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Dexamethasone and Insulin Regulation of Tumor Necrosis Factor and Interleukin 6 Production During Septic Shock

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DEXAMETHASONE AND INSULIN REGULATION OF TUMOR NECROSIS FACTOR AND INTERLEUKIN 6 PRODUCTION DURING SEPTIC SHOCK

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LIST OF ABBREVIATIONS

A23187	calcium ionophore
ACTH	adrenocorticotrophic hormone
APP	acute phase protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CD 18	cluster designation 18
CD 14	cluster designation 14
cDNA	complementary deoxyribonucleic acid
CRE	calcium response element
CRF	corticotrophin releasing factor
CYC	cycloheximide
DEX	dexamethasone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunoadsorbent assay
ETX	endotoxin
FBS	fetal bovine serum
FK565	chemotherapeutic agent

FFA	free fatty acids
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
GRE	glucocorticoid response element
H-7	1-(5-isoquinolinyl sulfonyl)-2-methyl-piperazine
IGF-I	insulin-like growth factor I
IL-1	interleukin 1
IL-10	interleukin 10
IL-6	interleukin 6
IL-8	interleukin 8
INDO	indomethacin
INFγ	interferon γ
INr	initiation region
INS	insulin
IPL	isolated perfused liver
kDa	kilodalton
KDO	2-keto-3-deoxyoctonic acid
KRB	Krebs' bicarbonate buffer
LAK	lymphocyte activated killer cell
LAL	limulus amoebocyte lysate
LBP	lipopolysaccharide binding protein
LIF	leukemia inhibiting factor

LPS	lipopolysaccharide
MDL201112	adenosine derivative
MHC	major histocompatibility complex
MP	methylprednisolone
MRE	multiple response element
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa B region
NK cell	natural killer cell
NO	nitric oxide
OKA	okadaic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEPCK	phosphoenolpyruvate carboxykinase
PGE_2	prostaglandin E_2
PGI_2	prostaglandin I_2
PKC	protein kinase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
RBC	red blood cell
RNA	ribonucleic acid
rTNF	recombinant tumor necrosis factor
SDS	sodium dodecanoate sulphate

SRE	serum response element
TATA	thymidine-adenosine-thymidine-adenosine
TGFβ	transforming growth factor β
TNF	tumor necrosis factor
TNF-R1	tumor necrosis factor receptor type 1
TNF-R2	tumor necrosis factor receptor type 2
UAUA	uracil-adenosine-uracil-adenosine
UTR	untranslated region

CHAPTER I

INTRODUCTION

Septic shock, as a consequence of an invasive, nosocomial, Gram-negative bacterial infection often signals the end of a hospitalized patient's life. The entire spectrum of pathophysiological events that occur in the evolving septic state are now known to be orchestrated by a sequential, interacting cascade of endogenous mediators from both the endocrine and immune systems. The endogenous metabolic dyshomeostasis of sepsis initiated early in the host response to infection has long been related to the production of stress hormones such as catecholamines, insulin, glucagon and cortisol. In addition, immunological mediators such as tumor necrosis factor, (TNFα), interleukin 1, (IL-1) and interleukin 6, (IL-6) have also been recently implicated to incite the sequence of pathophysiological steps characteristic of a fatal prognosis. Individually, the endocrine hormones and cytokines are able to disrupt cellular metabolism in the same tissue; however, the interaction between hormonal factors and the subsequent effects on the regulation of cytokine production have not been well established.

The performance of many organs such as the lung, spleen and liver are severely impaired during septic shock by the production of cytokines from resident populations of macrophages. The liver is considered to be a biologically strategic

organ in the septic cascade. It is necessary metabolically for the maintenance of blood and tissue euglycemia by its unique ability to convert gluconeogenic precursors to glucose. As the first organ to "see" and to process endotoxin originating from the bowel, the liver may also be a front-line, primary producer of cytokines from its resident population of macrophages, the Kupffer cells.

Endotoxin activation of second messenger pathways regulates the production of TNF and IL-6 by macrophages. The parallel increase in sepsis-induced hormones may also stimulate two or more second messenger pathways that converge with the endotoxin-mediated signal to either up or down regulate the production of a gene product. One may postulate that the merging of the endocrine and immune signals may propagate the vicious cycles that can ultimately trigger lethal shock and tissue injury through a process of signal convergence conflict.

The main focus of this thesis is to examine the interactive effects of insulin and glucocorticoids on TNF and IL-6 production during endotoxicosis. The specific aims include:

1) to assess the effects and interactions of insulin and glucocorticoids on morbidity and mortality during sepsis.
2) to evaluate the effects and interactions of insulin and glucocorticoids on metabolic parameters during sepsis.
3) to examine specifically, the interactions between glucocorticoid and insulin and their effects on the production of TNF and IL-6.

4) to examine possible mechanisms of glucocorticoid and insulin regulation of TNF and IL-6 production.

These aims will be accomplished by experiments at three biological levels of investigation. The effects of insulin and glucocorticoid during sepsis will initially be examined in an *in vivo,* endotoxic rat model in order that the response of the whole organism can be analyzed. The second model, the *ex vivo* isolated perfused rat liver will examine the contribution of the septic liver to the circulating levels of TNF and IL-6. The third level of examination investigates the endocrine regulation by the specific cells that produce TNF and IL-6 *in vitro.* Two isolated macrophage cell lines and two primary macrophage cultures will be tested as well as possible second messenger sites of regulation of glucocorticoid and insulin modulation of cytokine production.

This dissertation aspires to reinforce the physiological importance of hormonal alterations during septic shock to the survival of the organism. Also, this research will contribute to the growing body of evidence that significant communication exists between the endocrine and the immune systems during situations of physiological stress such as septic shock. The notion of "classical" hormones, such as insulin, as only a metabolic regulator is challenged as insulin can be shown to propagate immunological activity during endotoxicosis.

CHAPTER II

LITERATURE REVIEW

A. Introduction

The pathophysiological mechanisms of septic shock are multifarious, involving the direct and indirect effects of microbial agents and the activation of host endogenous mediators. The inappropriate activation and regulation of the cells of the endocrine system, especially the sympathetic-adrenal axis, the pancreas, and the cells of the immune system, which acts collectively as a "pseudoendocrine" system, are responsible for the release of mediators into the circulation in high concentrations. This excess of circulating endogenous factors act as lethal autotoxins which work collectively against the host. Corticosteroids and insulin are two endocrine hormones produced early after endotoxin stimulation. The cytokines, TNF and IL-6 are also produced in the early phases of the septic cascade.

This chapter is designed, to furnish the reader initially, with a basic understanding of the biology of endotoxin, TNFα and IL-6 and their roles during sepsis. Secondly, the literature pertaining to corticosteroids and insulin with respect to septic shock will be reviewed. The currently amassed literature

concerning these topics, especially TNFα and IL-6, is immense and grows exponentially on a monthly basis. Therefore, this review will be limited to those references which further the background and the rationale for the research described in this dissertation. This review cites literature up to and including references from February 1994.

B. Endotoxin

1. Structure

Lipopolysaccharide (LPS) is one of the amphiphilic macromolecules that constitutes the outer wall of Gram negative bacteria and is necessary for bacterial viability (272). Endotoxin is a generic term which includes LPS from all species of Gram-negative bacteria. LPS consists of four covalently interlinked segments as illustrated in fig. 1.

The O-specific chain is a carbohydrate polymer consisting of up to 50 oligosaccharide repeating units. LPS of wild type *Salmonella* or *Escherichia coli* exhibit a bimodal distribution of O-specific chains. The bulk of the O-specific chains are of high molecular weight and the remaining chains are of low molecular weight in 1-8 repeating units. The chemical composition of the sugar units and the degree of the polymerization gives heterogeneity to the molecule. The oligosaccharide units function confer the serological O-specificity of the LPS and the bacteria containing them (as reviewed in 266, 267).

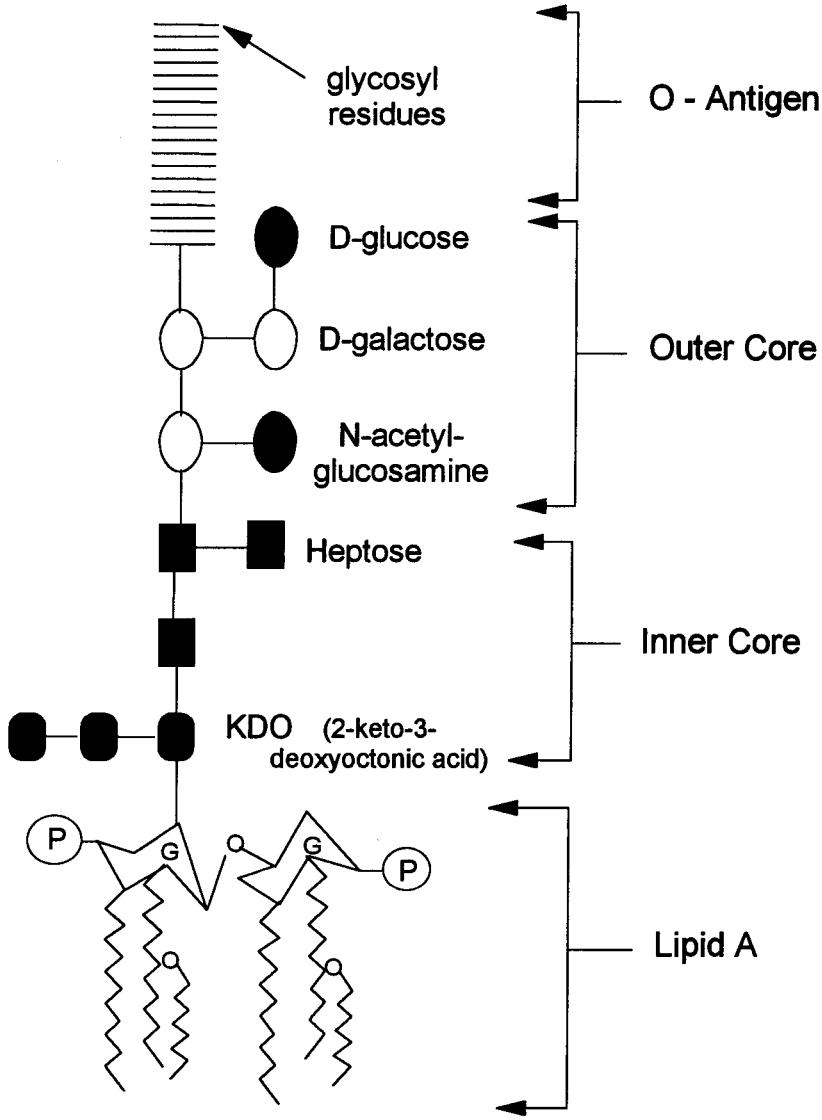


Fig. 1. Structure of *Salmonella sp.* lipopolysacharide.
(adapted from C.R. Raetz, 1991)

The core region is divided into the outer core, which binds to the O-specific chain and the inner core which binds to the Lipid A moiety. The structurally variability of the core region is limited. *Salmonella sp.* has only one core structure for all serotypes and *E. coli* has 5 core structures for over 100 serotypes. The outer core, is composed of common hexoses that form a branched pentasaccharide region. This region determines the outer core specificities and acts as a receptor site for bacteriophages. The inner core is the most conserved region of LPS and is characterized by uncommon heptose sugars arranged mainly in the L-glycero-D-manno configuration. Also present in the inner core is at least one α-linked pyranosidic or furanosidic, KDO, (2-keto-3-deoxyoctonic acid) region that occupies the lipid A proximal position. The inner core acts as a possible modulator of Lipid A biological activity and is essential for bacterial survival. bacteria with a defective inner core are not viable. KDO and LPS in general are essential for microbial growth and multiplication (as reviewed in 266, 267).

The Lipid A region is composed of gluco-configurated and pyranosidic D-hexosamine residues present as β(1-6)-linked disaccharide . This structure has not been identified in other natural occurring compounds and is unique to Lipid A. The disaccharide is linked to both a glycosidic and a nonglycosidic phosphoryl group and in ester and amide linkages and to medium to long chain (R)-3-hydroxy fatty acids (C10 to C28), some of which are acylated at their 3-hydroxyl groups. The lipid A molecule usually carries four mole equivalents of fatty acids. Lipid A represents the least conserved region of LPS among bacterial species. The

resulting R-3-acyloxyacyl residues are found in distinct evolutionary groups of Gram negative bacteria and is characteristic of Lipid A. Lipid A is essential for bacterial viability and virulence and for the remarkable spectrum of endotoxic activities. This region is responsible for initiating the classical complement cascade as well as binding to receptors on monocytes/macrophages and other host cells (as reviewed in 266, 267, 323).

2. LPS Stimulation of TNFα and IL-6 Production

As the main surface antigen (O-antigen) of Gram-negative bacteria, LPS is a potent stimulator of immune cells : neutrophils (86), lymphocytes (352), monocytes and macrophages (3,120). LPS is postulated to interact non-cytotoxically with these cells and the consequences are the synthesis and secretion of a variety of cytokines and proinflammatory mediators (311). The macrophage appears to play a pivotal role in endotoxin induced lethality (96,145,323,347).

