup>6</sup>Kinase are not well-characterized. However, Monfar et al. determined that at least, two possible unrelated pathways could be responsible for the activation of p70S<sup>6</sup>Kinase. One of them is rapamycin-sensitive and the other is wortmannin-sensitive (Monfar et al., 1995). The two pathways act independently for the activation of p70S<sup>6</sup>Kinase. These results and others from similar studies could be used to explain the significant, but partial decrease in insulin-induced SRE-Luc expression observed in our model upon

treatment with rapamycin (Banerjee et al., 1990; Shepherd et al., 1995; Monfar et al., 1995).

Since our laboratory previously showed that insulin and vanadate were capable of activating ERK1/2 MAPK's in primary rat hepatocytes (data not published), we decided it was important to assess their effect on JNK.

JNK, a member of the MAPK family has been implicated in the phosphorylation of c-Jun, which is a necessary step in AP-1 post-transcriptional modification. JNK as well as ERK1/2 have been implicated in the phosphorylation of the ternary complex factor Elk-1 (Cavigelli et al., 1995; Derijard et al., 1994; Whitmarsh et al., 1995), which is required for ternary complex formation and activation of SRE-mediated c-fos expression (Cavigelli et al., 1995; Gille et al., 1992, Gille et al., 1995). Cano et al. have determined that in some cell models Elk-1 activation requires both JNK and ERK MAPK's to elicit a complete nuclear response (Cano et al., 1995). These observations determined the second aspect of our study: assessment of JNK activation by insulin and vanadate in primary rat hepatocytes.

Miller et al. reported peak JNK activation (2.5 fold increase over unstimulated controls) 15 min after insulin treatment in Rat 1 fibroblasts overexpressing human insulin receptors (Miller et al., 1996). These activation levels, however, were not compatible with the activation levels we observed by insulin treatment in primary rat hepatocytes. In the hepatocytes, we measured only a small increase of JNK activation 10 min after treatment with insulin. The maximum activation of 1.40 fold was observed after 6h.

Some observations by Lopez-Ilasaca et al., suggested that activation of PI3K is a requirement for the activation of JNK by some growth hormones such as insulin (Lopez-Ilasaca et al., 1997). We decided to determine the relationship between PI3K, Ras and JNK, by using the inhibitors B581 and WT. We found that the use of Ras

and PI3K inhibitors decreased the activation of JNK by insulin. These findings are in agreement with the observations of Lopez-Illasaca et al., and complement our previous results that suggested that activation of both PI3K and Ras were involved in insulin induction of this plasmid expression.

Unlike insulin, vanadate did not increase JNK activation to any significant extent. Since in our laboratory, vanadate has shown activation of ERK1/2 MAPK's (data not published), it can be suggested that, in mediating its insulin-like effects, this element activates ERK1/2 and not JNK. It may also be suggested, since insulin induced JNK activity and not vanadate, that JNK is required for the induction of SRE-Luc.

The results obtained by determining the insulin and vanadate activation of JNK in primary rat hepatocytes suggested, once again, that vanadate and insulin did not operate in a similar manner with regard to the insulin signal proteins that they activate.

This study showed that insulin increased expression of SRE-Luc in a time-dependent fashion while vanadate did not. Use of inhibitors of the PI3K and MAPK pathways suggested that PI3K is necessary and the protein maybe located somewhere between Ras and MEK for the insulin-induced expression of this plasmid. The use of inhibitors also helped determine that MAPK pathway proteins maybe involved in the insulin-induced expression of the plasmid.

Additionally, since only insulin treatment resulted in JNK activation our results show yet another way that insulin and vanadate do not similarly act.

## Appendix A

### Abbreviations

Abbreviations: AP-1, activator protein-1; ATF2, activating transcription factor 2; BSA, bovine serum albumin; CHO, Chinese hamster ovary cells; CHO-IR, CHO overexpressing human insulin receptor; CRE, cAMP-response element; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; FRK, fos-regulating kinase; Grb2, growth factor receptor bound protein 2; IGF-1, insulin-like growth factor 1; IL, interleukin; IR, insulin receptor; IRS-1, insulin receptor substrate 1; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP K, mitogen-activated protein kinase-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MEK, MAP kinase/ERK-activating kinases PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; rsk, 90KDa ribosomal S<sup>6</sup> Kinase; SAPK, stress-activated protein kinase; SH2 and SH3, src homology domains; SIE, sis-inducible enhancer; S<sup>6</sup> K, 70KDa ribosomal S<sup>6</sup> kinase; SOS, son of sevenless; SRE, serum response element; SRF, serum response factor; TCF, ternary complex factor; tk, thymidine kinase; TPA, 12-tetradecanoyl-phorbol-13-acetate; TRE, TPA response element; UV, ultraviolet light; WT, wortmannin



Appendix B  
Research Protocol Approval

WESTERN MICHIGAN UNIVERSITY  
INVESTIGATOR IACUC CERTIFICATE

41

Title of Project: Regulation of gene expression by  
insulin, insulin-mimetics and other metabolites

The information included in this IACUC application is accurate to the best of my knowledge. All personnel listed recognize their responsibility in complying with university policies governing the care and use of animals.

I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. Technicians or students involved have been trained in proper procedures in animal handling, administration of anesthetics, analgesics, and euthanasia to be used in this project.

If this project is funded by an extramural source, I certify that this application accurately reflects all procedures involving laboratory animal subjects described in the proposal to the funding agency noted above.

Any proposed revisions to or variations from the animal care and use data will be promptly forwarded to the IACUC for approval.

\_\_\_\_\_ Disapproved \_\_\_\_\_ Approved ☒ Approved with the provisions listed below

Provisions or Explanations:

- ① specific name of database searched (eg Medline)  
to determine non-duplication
- ② specific room number where animals will be  
housed

IACUC Chairperson

8/26/97  
Date

Acceptance of Provisions

Signature: Principal Investigator/Instructor

9/1/97  
Date

IACUC Chairperson Final Approval

9/5/97  
Date

Approved IACUC Number 97-07-03

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