The cytokines secreted by macrophages in response to LPS include TNFα, IL-1 and IL-6 and are well documented; however, the receptor and signalling mechanisms by which LPS triggers cytokine production by macrophages remain unresolved (2,120,363). Within the past 5 years evidence has accumulated which indicates that several pathways are employed by LPS for the activation of TNFα production by macrophages (145).

As demonstrated with chemically synthesized constructs, Lipid A accounts

for the effects of endotoxin in animals and cultured cells (266). Several studies (96, 158, 319, 327, 352) suggest the existence of receptors for Lipid A on animal cells. The finding of acylated LDL or scavenger receptors which bind LPS on macrophages by Hampton et al., 1991 (146) suggested a possible site for macrophage activation. This binding facilitated the ability of cells to take up and dephosphorylate Lipid IVA at the 1 position thereby, greatly reducing the potency of endotoxin. However, Freeman et al., 1990, (119) found that although acylated LDL blocked the binding of LPS completely, it did not block the toxic effects of endotoxin and, therefore, may not be directly involved with the induction of cytokine synthesis.

CD18, a heterodimeric complex found on the surface of macrophages, has been identified to bind particulate LPS on the surface of cells (as reviewed in 352). The LPS-CD18 interaction results in the phagocytosis of particulate LPS. There is no strong evidence that CD18 participates in the LPS activation of TNFα production by macrophages and monocytes. Patients with CD18 deficiency demonstrate a normal pattern of TNFα production and a normal priming response to LPS. Also, antibodies directed against CD18 do not prevent monocyte synthesis of TNFα (352).

Lipopolysaccharide binding protein, LBP, is a 60 kDa glycoprotein often referred to as an acute phase protein because it is synthesized by hepatocytes (127). LBP is found in normal serum and increases in response to endotoxin and acute phase protein synthesis. LBP binds specifically to the Lipid A moiety of

LPS. LBP functions to opsonize the LPS particles and activate neutrophils and macrophages(127) LBP-LPS particles have been shown to bind avidly to macrophages. LPS-LBP has been shown to be 1000 fold more active in the induction of the production of TNFα and IL-1 than LPS alone (352). This complex also raises the levels of mRNA transcribed and the TNFα protein released.

The receptor for LBP-LPS has been demonstrated to be CD14 (352). CD14 is a 55 kDa glycoprotein originally described as a myeloid differentiation antigen on monocytes and macrophages (13,352). CD14 has also been found free in human plasma. LPS upregulates CD14 production (192). Marchant et al., 1992, (208) demonstrated that LPS stimulated an increase in CD14 within 30 minutes after LPS stimulation. Surface antibodies against CD14 on macrophages efficiently block LBP-LPS binding. When normal rabbit serum was depleted of LBP, a substantial inhibition of TNFα production was observed. The blocking of CD14 by monoclonal antibodies also blocked TNFα synthesis (352).

The results from these experiments support the model illustrated in fig. 2. The presence of endotoxin stimulates LBP synthesis. LPS can bind to CD18 or the scavenger receptor whereby the LPS can be phagocytosed by the macrophage and neutralized or processed for antigen presentation to T-cells. T-cells respond to presented LPS by differentiating and synthesizing interferon γ. Interferon γ synergizes with endotoxin to enhance TNFα synthesis. LPS binds to LBP to form a complex that can bind to CD14. By an unknown second messenger pathway, CD14-LPS-LBP interaction stimulates TNFα synthesis and release.

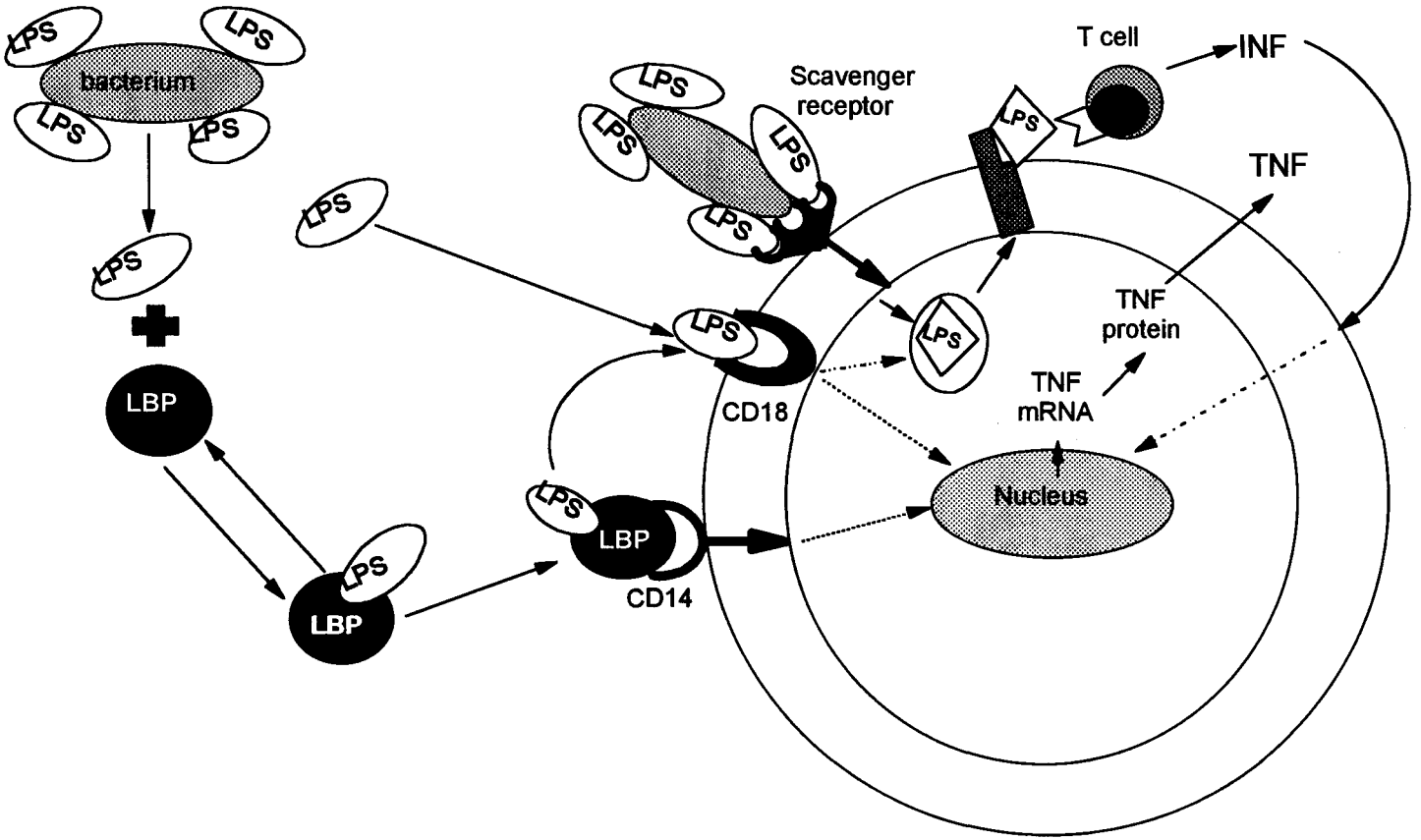


Fig. 2. Proposed role of LBP and CD14 in the activation of TNF production by macrophages.
(Raetz et al., 1990)

C. Tumor Necrosis Factor

1. Background

Over 100 years ago in separate clinical studies, Coley (67) and Chekhov (78) demonstrated that injections of endotoxin administered to cancer patients significantly reduced tumor growth. In 1943 Shear et al., (294) isolated the active component from Gram negative bacteria, a complex of lipid and sugar residues which they referred to as lipopolysaccharide. Shear believed that LPS directly induced the hemorrhagic necrosis of tumors observed in the Coley studies. O'Malley et al.,1962, (246) demonstrated that the serum from normal mice treated with *Serratia marcescens* LPS caused a dose dependent increase in the necrosis of tumors from tumor bearing mice. In 1975 Carswell et al. (62) using BCG stimulated murine serum, suggested that a serum factor produced by macrophages was responsible for the cytolytic activity towards tumors, and it was not LPS directly. In the mid 1980s research by both Beutler and Cerami described TNF and these authors are credited with pioneering cytokine biology. Agarwal et al.,1985, (5) succeeded in purifying and sequencing human TNFα and another tumorlytic protein produced by lymphocytes called lymphotoxin which is 30% homologous with TNF at the amino acid level and shares the same receptor. Several lines of evidence point to the macrophage as the principle source of TNF. Cerami and colleagues delineated the basic mechanism of cachexia associated with chronic disease states. Rouzer and Cerami, 1980, (277) demonstrated that

trypanosome-induced wasting in rabbits was accompanied by a paradoxical hypertriglyceridemia due to an endogenous mediator. Mice treated with LPS also demonstrated hyperlipidemia. These authors noted that endotoxin stimulated macrophages to produce a hormone capable of suppressing lipoprotein lipase essential for clearing plasma triglycerides. Beutler et al., (41) purified cachectin to homology and observed the murine cytokine had strong homology with reported human TNFα.

2.Structure

TNFα is the product of a single gene that resides within the major histocompatibility complex (short arm chromosome 6 human; chromosome 17 mouse). The gene consists of four exons and spans 3 kilobases. The TNFβ or lymphotoxin gene is present downstream on the same gene, separated by 1100 base pairs. TNFβ is subject to an entirely different form of regulation suggesting that TNF promoter/enhancer region lies downstream in this 1100 base pair region (5,322).

The TNFα gene encodes a protein of 26 and 17 kDa molecular weight as determined by SDS PAGE (322). The cDNA and the genomic sequences are highly conserved among species. Table 1 summarizes the structural protein properties of TNF among different species. Human TNF is produced as a precursor molecule with a presequence region of approximately 79 amino acids (5). The precursor molecule also contains a conserved region 26 amino acids in

Table 1--TNF protein comparisons between species.

	Leader peptide	Mature peptide			
Species	Amino	Acids	Isoelectric Point	Glycosylation Sites	Reference
Human	79	157	5.3	no	120
Mouse	76	156	3.9	yes	135
Rabbit	80	154	4.0	no	135
Rat	78	156	n/d	yes	161

n/d = not determined

length and a hydrophobic region possibly important for secretion or processing. The very long leader sequence is thought to function as a transmembrane domain. The mature TNF consists of 157 amino acids. TNF has two cysteine residues conserved at positions 69 and 101. These residues are involved in a single intermolecular disulfide bond. This bond is important for biological activity as gene mutations that lack this disulfide bond have decreased cytotoxicity, macrophage activation and inhibition of lipogenesis without alterations in secondary or tertiary structure (5). Human TNF does not contain a glycosylation site which has been found at position 7-9 of murine TNF. The function of the glycosylation site is unknown as both peptides display equivalent biological activity.

The active form of TNF consists of dimer, trimer or higher oligomer complexes. Variations in conformation have been attributed to the methods of analysis and species differences. Smith and Baglioni, 1987, (299) have demonstrated that the human and murine forms that bind to cellular receptors and evoke cell death in L929 cells exits as a trimer. Crystallographic analysis has revealed that each subunit an antiparallel β sandwich forms a trimeric molecule through edge to face packing (322).

3.TNF Receptor

Two distinct receptors, R1 and R2 have been found for TNF (227,276). They have a molecular mass of 55 and 75 kDa, respectively, and both bind

lymphotoxin.

The amino acid sequence of the 55 kDa receptor contains 426 amino acids with a single membrane span. The extracellular domain contains 182 amino acids and the intracellular domain is 221 amino acids. N-linked glycosylation is responsible for the deviation of the predicted mass from the cDNA and the actual mass (276).

The 75 kDa receptor is also a single membrane spanning protein comprised ofl 439 amino acids. The extracellular domain is 235 amino acids and the intracellular domain is 174 amino acids. Both N and O linked glycosylation sites have been found on the R2 receptor (276).

Both R1 and R2 are considered distinct receptors as they share only 28% homology in the extracellular domain and no homology in the intracellular domain. This suggests that different signalling pathways exist for both receptors. The observation that the intracellular domains also do not share homology with any known protein further complicates the definition of TNF transduction pathways (276).

Most tissues express both types of TNF receptors (17,126). With the use of monoclonal antibodies, Tartaglia et al., 1993, (315) demonstrated that the binding of TNF to TNF-R1 results in the induction of NF-κB , MnSOD and cytotoxicity. The binding of TNF to TNF-R2 results in thymocyte proliferation and differentiation (322). As illustrated in fig. 3, TNF binding to receptors exerts multiple effects on many cell types. As has been demonstrated with other cytokine

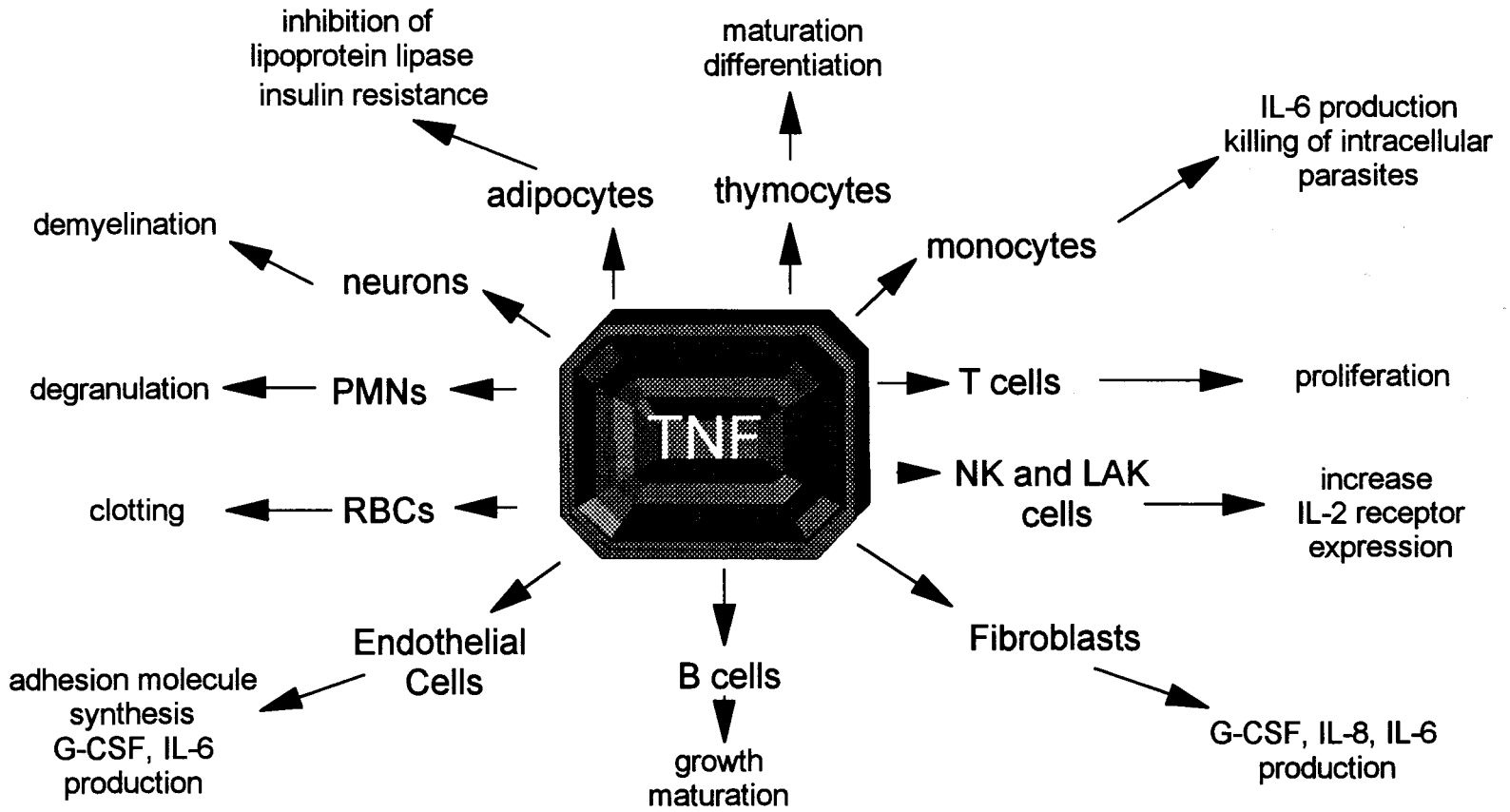


Fig. 3. Cellular effects initiated by TNF. (Adapted from Vasselli, 1992 (334))

receptors, the TNF-R1 and TNF-R2 are shed from the surface of cells and function as soluble TNF binding proteins in plasma (33,301,276). The release of these receptors has been postulated to bind excess TNF in plasma. Treatment with recombinant TNFRs partially protected mice from LPS induced lethality. The production of both receptors by cells is increased by trauma, TNF and LPS (312). Spinas et al., 1992, (301) demonstrated that three hours after *E.coli* administration to human volunteers, a four to five-fold increase in TNF-R1 and TNF-R2 concentrations in plasma was observed. Pretreatment of the volunteers with ibuprofen slightly increased concentrations of TNF receptors. Bemelmans et al., 1993 (33) have also suggested that the protective effect of LIF during endotoxic shock may occur via the ability of LIF to stimulate an increase in TNFR production.

4. Regulation of TNF Production

a) Molecular Regulation

TNFα is synthesized by many cell types including macrophages, monocytes, lymphocytes, NK cells, astrocytes, microglial cells, Kupffer cells, fibroblasts, endothelial cells (as reviewed in 334) and eosinophils (72). A wide variety of infectious and inflammatory agents can trigger TNF synthesis including LPS, staphylococcus/streptococcus exotoxin, enterotoxin, toxic shock syndrome toxin, mycobacteria, viruses, C5a, fungal, parasitic infection, hemorrhage, IL-1 and TNF (as reviewed in 334). With such a diverse group of stimulatants of TNF,

regulation of TNFα is under stringent regulation. Over the last 5 years, Beutler and coworkers, have contributed greatly to the current understanding of TNFα regulation at the gene level (41,42,43).

The TNFα promoter region is responsive to LPS stimulation within primary macrophages. However, the intracellular journey from the membrane to the gene remains uncharted. Four NFκB regions have been identified within the promoter region that appear to be necessary for LPS enhancement of expression. Mutations that remove two or more of these sites abolish LPS stimulated TNFα production (42). However, several genes unresponsive to LPS contain the same NFκB region, suggesting that other factors or flanking regions on the gene are also required. A purine rich motif (PU box) that exits within the promoter region has the potential for determining tissue specific expression. SP-1 and TFIID regions confer "housekeeping" functions related to the initiation of transcription (41,42).

TNFα is regulated at the level of transcription. Several studies (41,271,324,339) have demonstrated that TNFα mRNA is significantly increased after LPS stimulation up to 100 fold. The TNFα protein, surprisingly is increased by 10,000 fold. Therefore, TNFα is also regulated at the level of translation (43). The identification of post transcriptional regulatory regions within the cDNA for the mouse TNF offered further explanation to the disparity between mRNA and protein levels. Caput et al., 1986, (59) noted repeated and intercleaved octomeric units of TTATTAT in the 3' untranslated regions of the cDNA for both murine and

human TNF cDNAs. This region has also been found in the UTR 3' regions specifying other cytokines (IL-1, IFNγ, several proto-oncogenes, GM-CSF) and more recently the inducible form of nitric oxide synthase (iNOS) (99). Shaw and Kamen, 1986, (293) identified this TTATT region as a pentameric unit and thus identified it to exist in a larger population of cytokine genes. From experiments in which this sequence was spliced into the UTR 3' region of the rabbit β globulin gene, Shaw and Kamen determined that the rate of transcription was not altered; however, the normal β globulin mRNA, which is extremely stable had a half-life of only 15 minutes (293). They postulated that the UA rich region confers message instability. This finding appears to be cell specific as transfected lymphocytes with modified message did not express mRNA instability (293).

The observation of superinducibility may also be ascribed to the UA rich regions. Cells treated with an inhibitor of translation, such as cycloheximide, overexpress mRNA (65,247). The overexpression of mRNA thus results in the over production of the protein coded by the specific mRNA. This is also observed in modified δ globulin mRNA (293). Superinduction has been accredited to unstable repressors of transcription which are destroyed and unreplenished in the presence of cycloheximide (43,65,247). Superinduction may also be ascribed to the existence of labile ribonucleases that specifically target such mRNA sequences. Beutler et al., 1992, have recently demonstrated that RNAase A found in macrophages selectively hydrolyses the UA dinucleotide linkages found in TNF mRNA (44).

b) Regulators of TNFα Production

Over the last 8 years, many researchers have investigated numerous pharmacological and physiological compounds that alter TNFα production in many cell types. Table 2 is a compilation of the compounds and agents that have been examined as regulators of TNFα. As illustrated in table 2 the number of substances tested is extensive and, therefore, in the interest of brevity, two stimulatory compounds, IFNγ and PMA as well as two inhibitors, PGE_2 and IL-10 will be discussed in the context of this review. Glucocorticoid inhibition of TNFα production will be discussed in detail in a later section of this chapter.

Interferon γ also called macrophage activating factor is produced and secreted by T lymphocytes and large granular lymphocytes in response to antigens and T cell mitogens (212). IFNγ stimulates macrophages to increase antimicrobial activity and secrete various inflammatory mediators. IFNγ synergizes with LPS to produce augmented levels of TNFα both *in vivo* and *in vitro*. Doherty et al.,1992, (85) demonstrated that TNFα and IFNγ, when administered to mice, were well tolerated individually ; however, when given together, the combination caused a significant increase in mortality associated with increased plasma IL-6 concentrations. The C3H/HeJ mouse carries the *lps*^{d} mutation which confers endotoxin resistance and the inability to produce TNFα in comparison with the wild type strain (309). Adi et al.,1992, (4) have shown that administration of IFNγ to C3H/HeJ mice restores TNFα production by both the liver and the spleen. Matic et al.,1992, (212) have also shown that IFNγ relieved the suppression of TNFα

Table 2--Summary of pharmacological and biological modulators of TNF production.

INCREASES TNF	cycloheximide (247) PMA (66) A23187 (345) okadaic acid (310) indomethacin (209) calcyclin A (310) calphostin (310) staurosporine (66) H-7 (66) FK565 (150) taxol (280)	LPS (334) interferon γ (212) growth hormone (91) ACTH (292) nitric oxide (99) pertussis toxin (361)
DECREASES TNF	MDL201112 (250) cyclosporin (238) ibuprofen (196) cimetidine (196) diphenylhydramine (196) taurine Cl (249) N-acetylcysteine (255) glutathione (255) E330 (228) puromycin (150) actinomycin D (365) polymyxin B (66) pentoxyphylline (148) thalidomide (281)	adenosine (250) TNF inhibitor (251) PGE_2 (209) glucocorticoids (339) interleukin 6 (3) interleukin 1 (195) TGF β (88) interleukin 10 (113) G-CSF (138) LIF (33) arachidonic acid (174) TNF receptor (301) fatty acids (344)

synthesis induced by LPS induced tolerance in macrophages. They suggested that IFNγ opposes endotoxin tolerance through its action on a PKC dependent pathway (212).

Glucocorticoids are potent inhibitors of the synthesis of many cytokines. The inhibition of TNFα by glucocorticoids occurs both at the transcriptional and the posttranscriptional level (43). Interferon γ causes a reversal of dexamethasone-induced suppression of TNFα message and to a lesser extent TNF protein production (205). IFNγ stimulation of TNFα synthesis and reversal of dexamethasone suppression also occurs at a transcriptional level. Dunham et al.,1990, (88) have also found that IFNγ was able to relieve TGFβ suppression of TNFα synthesis.

Another potential mechanism of IFNγ control is by regulation of TNFα receptor synthesis. Aggarwal et al.,1985, (5) have shown that IFNγ increases TNFα receptor number in several cell lines without increasing TNFα receptor affinity.

LPS has been shown to stimulate phospholipase C and to activate protein kinase C in macrophages (40,66,343). PMA is the most potent tumor promoter known and is a stimulant of PKC, which induces adherence and the synthesis of superoxide and peroxide radicals by monocytes (214). In murine Kupffer cells LPS stimulation of TNFα and IL-1 is attenuated by the PKC inhibitor, H-7 (345). PMA alone will not stimulate TNF production, however, in combination with LPS, PMA has been demonstrated to augment both TNFα and IL-1 production by

macrophages (129,259). Coffey et al.,1992, (66) observed a paradoxical stimulation and inhibition by PMA and H-7 based on the concentration used to treat human monocytes *in vitro.* Concentrations of PMA greater than 3nM inhibited TNFα production, whereas, concentrations between 0.3 and 3 nM augmented LPS induced TNFα production. H-7 addition (10-30 μM) augmented TNFα production, and higher concentrations of H-7 inhibited LPS induced TNFα production. The disparity in this report was suggested to be the result of activation of different PKC isozymes that may mediate stimulatory as compared to inhibitory effects of PKC on TNFα production (66).

LPS stimulates the production of prostaglandin E_2 and prostacyclin I_2 (78). *In vitro*, PGE_2 production occurs after TNFα production and once initiated TNFα production begins to plateau and decline, suggesting that PGE_2 acts to inhibit TNFα synthesis. The addition of cycloxygenase inhibitors such as indomethacin, results in augmented TNFα production and results from several studies demonstrated that addition of PGE_2 or prostacyclin to LPS stimulated macrophages results in a dose dependent reduction in TNFα production (180,345). PGE_2 blocks TNFα production at the level of transcription (345). PGE_2 increases the intracellular cAMP concentration because its effects can be mimicked by the addition of dibutyrl cAMP, forskolin and phosphodiesterase inhibitors. Feedback control of TNF production is likely at a local level in *in vitro studies* as the role of PGE_2 *in vivo* is not as clear (78). Administration of PGE_2 *in vivo* has been shown to have little effect on preformed TNFα and the addition

of PGE_2 to LPS treated animals causes toxic responses (78). In fact, the use of cycloxygenase inhibitors has been shown to be beneficial in reducing the toxic effects of TNFα and LPS (78). Marcinkiewicz, 1991, (209) has shown that PGE_2 and PGI_2 effectively inhibit TNFα and enhance IL-6 levels in peritoneal cells.

Interleukin 10 was first described as a cytokine produced by subsets of T cells. IL-10 inhibits macrophage APC-dependent cytokine synthesis by Th1 Th cells. Ralph et al.,1992, (268) found that IL-10 is also produced by melanoma, epidermoid and fibroblast cell lines. IL-10 is also produced by LPS stimulated monocytes and inhibits IL-1, TNFα, IL-6, IL-8, G-CSF and class II MHC in purified monocytes (268). Gerad et al., 1993, (132) demonstrated in mice that IL-10 pretreatment reduced TNF levels by 92%, reduced LPS hypothermia and significantly decreased mortality. Recently, Corradin et al., 1993, (71) demonstrated that IL-10 stimulated induction of NO synthase mRNA in LPS/IFNγ cells and increased NO synthesis.

5. TNFα: Role in septic shock.

TNFα has been implicated in the pathogenesis of many disease states such as ARDS, reperfusion injury, graft vs. host disease and rheumatoid arthritis. The contribution of TNFα to the pathology of these diseases is reviewed in several recent review articles (78,126,134,334).

More literature exists on the role of TNFα in septic shock than any other cytokine. TNFα was implicated many years ago as causing many of the symptoms

of Gram-negative sepsis.

Endotoxin administration to human volunteers results in a rapid and early increase in plasma TNFα concentrations as illustrated in fig. 4. The pattern and time course of cytokine appearance *in vivo* is remarkably consistent among species (116, 182, 271, 337). In comparison to the other cytokines, IL-1 and IL-6 which appear 2 to 3 hours after endotoxin, TNFα is measurable in the circulation within the first 30 minutes (116) . When injected into human volunteers, endotoxin stimulates increases in TNFα within one hour which thereafter decline to baseline after 3 hours (116). The TNFα burst is an early event during endotoxemia as Galley et al., 1993 (128) have shown that the increase in TNFα message occurs within 5 minutes after stimulation of human monocytes with LPS. Remick et al. 1989, (271) have also shown that the peak expression of message by peritoneal macrophages occurs one hour after LPS injection.

Clinical studies have implicated TNFα as an important mediator in septic shock. Detectable levels of TNFα are found in the serum of patients with meningococcal disease and sepsis. Patients with serum levels greater than 0.1 ng/ml subsequently died (as reviewed in 78). Recently, Casey et al. 1993. (60) observed that 45% of patients with sepsis syndrome exhibited increases in plasma TNFα concentrations. Increased plasma TNFα levels did not, however, correlate with death of these patients. This finding has been confirmed by several studies. The discrepancy may exist due to the inclusion criteria in the study or due to other injuries. Hemorrhagic shock and trauma dramatically alter the

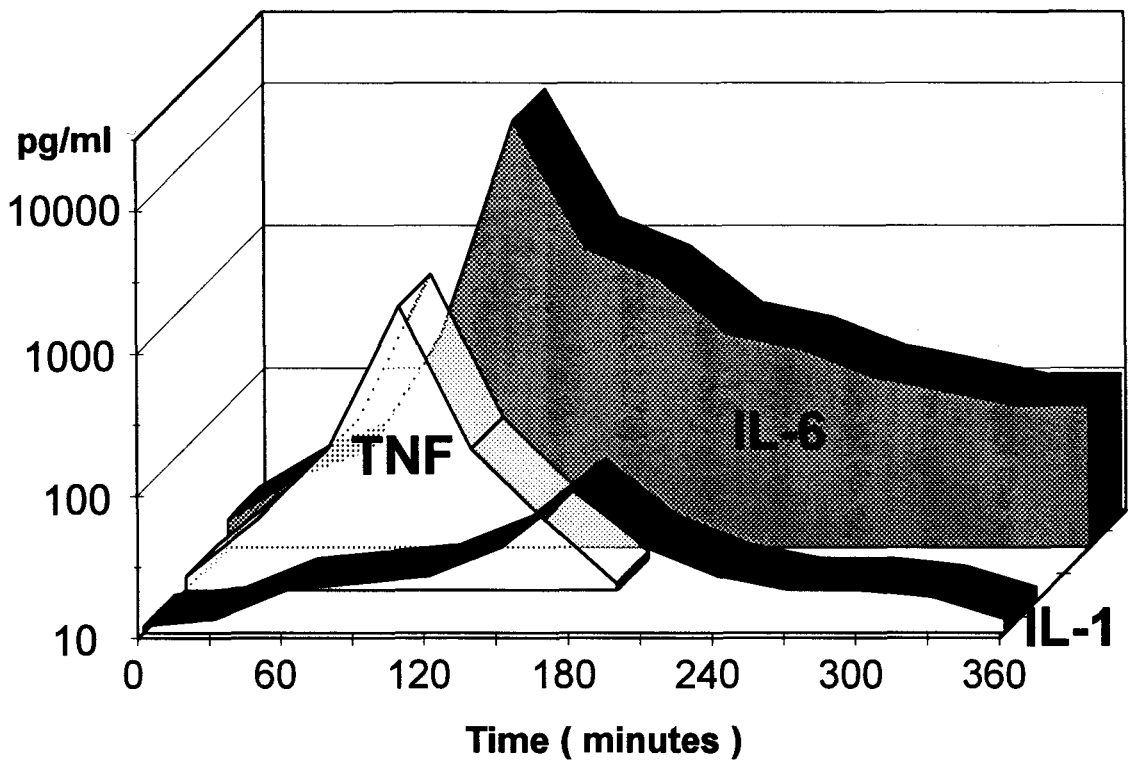


Fig. 4. Time course of circulating cytokine production following endotoxin infusion in human volunteers. (Adapted from Fong et al., 1990 (116))

cytokine profile as compared to that found in pure gram negative sepsis (60).

The administration of highly purified recombinant TNF*α* causes shock and injury in every mammal studied. The list of species tested includes rat, mouse, rabbit, dog, pig, sheep, cow, monkey, baboon, and man. TNF*α* administration causes a syndrome almost indistinguishable from septic shock. Although other cytokine, IL-1 and IL-6 can induce some of the symptoms of septic shock, TNF*α* administration is the best reproduces the septic state, not only because of its own pathological activities but also because of its ability to induce the production of other cytokines that are pathological. Hypotension, fever, hypoglycemia, increases in stress hormones and acidosis are only a few of the symptoms observed after TNF*α* treatment. Sakurai et al.,1993, (278) recently demonstrated that high doses of TNF*α* injected into dogs caused a fall in arterial pressure, pulmonary artery pressure and cardiac index. They also concluded that TNF*α* caused a shift towards carbohydrate as an energy substrate by decreasing the availability of FFAs and decreasing lipid oxidation, while simultaneously increasing glucose production and clearance (278). Others have also shown that TNF*α* increases glucose oxidation especially in non-insulin dependent tissues such as Kupffer cells (223,224).

TNF*α* has been implicated as the factor responsible for the lethal hypoglycemia observed during sepsis (337). Chajek-Saul et al., 1990, (63) demonstrated that rTNF*α* injected into adrenalectomized rats resulted in increased endotoxin sensitivity . Pretreatment with dexamethasone or glucose protected

these animals from death (63) A significant reduction in PEPCK activity was also observed (63). Satomi et al.,1985, (284) also found a strong negative correlation between plasma glucose concentration and plasma TNFα.

Tumor necrosis factor has been shown to alter liver enzyme profiles. Intraperitoneal injections resulted in a 20-50% reduction in hepatic aminotransferases and a 50-200% increase in alkaline phosphatase (354). Yasmineh et al 1992. (355) demonstrated that TNFα administration in the rat had differential effects on the enzymes of gluconeogenesis. A significant reduction in kidney 1,6 diphosphatase, PEPCK and glucose-6-phosphatase was observed after TNFα administration (355). Hill and McCallum, 1992, (161) confirmed the findings of this group by demonstrating that PEPCK is transcriptionally negatively regulated by TNFα in mice and in H 4 II Reuber hepatoma cells.

Protection against endotoxic or bacteremic shock has been achieved by passive immunization with anti-TNFα antibodies and anti-IL-6 antibodies. Controversy exists as to the efficacy of TNFα antibodies in septic therapy. Polyclonal antibodies administered to endotoxic mice and monoclonal antibody therapy in baboons have been shown to be efficacious in preventing endotoxic shock (as reviewed in 78). Recently Zanetti et al. 1992, (360) demonstrated that antibodies against TNFα administered to a 100% mortality model in mice was extremely effective in reducing mortality as well as reducing the plasma concentrations of TNFα, IL-1 and IL-6. However, Eskarandi et al. 1992, (97) demonstrated that in a CLP sepsis model and during endotoxemia that anti-TNFα

Abs failed to prevent lethality. Results from clinical trials are currently ongoing, and anti-TNF Abs may possibly prove to be a useful clinical modality (89). As TNFα antibody therapy alone was shown to be ineffective in preventing death from sepsis syndrome, Mullen et al.,1993, (233) using a septic porcine model concluded that combination therapy with ibuprofen and antiTNFα antibody therapy provided greater protection from acute lung injury and hemodynamic failure.

With the recent identification of soluble receptors for TNFα found in the plasma and urine of patients (312), another clincal modality is currently under review. Spinas et al.,1992 (301) have shown that partial protection against endotoxin induced lethality in mice can be achieved with TNFαR therapy. The TNFαR is thought to act like a "sponge" and neutralize the bioavailable TNFα present in the plasma.

D. Interleukin 6

1. Background

Interleukin 6 is probably unique among cytokines because it was cloned inadvertently long before the discovery of its major biological activities (76). Weisenbach et al., 1980, (346) isolated two cDNA clones derived from 1.3 kb mRNA in a fibroblast cell line in search of the sequence for interferon β, calling this species interferon β2. Content et al., 1982 (68) cloned the same 1.3 kB mRNA species concluding that the protein synthesized which they named 26K

had no antiviral activity and was unrelated to interferon β. Simultaneously Teranishi et al., 1982, (316) observed that the activated T-cells produced a B cell differentiation factor called BSF-2. The cloned sequence was found to be identical for the sequence described for 26K (316).

The existence of growth factors for plasmacytoma and hybridomas although unsuccessfully characterized, had been known since the 1970s. With the availability of stable growth factor dependent cell lines, these factors were easily characterized (76). Van Snick et al. (331) purified a factor from helper-T cells known as IL-HP1, and Nordan et al. 1986 (241) purified PCT-GF from the supernant of cultured macrophages. Van Damme et al., 1987, (328) purified a human hybridoma/plasmacytoma growth factor (HPGF) from the medium conditioned by IL-1 osteosarcoma cell line. It was eventually determined that these hybridoma growth factors all shared sequence homology with IFN-B2 26K and BSF-2 and were collectively referred to as IL-6 (331). Later, Gauldie et al., (130) discovered that antibodies to IL-6 blocked the activity of a monocyte derived protein termed HepSgF which has been shown to activate acute phase synthesis by hepatocytes. As described in a review by Van Snick (331) , IL-6 existed in the scientific world for many years by the following aliases:

IFNβ2 = 26K = BSF2 = PCT-GF = ILHP1 = HPGF = CDF = IL-6

2. Structure

The interleukin 6 gene has been cloned from human (10), mouse (314), rat

(48) and more recently from the pig (273). IL-6 is a single copy gene and in all species contains 5 exons and four introns with conservation among species of the intron exon borders. The interleukin 6 gene is located on chromosome 5 in the mouse and on chromosome 7 in man (10, 314). The cDNA share homology as illustrated in the table 3 with subsequent differences in the amino acid sequence of the protein. Messenger RNA transcripts are initiated from several cap sites on the human gene. The rat gene has 2 cap sites, however, only one site appears to be utilized during transcription (48). From the human gene, two species of mRNA are transcribed that are 1.3 kilobases long (10). Two polyadenylation signals which are 78 bases apart are utilized. In the mouse, however, only mRNA is produced from similar transcriptional signals (314). The rat gene codes for two species of mRNA, 1.2 and 1.35 kilobases in length (48). The transcript content of AUUUA regions which confer stability of the message, varies among the species (76).

The IL-6 gene codes for a 211 amino acid protein in both the rat and the mouse and a 212 amino acid protein in human (10,48,314). The signal peptide is cleaved to yield a mature 183-185 amino acid protein. Human IL-6 has 2 N-glycosylation sites and several O-glycosylation sites. The mouse and rat IL-6 proteins are not glycosylated. The absence of glycosylation sites in rodent IL-6 may account for the inability of mouse IL-6 to bind to the human IL-6 receptor (6,7,28). All species studied to date have four cysteine residues at conserved sites on the protein. The rat has an additional free cysteine at position 103 whose

Table 3-- Comparison of species similarities with respect to the cDNA and the protein for IL-6 (76).

Comparison	cDNA homology	Amino acid homology
rat to mouse	92%	93 %
rat to human	65%	58%
mouse to human	68%	42%
pig to human	83%	62%
pig to mouse	61%	42%

function is yet to be determined (48). The N terminal region of the peptide is not necessary for bioactivity. Residues 1-28 can be removed without substantial alterations in action. The removal of as few as two amino acid residues from the carboxyl terminus of the protein results in significant loss of bioactivity (6,76).

3. IL-6 Receptors

An abundance of cell types express surface IL-6 receptors (6,300) . Petersen et al. 1990 (253) found that I^{125} labelled IL-6 was quickly taken up from the circulation by the spleen. Approximately 60% of this binding was found to be by spleen macrophages. IL-6 was also shown to bind avidly to rat peritoneal macrophages *in vitro* (253).

The IL-6 receptor has a molecular mass of 80 kDa and is highly glycosylated (6,28,313). It is structurally unique in comparison to other cytokine receptors. The extracellular portion consists of two domains. One domain is a member of the cytokine receptor family with a distinct region, and the other domain is a member of the immunoglobulin gene superfamily (28). The cytoplasmic domain is 82 amino acids in size and contains no known sequences that might mediate signal transduction (184). Taga et al. 1989, (313) determined that signal transduction by the IL-6 receptor occurs via the interaction with a 130 KDa protein (gp130) at the extracellular domains. The structure of gp130 is similar to the G-CSF receptor, and IL-6R-gp130 association results in the transformation from a low to a high affinity receptor (184). Other cytokines such as IL-3, GM-CSF

and the IL-5 receptor also function in a similar manner. Signal transduction by the interaction with gp130 may explain the functional pleotrophy and redundancy of the many cytokines. The signal transduction mechanism for gp130 is unknown; however, evidence of tyrosine kinase autophosphorylation has been documented (184).

4. Regulation of IL-6 Production

a) Molecular Regulation

The investigation of the molecular regulation of IL-6 production is a very recent area of study. Stimulation of IL-6 production has been demonstrated by at least three different intracellular pathways. The production of cAMP, diacylglycerol and an increase in intracellular of calcium are implicated in the upregulation of IL-6 synthesis (76) . Tanabe et al. 1988, (314) have described a highly conserved region in both the human and mouse IL-6 genes approximately 350 base pairs upstream of the initiation site that acts as the IL-6 promoter region. The different regions of the IL-6 promoter region are illustrated in fig. 5. Within the promoter is the MRE region which contains several smaller segments that are responsible for activation by different substrates. The SRE is found in the -113 to -225 region and is necessary for oncogene activation by *c-fos* (7). The region at -145 to -158 is known as the NFIL-6 region. NFIL-6 is a nuclear factor whose synthesis is stimulated by LPS, IL-1, and IL-6. NFIL-6 belongs to the C/EBP family of nuclear binding proteins (7). This group of proteins includes

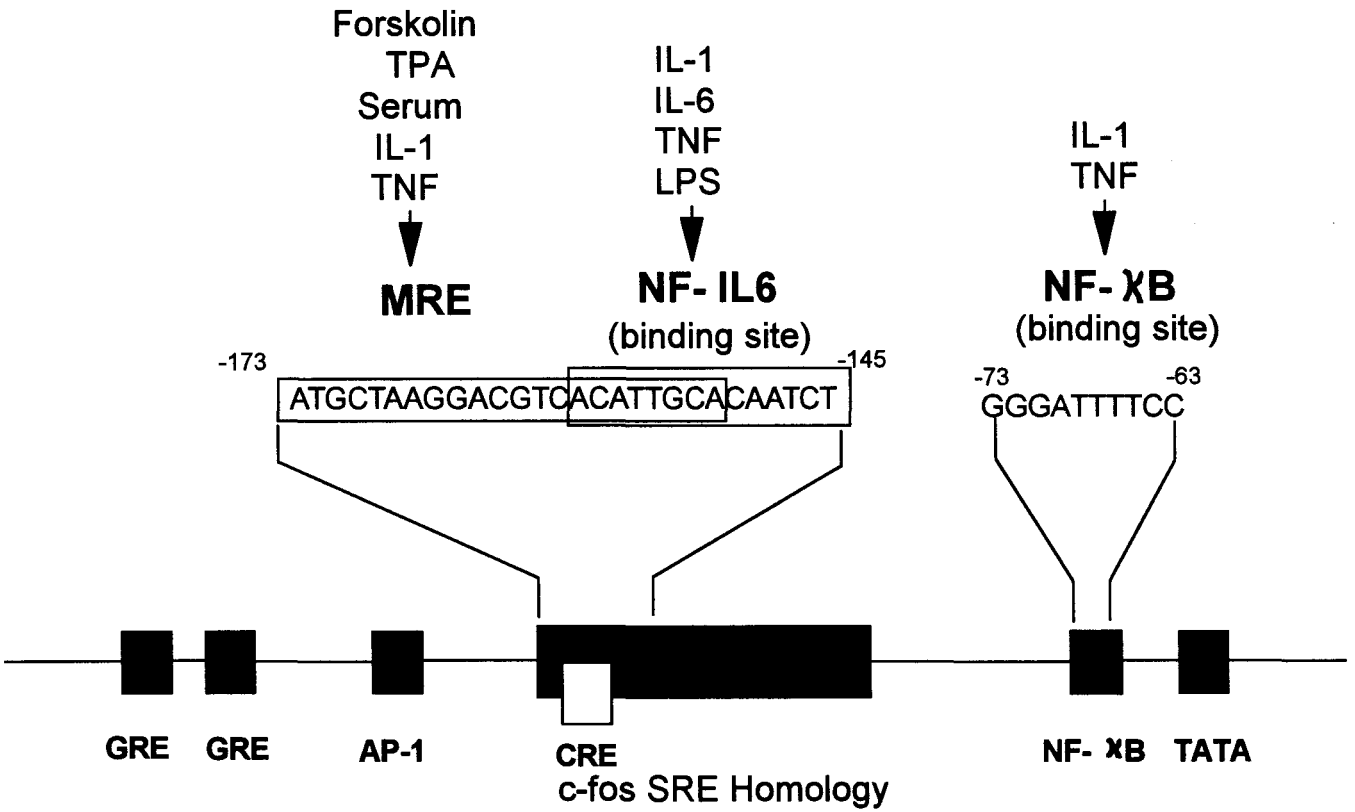


Fig. 5. Functional regulatory regions in the promoter of the IL-6 gene. (Akira et al., 1990 (6))

C/EBP, IL-6DBP, Ig/EBP-1 and NF-IL6β. They recognize the same nucleotide sequence in a gene but exhibit distinct patterns of expression among different cells (7). The CRE is also found in the MRE region. The NFκB region is found between the -63 to -73 positions and is necessary for induction of transcription (7). Matsusaka et al., 1993, (213) recently described the synergistic activation by NF-IL6 and NFκB of the IL-6 promoter.

Negative regulation of IL-6 synthesis by glucocorticoids has been described by Ray et al.,1990, (269). DNase footprinting demonstrated that the glucocorticoid receptor bound across the MRE, TATA box and the INr. At least two GRE regions have been described in the IL-6 promoter (7). Two AP1 sites have also been described on the promoter, however, the function of these sites remains unresolved (7).

b) Regulators of IL-6 Production

Most nucleated cells studied, including transformed cells, are capable of producing IL-6 (76,154). Since IL-6 has not yet achieved the same degree of research notoriety as TNF, the substances tested to date as possible regulators of IL-6 synthesis are limited. Also, activation of IL-6 synthesis by endotoxin, growth factors and pharmacological compounds is dependent on the cell type examined (76,288). In fibroblasts the most potent stimulator of IL-6 production is IL-1 and TNFα. In macrophages the most potent stimulator of IL-6 is LPS and the phorbol ester, PMA (76).

IL-6 production *in vivo* is significantly augmented by LPS induced TNF production (290, 326, 338). Many studies *in vitro* have also concluded that the production of TNF is closely followed by the production of IL-6 (95, 116, 290). Therefore, substances which alter TNF production also alter IL-6 production in the same direction. Shalaby et al.,1989, (290) demonstrated *in vivo* that injections of both TNF and IL-1 could stimulate IL-6 production by mice. The administration of both TNF and IL-1 resulted in a synergistic increase in IL-6 production. Administration of TNF antibodies also partially attenuated IL-6 production in LPS stimulated mice (290). However, reports from *in vitro* studies with transformed cell lines (211) and isolated primary liver endothelial cells (101) indicate that IL-6 production is not coupled to the presynthesis of TNF.

The activation of protein kinase C by PMA results in IL-6 production by macrophages (76,283). Mengozzi et al. 1991, (220) have also demonstrated that PMA could reverse LPS tolerant suppression of IL-6 production, but not TNF production in mice. They also observed that IFNγ could also partially restore IL-6 production in this model (220). Recently, Sironi et al.,1993 (296) found that PMA could also reverse LPS tolerance in a mouse glioma cell line. The restoration of cytokine production by PMA was also specific for IL-6 as TNF production was not restored by PMA treatment. From this study the authors concluded that down regulation of IL-6 production during LPS tolerance occurred at the level of transcription by down regulation of PKC or another PMA-induced signalling pathway (296).

5. IL-6 Function and Regulation during Sepsis

As illustrated in fig. 6, IL-6 is a polyfunctional cytokine that plays a central role in many host defense mechanisms. The impact of IL-6 on the cell types illustrated can be found in a recent comprehensive report by Van Snick (331).

IL-6 has been implicated *in vivo* to be one of the mediators of the host response to trauma, infection and sepsis, as several authors have demonstrated that LPS administration stimulates the production of IL-6 (15,16,24, 46, 81, 94, 115, 116,168,181,185) . Fong et al., (116) demonstrated that *E.coli* administration to human volunteers stimulated IL-6 production. Moreover, recent clinical studies (80,244,359) have described a strong correlation between elevated plasma IL-6 concentrations and both postoperative complications and increased mortality of septic patients. Patients with *Pseudomonas pseudomallei* sepsis, whose serum IL-6 levels were greater than 1 ng/ml had a 75% increased mortality rate (123). Casey et al. 1993, (60) demonstrated similar results. Septic syndrome patients who died had significantly elevated plasma IL-6 concentrations. Plasma tumor necrosis factor and IL-1 concentrations did not correlate with an increased mortality rate (60).

IL-6 administration to animals results in the physiological sequelae observed during sepsis, such as acute phase protein synthesis, fever, hypoglycemia and hypotension (27).

IL-6 is the cytokine predominately responsible for the production of acute phase proteins (APP) by the liver (27,53,155). LPS stimulates Kupffer cells and

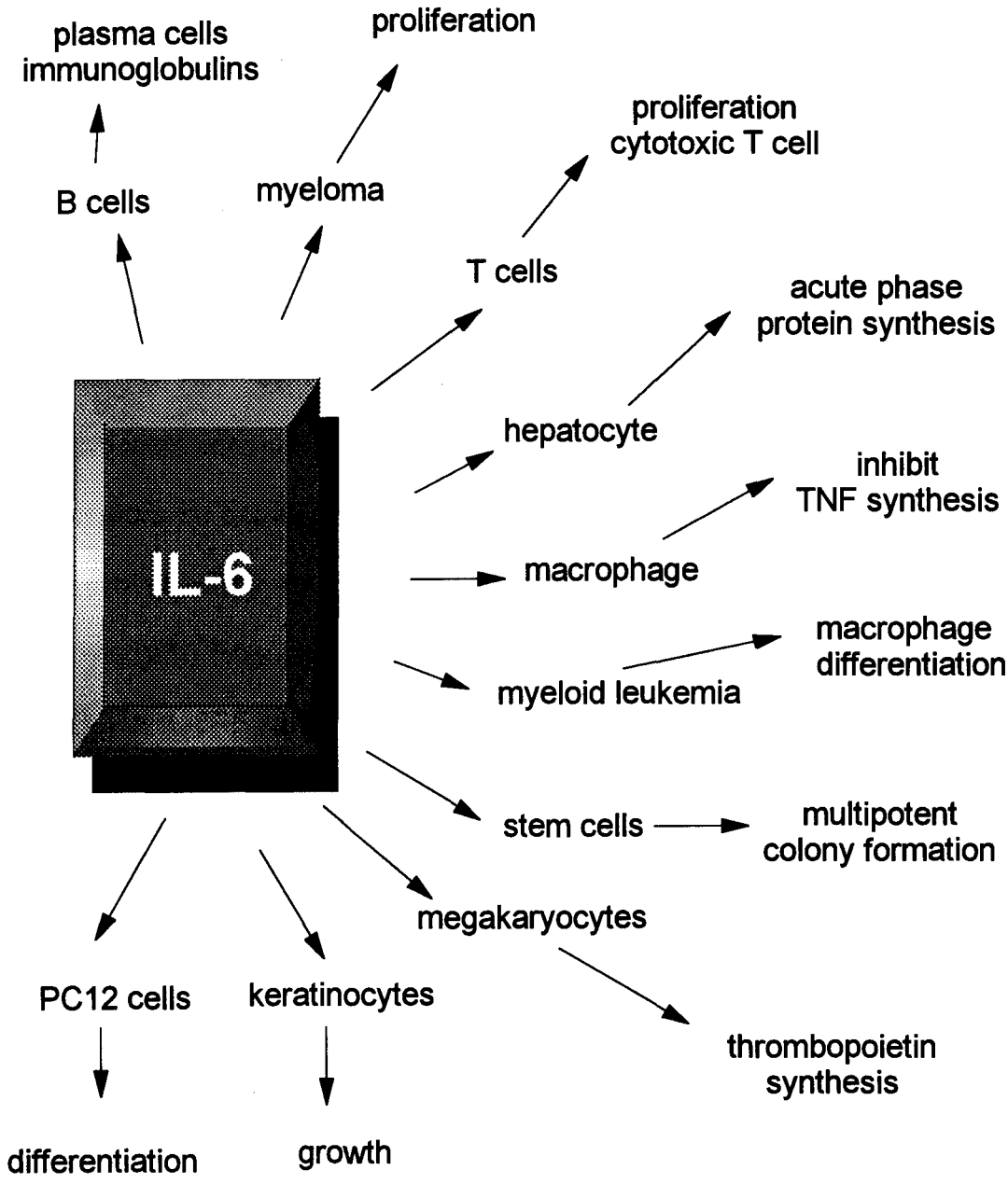


Fig. 6. Polyfunctional nature of IL-6. (Akira et al., 1992).

the hepatic endothelial cells to produce IL-6, and a paracrine activation of APP synthesis by hepatocytes is initiated (76). The proteins specifically induced by IL-6 include cysteine proteinase inhibitor, α_2-macroglobulin, fibrinogen, α_1-antiproteinase inhibitor, haptoglobin, α_1-antichymotrypsin, ceruloplasmin and C_1 esterase. In combination with IL-1, IL-6 also stimulates the synthesis of other acute phase proteins such as C3 and C-reactive protein (103,210). Maximal production of APP by hepatocytes requires the presence of glucocorticoids. Glucocorticoids enhance the production of APP by stimulating the upregulation of IL-6 receptors on hepatocytes. IL-6 also inhibits the production of corticosteroid-binding protein, CBG, thus increasing the availability of the glucocorticoid to the hepatocyte (23). Regulation of APP by IL-6 is via increased transcriptional activity (76,131). An IL-6RE has been described by Baumann et al.,1990 (26) in the 5' promoter region of the α_2 macroglobulin gene. This region contains nucleotide sequences similar to that described to bind NFIL-6 (6,7). Perlstein et al., 1991, (256) reported that interleukin 6 administration directly into the CNS of conscious rats stimulated the production of ACTH. The stimulation of ACTH production occurred through increased CRF secretion by the hypothalamus (235, 256). ACTH secretion would subsequently stimulate cortisol production by the adrenals and augment APP production by the liver.

The cells of the central nervous system, primarily astrocytes and microglial cells produce interleukin 6 (58,296) . Elevated IL-6 concentrations in the cerebral spinal fluid of patients with meningitis and sepsis have been documented (80).

The ability of IL-6 to cross the blood brain barrier has not been described. The production of IL-6 within CNS has been postulated to be responsible for the induction of LPS induced fever and anorexia. Damas et al., 1992 ,(80) found a strong correlation between increased body temperature and plasma IL-6 levels. LeMay et al., 1990 (197,198) have shown that intracerebroventricular injections of IL-6 to rats stimulated a dose-dependent increase in body temperature. Identical doses administered either i.p. or i.v. did no cause fever. Prostaglandin synthesis mediated the fever observed as indomethacin completely blocked IL-6 induced fever. A direct link between IL-6 and neural degeneration was recently described by Campbell et al., 1993 (58). Transgenic mice that overexpressed IL-6 in the central nervous system, exhibited severe neurodegenerative disease, tremors, ataxia and seizures (58).

Alterations in carbohydrate and protein metabolism are characteristic observations in patients with sepsis (122). TNF and IL-1 have been implicated as mediators of hypoglycemia and weight loss in these patients (93). Gershewald et al. 1990. (133) observed that immunization against IL-1 type I receptor attenuated not only cachexia and anorexia but also significantly reduced plasma IL-6 concentrations. The reduction of plasma IL-6 concentrations could also have been responsible for the reduction of symptoms observed. Oldenburg et al.,1993, (245) found that IL-6 receptor blockade prevented weight loss and anorexia to the same extent as that observed with IL-1 blockade. They concluded that the influence of IL-1 on cachexia is in part mediated by IL-6. Recently, Strassmann

et al. 1993, (308) illustrated that the administration of anti-IL-6 antibodies *in vivo* significantly decreased LPS induced hypoglycemia and weight loss. However, the administration of TNF antibodies reduced LPS induced hypertriglyceridemia and had a lesser effect on LPS induced hypoglycemia.

E. Glucorticoids and Insulin Physiology during Sepsis

Whether endotoxin directly stimulates endocrine cells or the stimulation of cytokine production and inflammatory mediators indirectly initiates the endocrinological stress responses, remains unknown (318). The hypothalamic-pituitary-adrenal axis responds primarily to endotoxin induced hypotension by increasing plasma concentrations of ACTH, catecholamines, cortisol and aldosterone (295,318). The pancreas responds to increased plasma cortisol and glucose concentrations by increasing plasma insulin and glucagon concentrations (31,295,318).

1. Glucocorticoids and Sepsis

Glucocorticoids are important to the natural host defense against endotoxin. Sepsis increases plasma glucocorticoids, such as corticosterone and cortisol (167, 295) A measurable increase in plasma corticosteroids occurs within the first one half hour after an endotoxin challenge (116, 248, 257). At the level of the pituitary gland, ACTH also increases in response to endotoxin. This increase ultimately

results in an increase in plasma cortisol. During physiological stress, such as sepsis, glucocorticoids are important for providing amino acids as substrates for gluconeogenesis in the liver and for the up regulation of enzymes necessary for gluconeogenesis (36,37). Cortisol also acts to maintain blood pressure and cardiac output as well as modulating an activated immune system.

Several aspects of glucocorticoid physiology are impaired by endotoxin and sepsis. Under normal conditions, the increases in plasma glucocorticoids observed during stress initiate feedback inhibition at the level of the hypothalamus and the pituitary by inhibiting CRF and ACTH (167). However, recently, Perrot et al. 1993., (257) demonstrated in a group of septic patients in which elevated cortisol and β lipotrophin concentrations were refractory to the suppressive effects of dexamethasone infusion. This finding suggests that other factors are interfering with the normal feedback mechanisms (257).

Corticosteroids exert their activity by binding to cytoplasmic glucocorticoid receptors (22,25). The binding of cortisol and corticosterone results in a structural and conformational change in the receptor converting it from the inactive to the active form (22, 25). The activated receptor then translocates to the nucleus where it binds to the GRE of a particular gene to either upregulate or inhibit transcription. The positive or negative modulation of transcription is determined by the interaction of the glucocorticoid-GRE complex with other nuclear regulatory proteins such as AP1 or NFκB (25).

Endotoxin wields a dichotomous impact on glucocorticoid receptors

depending on the cell type influenced. During an endotoxin challenge, several authors (8,159,304,305,332) have shown that glucocorticoid receptors in liver and cultured hepatocytes decrease. McCallum et al., 1983 (215) hypothesized that the hypoglycemia observed during sepsis occurs because of the inhibition of glucocorticoid mediated enzyme induction. This is due to the down regulation of cytosolic glucocorticoid receptors (215). Ali et al., 1990, (8) found that 24 hours after subcutaneous *E.coli* injection glucocorticoid receptor binding declined by 40%. The glucocorticoid receptor mRNA declined by 30%. Transformation of the receptor from the inactive to the active form was unaffected during sepsis (8).

In other cell types, for example, macrophages, glucocorticoid receptors are increased. Salkowski et al. 1992. (280) demonstrated that in RAW 264.7 and primary macrophages, endotoxin stimulated an increase in glucocorticoid receptor number without altering receptor affinity. The observed increase was present as early as 4 hours after stimulation and was maximal at 12 hours after stimulation (280). These findings suggest that LPS sensitizes the macrophage for both the positive and negative regulation of cytokine production.

In spite of increased plasma glucocorticoid concentrations during septic shock, the induction of hepatic gluconeogenic enzymes, glucose-6-phosphatase, fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (PEPCK) are decreased during endotoxicosis (11,12,83,160). The pharmacological administration of synthetic glucocorticoids such as dexamethasone has proven effective in restoring gluconeogenesis and euglycemia when administered prior

or concurrently with endotoxin (37,38,167,358). The benefits of glucocorticoid treatment are negated when endotoxin is administered. Berry and Smythe observed that glucocorticoid mediated protection could be removed by the administration of actinomycin D and other inhibitors of protein synthesis (295). They proposed that protection was related to the ability of corticosteroids to induce the synthesis of certain liver enzymes (38). PEPCK converts oxaloacetate to PEP, is considered to be the rate limiting step in gluconeogenesis (140), and is subject to regulation by several hormones including insulin, glucocorticoids, thyroxine and glucagon (140). Granner et al., 1990 (141) have confirmed that distinct regulatory sites exist on the PEPCK gene for the positive modulation by corticosteroids (176, 254) and the negative modulation by insulin (242,243). Therefore, the effect of glucorticoids may act to increase PEPCK transcription in normal and in stress situations such that euglycemia may be maintained in the organism.

PEPCK activity is significantly altered by endotoxin. Berry introduced the concept that LPS induced mediators released from immune cells, primarily macrophages, were culpable for antagonizing the positive effects of glucocorticoids (229, 295). The inhibitory activity derived from LPS-stimulated macrophages was termed , GAF, glucocorticoid antagonizing factor (230). Berry further investigated the properties of the antagonizing factor of endotoxin. He determined that GAF possessed protein-like properties and was derived from macrophages with a molecular weight of 150,000 (230,295). Berry also

postulated that glucocorticoids may act to control GAF production by macrophages (295). Although GAF was never identified specifically as one of the many cytokines known today, some scientists have postulated that GAF could have been a oligomeric complex of TNFα (295). Recently, Hill and McCallum, 1991 (160) demonstrated that rats treated with endotoxin exhibited lower rates of PEPCK transcription. They also found that both TNFα and IL-6 altered PEPCK transcription rates (160).

2. Glucocorticoid Regulation of Immune Function

For many years physicians have known that clinically, glucocorticoids posses the ability to reduce inflammation in conditions such as arthritis, trauma and injury (142). The reduction in inflammatory activity is attributed to the ability of corticosteroids to influence the activity of almost every immune cell type (142). Glucocorticoids in pharmacological doses suppress hematopoiesis, induce apoptosis in T cells and reduce neutrophil chemotaxis and phagocytosis (22,31,142). Glucocorticoids also have a potent effect on monocyte and macrophage activity. Increases in plasma cortisol cause decreased differentiation, margination, emigration/ and phagocytosis by macrophages (151,171, 236,297,335).

The research conducted over the last ten years has uncovered the fundamental rationale of corticosteroid's powerful anti-inflammatory activity. Glucocorticoids block the production of many cytokines including, IL-1, IL-2, G-

CSF, INFγ, IL-6 and TNFα (39,75,175,189,205,219,234,239,252). Glucocorticoids have the ability to alter cytokine production by binding directly to the regulatory elements of the cytokine gene and altering transcription or by altering the rate of translation (22). Indirectly, glucocorticoids alter cytokine production by regulating the production of other biological modifiers of cytokines such as prostaglandins (236).

TNFα stimulates the production of other cytokines such as IL-1β, IL-2, INFγ and IL-6 by macrophages and other immune cells. Glucocorticoids have been shown to significantly suppress TNFα production both *in vitro* (252) and *in vivo* (21,39, 248,271,339) . Remick et al. 1989, (271) investigated the *in vivo* dynamics of TNFα mRNA production and the effects of dexamethasone suppression in mice. They determined that TNFα protein and mRNA are rapidly induced following an endotoxic challenge. Time course studies indicated that 4 hours pretreatment with dexamethasone (4 mg/kg) significantly suppressed TNFα; however, post treatment as short as 20 minutes after endotoxin administration did not alter TNFα production in comparison with endotoxic control mice. Pretreatment with dexamethasone did not entirely suppress mRNA, indicating only partial inhibitory effects at the level of transcription (271).

Dexamethasone can also abolish TNFα synthesis without entirely eliminating TNFα mRNA accumulation (44, 365) . At the level of transcription, glucocorticoids exert strong inhibitory influence by reducing mRNA by 80% (42,44). However, the effect appears to be more effective at the level of

translation. Greater than 99% inhibition can be achieved at the protein level in peritoneal macrophages (44). Using nuclear runoff assays which measure the rate of message degradation, Remick et al., 1989, (271) determined that the slope of message degradation over time was sharply increased in the presence of dexamethasone. TNFα, other cytokines and fast inducible genes such as NO synthase contain UA rich regions in the 3' untranslated region of the gene. This region is believed to confer message instability. Using TNFα promoter:CAT constructs as well as CAT-TNFα 3' UTR constructs in transfected cells, Han et al. 1991 (147,148) demonstrated that dexamethasone treatment hinders the expression of TNF. Both regulation in the promoter and in the 3' untranslated region of the TNF gene was observed.

Glucocorticoids significantly depresses IL-6 production in macrophages, fibroblasts and endothelial cells (144,340). Waage et al.,1990, (340) showed that dexamethasone completely abolished IL-6 production in isolated human monocytes and RAW 264.7 macrophages. Dexamethasone substantially reduced IL-6 mRNA levels, indicating that the suppressive effect occurred primarily at the level of transcription (340). Akira et al. 1992. (7) have found GRE responsive elements in the promoter region of the IL-6 gene. Dexamethasone binding to its receptor occludes the inducible elements of the MRE enhancer region and the TATA box (start site) of the IL-6 gene (269). The presence of unstable UAUA regions on IL-6 mRNA is currently controversial. From experiments involving translational inhibitors of cytokine synthesis such as cycloheximide, IL-6 does not

appear to be regulated at the level of translation (65). Therefore, the inhibitory action of dexamethasone with respect to IL-6 production is currently limited to the transcriptional level (65).

3. Glucocorticoids and Clinical Sepsis

Controversy still exists in clinical studies as to the efficacy of glucocorticoid in the prevention of septic shock induced mortality (289, 298,). Authors have demonstrated in animal models (18,39,164) that pretreatment with glucocorticoid significantly reduces endotoxin-mediated mortality. Also, the adminstration of a glucocorticoid receptor antagonist, RU3486, has been shown to increase mortality during experimental endotoxicosis (50,153,216). Hinshaw and coworkers, in a series of papers, examined the protective effects of corticosteroid administration in LD_{100} canine and baboon models septic shock (77,162,163,164,165,348). They concluded that high dose corticosteroid administration prior or early in the septic trial protected the animals from hypotension, hypoglycemia, tissue necrosis and mortality. The prognosis for survival was especially good when the steroids were administered early and in conjunction with antibiotics (167).

Although these and numerous other authors have demonstrated the protective effect of corticosteroid therapy in animal models, the efficacy of corticosteroid use in clinical sepsis remains unproven (298). Early studies by Schumer et al., 1976, (289) found that the administration of corticosteroids provided protection to septic patients. However, in two later and rather

significantly cited clinical trials (47,166), the use of high dose methylprednisolone was shown to be ineffective and partially detrimental to septic patients. The factors that have been ascribed to the differences observed between the human and the animal studies include species differences, health and immune status of the clinical patients, preshock complications, the choice of the steroid and the dosage, inotropic and metabolic support, and primarily the failure of early recognition of sepsis (21,167,298). One of the distinct conclusions of all animal research was the early administration of corticosteroids, primarily for the reduction of TNFα synthesis. One of the inclusion criterion for clinical trials by both Bone and Hinshaw was the appearance of fever. The clinical appearance of fever is preceded by cytokine production (117,197) and therefore, corticosteroid therapy would be ineffective. Unfortunately, the early and possibly corticosteroid modifiable stages of clinical sepsis cannot be detected with present clinical techniques (167).

4. Insulin and Sepsis

A marked disturbance in substrate metabolism is one of the classic characteristics of Gram negative septic shock (107). Changes in plasma energy substrates such as glucose and amino acids have been reported to occur after lethal doses of endotoxin in several mammalian species used as experimental models of septic shock (4,116,122,350,353). The alterations in plasma metabolites have been attributed to the presence of endogenous mediators and

elevated circulating hormones.

Several authors have reported that endotoxin administration alters plasma concentrations of glucagon and insulin (11,12,83, 104, 105, 187, 270, 306). The *in vivo* response to endotoxin is associated with a profound hyperglycemia and hyperinsulinemia which is often followed by a lethal hypoglycemic phase (107). Buchanan and Filkins, 1976, (52) found that serum insulin levels were significantly elevated 90 minutes post intravenous endotoxin. In response to a glucose challenge, insulin values were inappropriately elevated when compared to the control values (180 vs. 55 μU/ml). Although insulin levels return to pre-endotoxin values by eight hours, the values are significantly elevated above control values between two and six hours post endotoxin (52). Knowles et al. 1986, (187) observed that rats administered *S. typhimurium* endotoxin exhibited increases in plasma insulin and glucose concentrations. In rats made septic by cecal ligation and puncture Ardawi et al. 1989, (11) 1990, (12) also measured significantly elevated plasma insulin and glucagon concentrations.

As stated above, within the first two hours after endotoxin administration, a hyperglycemic, hyperinsulinemic phase has been observed in several species (11,104,107). Even in the presence of significantly elevated plasma insulin, peripheral tissues such as muscle and adipose are unresponsive to insulin-stimulated glucose uptake and thus, plasma glucose remains elevated (122,187,221,222). This observation has been classically cited as "sepsis-induced insulin resistance" (193,221). Both sepsis and burn injury produce clinical states

characterized by a poor response to insulin and a glucose intolerance. Shangraw et al. 1989. (291) investigated the difference between septic and postburn insulin resistance. They concluded that septic and postburn insulin resistance differ in that peripheral tissue glucose uptake in sepsis is refractory to pharmacological insulin stimulation. Nonseptic burn injury patients exhibited a normal response to insulin administration and insulin-induced potassium uptake is unchanged in both conditions (291).

The role of endotoxin as the direct stimulator of insulin secretion remains controversial. The hyperglycemia and possibly other endogenous factors have been suggested as key factors responsible for glucose dyshomeostasis (4,82,104, 110,111,112) . The hyperinsulinemia observed during sepsis is not always related to the increase in plasma glucose concentrations. Cornell, 1989, (70) demonstrated that low dose endotoxin stimulated hyperinsulinemia and glucagonemia without hyperglycemia. Rayfield et al.,1977, (270) observed fever and subsequent increases in plasma insulin and glucagon with little change in plasma glucose concentrations during acute nonlethal endotoxicosis in fasted human subjects. Yelich and Filkins,1980, (356) demonstrated that the liver's capability of removing endotoxin from the circulation was unimpaired during endotoxicosis and that the mechanism for increased plasma insulin was due to hypersecretion by the endotoxic pancreas. In subsequent studies, these authors also observed that the media from inflammatory exudate cells stimulated insulin release from the isolated perfused pancreas (111,357). The acronym, MIRA,

(macrophage insulin releasing activity) was attributed to the monokine producing this response (108). MIRA eventually was purified and found to be interleukin 1 (108) . Cornell 1989 (70) demonstrated that the cytokine IL-1 was the mediator of the hyperinsulinemia observed during endotoxicosis. Hyperinsulinemia was observed 30 minutes after injection of recombinant human IL-1 into rats (70).

The lethal phase of hypoglycemia observed in the study by Buchanan and Filkins and other authors, occurs two to six hours after endotoxin administration when insulin levels are not markedly elevated (52). The hypoglycemia is the result of a negative balance between the production of glucose by gluconeogenesis and the disposal of glucose by the peripheral tissues (107). The prior hyperinsulinemic phase may have altered both the tissue sensitivity to insulin and the liver enzymes responsible for gluconeogenesis (107). Filkins and Figlewicz 1979, (109) demonstrated that the epididymal fat pads from endotoxic rats manifested increased basal glucose oxidation. Moreover, an enhanced increase in glucose oxidation of the endotoxic tissues was observed to several insulin doses (109). Lang and Dobrescu,1991 (193) observed that injections of live *E.coli* into rats increased whole body disposal of glucose by 53% in comparison to controls. Sepsis induced glucose uptake by the liver, spleen, lung, ileum and skin under hypoglycemic-insulinopenic conditions was augmented (193). This observation suggests that the non-insulin dependent tissues (ie. macrophages and Kupffer cells) requirement for glucose is enhanced during sepsis (55, 73,74). Insulin may not directly alter glucose uptake in these tissues; however, insulin may alter other

factors related to the enhanced septic glucose utilization (73,74,333).

During sepsis hepatic gluconeogenesis is severely impaired in the liver and in the kidney (9,106,173,187,188). Ardawi et al., 1990, (12) observed that the maximal activities of glucose-6-phosphatase, fructose-1,6-bisphosphatase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase (PEPCK) were markedly decreased in kidneys obtained from septic rats. PEPCK activity in the liver is severely depressed during sepsis. McCallum et al. 1983 (215) observed that following endotoxin challenge hepatic PEPCK activity dropped rapidly and was consistent with the overall hypoglycemia which subsequently appeared. The upregulation of PEPCK activity by glucocorticoid therapy was negated by endotoxin pretreatment (215). Recently, Hill and McCallum, 1991, (160) have shown that the PEPCK transcription rate was significantly reduced by endotoxin pretreatment. Insulin is a negative modulator of the PEPCK gene by affecting a 15 base pair sequence (242,243). This group has also shown that insulin and phorbol esters exert dominant regulation, since both prevent stimulation of PEPCK transcription in the presence of the inducers cAMP or glucocorticoid. Although phorbol esters and insulin de-induce PEPCK gene transcription through distinct signal pathways, the final target of both substances is the same DNA element (243). The hyperinsulinemia observed during sepsis may exert dominant suppression of PEPCK activity, thus causing hypoglycemia. Recently, Hill and McCallum 1992, (161) challenged the role of insulin as a modulator of PEPCK activity during sepsis. Using diabetic rats, they found no alterations in PEPCK

activity suggesting that a cytokine mediator was responsible for the alterations in PEPCK transcription observed.

Insulin can exacerbate endotoxin lethality. Buchanan and Filkins, 1976, (51) have shown that an exogenous dose of insulin given at the same time as endotoxin can increase mortality from 20 to 97%. Tolbutamide, a stimulator of endogenous insulin secretion can also increase mortality (51). Endotoxin in combination with phorbol esters (PMA) also results in an increase in mortality (177). The animals in the above studies are hypoglycemic and have elevated lactates prior to death (178). Satomi et al., 1985, (284) have observed that glucose administered to correct the endotoxin-induced hypoglycemia is ineffective and death is quickened. Insulin may produce or affect other mediators that initiate hypoglycemia and are unresponsive to restoring euglycemia.

5. Insulin Regulation of Immune Function

Does insulin regulate immune function and cytokine production ? Cornell, 1989, has documented that cytokines, primarily IL-1 stimulate insulin secretion (70). However, whether insulin can modulate cytokine secretion normally or during sepsis remains unknown. Several pieces of indirect evidence and a few *in vitro* studies suggest a possible role for insulin as a cytokine modulator.

Insulin receptors have been classically associated with metabolically active cells such as hepatocytes, myocytes and adipocytes (362). With the use of radiolabelled insulin as a ligand, insulin receptors have been identified on

erythrocytes, platelets and lymphocytes (321). Several authors (20,29,30,321,364) have demonstrated that insulin receptors are present on monocytes. Tsibris et al.,1980, (321) found that human monocytes contain approximately 50,000 insulin receptors per cell, suggesting that insulin may be important for the metabolism and function of this cell class. Bautista et al.,1987 (29), 1989 (30) also found insulin receptors on the surface of peritoneal macrophages and demonstrated down regulation of insulin receptors in *Propionibacterium acnes* activated macrophages. Bierger et al.,1980, (45) demonstrated that insulin binding to monocyte receptors can initiate a specific reaction increasing hexose monophosphate enzyme activity and antimicrobial activity .

Certain hormone receptors associate with MHC Class I antigens (201). The insulin receptor and MHC Class I appear to be situated in close proximity in the cell membrane as evidenced by coimmunoprecipitation the presence of insulin in the media also increases MHC I and insulin receptor association on monocytes (201). Insulin receptor affinity appears to correspond with MHC class I alleles. Helderman et al., 1992, (156) have discovered a monocyte insulin regulatory protein (MIRRF), a small molecular weight peptide that stimulates the upregulation of insulin receptors on lymphocytes and is derived from monocytes stimulated with insulin. Insulin is necessary for lymphocyte proliferation and activation (201).

Insulin has been shown to act as a growth factor, as some cell lines require it for growth and survival *in vitro* (69,100,122). Freund et al., 1993 (122) demonstrated that insulin and IGF-1 can increase mitogenesis and glucose

metabolism in the multiple myeloma cell line RPMI 8226. The regulation of mitogenesis and glucose metabolism by insulin was postulated to potentiate malignancy (122).

The immunologic activities of peripheral leukocytes is altered by insulin (264). The polymorphonuclear cell functions such as adherence, chemotaxis, phagocytosis and superoxide production are impaired in diabetic patients (135). The *in vivo* administration of insulin to diabetic patients improves immunological activity. Cavot et al. 1992 (61) demonstrated *in vitro* that increasing concentrations of insulin from 40 to 320 nM increased PMN chemotaxis.

Insulin increases PGE_2 binding to P388D1 macrophages (265). PGE_2 is a potent inhibitor of macrophage TNF production. However, Doherty et al., 1992, (84) observed that murine peritoneal macrophages in the presence of LPS (10 μg/ml) and insulin concentrations from 0.2 μU/ml to 400 μU/ml increased TNFα production as assessed by L929 bioassay and ELISA above that of controls. IL-6 bioactivity was increased by insulin and LPS. Insulin had no effect on IL-1 production. The transcription of TNFα was slightly reduced in the insulin treated cells suggesting the site of regulation is post-translational. IL-6 gene transcription was unaltered from the control (84). Recently, Halan and Minowada, 1992, (172) reported that insulin, IGF-1 and IGF-II stimulated IL-1β production in LPS stimulated monomyelocytic cell lines, although none of the hormones stimulated IL-1 production alone. This affect appears to specific to monocytic cell lines as T-cell lines tested were unresponsive to LPS and insulin treatment (172).

The effects of insulin on cytokine production may be due to its ability to cross react with the IGF-1 receptor, which has been found on macrophages (179, 275, 364). Rom and Paako, 1991, (275), have demonstrated that human alveolar macrophages express IGF-1 receptors only when activated with LPS or asbestos. The structural similarity between the IGF-1 and the insulin receptor indicates that insulin can cross react weakly with the IGF-1 receptor (124, 179, 364). Insulin-like growth factors (IGF-1) and (IGF-2) are peptide hormone homologous to insulin that have rapid insulin-like and slow-growth promoting actions *in vivo* (232,240,282). The circulating plasma pool of IGF-1 is derived primarily from hepatocytes under the influence of pituitary growth hormone (282); however, other cells such as macrophages, endothelial cells and fibroblasts can produce IGF-1 under the influence of growth hormone and other currently unknown stimuli (14,91,170,275).

Currently the role of IGF-1 as a mediator of cytokine production or the role of IGF-1 in sepsis remains sparsely documented. Edwards et al., 1991 (90,91) have demonstrated that GH serves a protective action against the lethal effects of *Salmonella typhimurium in vivo* . In the above studies plasma IGF-1 was not measured, and it could not be concluded whether the protective action of GH was direct or indirectly modulated by IGF-1. They also demonstrated that hypophysectomy in rats altered TNFα production (90). GH treatment of endotoxic macrophages *in vitro* has been shown to enhance TNFα production (92). One preliminary report has shown that plasma IGF-1 is not increased in septic patients

in response to a 48 hour infusion of GH (79). Fu et al., 1990, (125) have demonstrated *in vitro* that GH and IGF-1 are both potent signals in polymorphonuclear cells (PMNs) superoxide generation. PMNs have also been shown to synthesize GH and IGF-1 and may be able to activate superoxide generation in an autocrine fashion (125).

6. Insulin and Clinical Sepsis

The hyperinsulinemia characteristic of experimental septic shock is not always detected in the hospitalized septic patient (64,116,122,183). The observation of hyperinsulinemia appears to depend on several factors such as severeity and stage of sepsis, the plasma glucose concentrations, the administration of dextrose or total parenteral nutrition (203) and the timing of the blood samplings (336). However, most clinicians agree that the septic patient does have an altered sensitivity to insulin (237,291). In spite of plasma insulin concentrations that should theoretically prevent lipolysis, stimulate peripheral glucose uptake and prevent protein breakdown, the opposite scenario of hypertriglygeridemia, hyperglycemia and protein catabolism is often documented (64, 122, 270). Freund et al. 1978 (122) found in a group of 15 septic patients a significant elevation in plasma aromatic amino acids, phenylalanine and tyrosine concentrations at an insulin/glucagon ratio of 3.6. Sauerwein et al. 1991 (285) concluded as well that significantly higher insulin concentrations were necessary to obtain equal values for endogenous glucose production and glucose tissue

uptake in septic patients compared to control subjects. The cellular mechanisms for the sepsis-induced altered sensitivity to insulin remain unknown; however, from these and other clinical studies the authors have determined that muscle tissue exhibits a greater degree of insulin resistance in comparison to adipose tissue (284,285,286).

Insulin administered therapeutically has profound systemic effects, especially with respect to plasma potassium concentrations (291). Therefore, in contrast to glucocorticoids, insulin has not been routinely used as a pharmacological modality in patients with shock. Insulin administration is indicated only if the patient exhibits significant hyperglycemia. Bronsveld et al. 1985 (49) observed that an infusion of the combination of glucose-insulin-potassium (GIK) to septic patients in cardiac failure significantly improved survival. They concluded that because GIK increased cardiac output and decreased systemic vascular resistance, and improved 0_2 consumption, its administration may be considered when conventional volume loading and vasoactive medication have failed (49) . Insulin administration to septic patients may reduced muscle catabolism. Mitchell and Norton,1990, (226) demonstrated in an *in vitro* system that insulin could protect against the muscle proteolysis induced by plasma from septic patients. The clinical use of insulin as an anabolic agent has not been seriously explored in comparison to other anabolic hormones such as IGF-1 (87,232) and growth hormone .

F. Summary

Insulin and glucocorticoids circulate within the organism in a precarious balance regulating metabolic activity. The fundamental determinant of homeostasis is the appropriate "ying and yang" of these hormones. Under the influence of physiologic stress, such as sepsis, the balance between these two hormones is significantly disturbed, thus inducing an ominous metabolic dyshomeostasis. The ability of elevated circulating glucocorticoids to induce gluconeogenesis and restore euglycemia as well as to modulate and supress immune activity is profoundly impaired during septic shock.

The findings of Granner (242,243) are important to the research proposed in this dissertation for two reasons:

1) since insulin is a dominant regulator of PEPCK activity, one could hypothesize that the elevated insulin concentration observed during sepsis may overide the stimulatory activity of glucocorticoids, thereby inducing hypoglycemia and death.

2) the control of PEPCK gene expression is modulated in opposite directions by two hormones, and this glucocorticoid-insulin regulation of gene expression is not limited to PEPCK (204). Glucocorticoids suppress TNF and IL-6 production. Sepsis-induced insulin production may also modulate the production of TNF and IL-6 in a manner similar to PEPCK.

Therefore, the purpose of the following studies is to examine the ability of

insulin to alter the metabolic status of the animal as well as the production of TNF and IL-6 in endotoxic and dexamethasone-protected models of septic shock.

CHAPTER III

MATERIALS AND METHODS

A. Animals

Male Holtzman viral antibody free rats were used for all experiments. The body weight range for the initial in vivo studies was 400 to 450 g. The liver perfusion studies used rats of 500 to 600 g in order to facilitate the surgical procedures. The rats were obtained from Harlan (Madison,WI) or Sasco (St. Louis, Mo). They were housed in pairs in plastic containers covered with filtered hoods in order to minimized airborne infections. Environmental conditions were a temperature of 24°C, a humidity of 45-50% and a 12 hour light and dark cycle (7:00 am to 7:00 pm CDT). The rats had ad libitum access to Purina rat chow and fresh water. Prior to the initiation of experiments, the rats were acclimatized to the facility for at least 7 days. The rats were moved to the laboratory from the animal facility the evening before the experimental procedures in order to reduce stress-induced alterations in plasma hormones that would result during transportation. All experiments were initiated between 10:00 am and 2:00 pm. When fasting was required, food, but not water was removed at 4:00 pm on the day prior to the experiment.

B. Cells

1. Cell Lines

The cell lines used for experimentation were obtained from the laboratory of Elizabeth Kovacs. PhD, Dept. of Cell Biology, Neurobiology and Anatomy, LUMC. The ANA1 cell line (75) is a murine macrophage cell line obtained from the bone marrow of the C57/BL mouse which was transformed with a J2 retrovirus expressing v-myc/v-raf oncogene (L. Varesio, Laboratory of Molecular Immunoregulation, National Cancer Institute, Fredrick, MD). RAW 264.7 cells are macrophages from the BALB/c mouse, which were transformed with Abelson leukemia virus and supplied by American Type Culture Collection, Rockville, MD.

Two cell lines were used to bioassay the cytokines TNF and IL-6 in plasma, media and perfusate samples of rat origin. IL-6 was measured using the B9 cells, obtained from Dr. Jack Gauldie, Department of Pathology, McMaster University, Hamilton, Ontario, Canada. B9 cells are a murine B-cell hybridoma cell line dependent on IL-6 for growth (1,194). TNF was measured using the L929 cytotoxicity bioassay. L929 mouse myeloma cells are an adherent fibroblast cell line, obtained from the American Type Culture Collection, Rockville, MD. The cytokine bioassays are described in detail in the assay section of this dissertation.

2. Primary Cells

Primary macrophage cultures were also used for several experiments.

Peritoneal macrophages and Kupffer cells were obtained from male, fed Holtzman rats which had body weights of 400 to 450 g. The procedures required for the isolation of these cells are described in detail later in this dissertation.

C. Agents

1. Endotoxin

Lyophilized *Salmonella enteritidis* lipopolysaccharide Boivin, lot # 764190, was utilized for all experiments and was purchased from Difco, Detroit, MI. The endotoxin was suspended in 0.9 % sodium chloride prior to injection. For cell culture studies, the endotoxin was serially diluted to the desired concentrations in sterile calcium and magnesium free phosphate buffered saline (PBS)(Gibco, Grand Island, NY).

2. Dexamethasone

For the in vivo and liver perfusion experiments, dexamethasone 21-acetate (Sigma, St. Louis, Mo.) was suspended in 0.9% sterile sodium chloride and sonicated for 5 minutes using a Bransonic 12 sonicator (VWR Scientific, USA).

For cell culture studies, sterile dexamethasone sodium phosphate (Sigma, St. Louis, Mo) at an initial concentration 4 mg/ml, was then diluted to the required concentrations using sterile phosphate buffered saline without calcium or magnesium (Gibco, Grand Island, NY).

3. Insulin

Humulin R (Lilly, Indianapolis, IN), human insulin of recombinant origin was used in all experiments. For in vivo experiments, insulin was diluted in 0.9% sodium chloride and 0.5% bovine serum albumin (BSA) (Sigma, St. Louis, Mo.). For cell culture experiments, insulin was initially diluted in 0.5% BSA and further diluted in sterile PBS . The endotoxin content of the insulin was determined to be below the detectible limits (< 0.001 ng/ml) of the Limulus Amoebocyte Lysate Assay (see assay section).

4. Tolbutamide

Tolbutamide, (Upjohn, Kalamazoo, MI), tolbutamide sodium , was used as a stimulator of endogenous insulin secretion. The tolbutamide was diluted for injection in sterile 0.9% sodium chloride.

5. Tumor Necrosis Factor

Recombinant murine tumor necrosis factor (Genzyme, Cambridge, MA) was used as the TNF standard in both the L929 cytotoxicity assay and the ELISA. The TNF was reconstituted with endotoxin free water and diluted further with sterile PBS.

Recombinant murine TNF was also used to test the stability of TNF in the perfusion apparatus. TNF was reconstituted with endotoxin free water (Sigma, St. Louis, Mo) and added to 100 ml of KRB with 5% albumin for this procedure.

Endotoxin content of the reconstituted TNF was determined to be less than 0.01 ng/ml by the Limulus Amoebocyte Lysate test (Whittaker, Walkersville, MD).

6. Interleukin 6

Recombinant human interleukin 6 (Endogen, Boston, MA) was added to the B9 cells culture media in order to maintain growth. Recombinant rat IL-6, (Dr. J. Gauldie, McMaster University, Hamilton, Ont., Canada) was utilized to generate the standard curve in the B9 proliferation bioassay. The IL-6 was diluted in Iscove's Minimal Essential Media with 5% fetal bovine serum (FBS). Endotoxin content was determined to be less than 0.01 ng/ml by LAL testing.

7. Phorbol myristate acetate (PMA)

PMA (Sigma, St. Louis, Mo) was utilized as a stimulator of protein kinase C (PKC) in tissue culture experiments involving the ANA1 and RAW 264.7 macrophages. PMA was initially dissolved in dimethyl sulfoxide (DMSO) and diluted further in PBS.

8. 1-(5-isoquinolinyl sulfonyl)-2-methyl-piperazine (H-7)

H-7, (Calbiochem, San Diego, CA), an inhibitor of protein kinase C and other kinases was utilized in tissue culture experiments involving ANA1 cells, RAW 264 cells and peritoneal macrophages. H-7 was dissolved in DMSO at a concentration of 1 mg/ml. Further dilutions were performed using sterile PBS.

9. Indomethacin

Indomethacin (Sigma, St. Louis, Mo), an inhibitor of cycloxygenase and thus, prostaglandin synthesis, was utilized in cell culture experiments involving the ANA1, RAW 264.7 and peritoneal macrophages. Indomethacin was dissolved in 70% ethanol at a concentration of 1 mg/ml. Further dilutions were performed with sterile PBS.

10. Insulin-like Growth Factor 1 (IGF-1)

IGF-1 was used in cell culture experiments to determine if insulin was acting similar to this growth factor. Lyophilized recombinant human IGF-1 (Gropep, Adelaide, Australia) was reconstituted to 1 mg /ml with sterile saline containing 3% BSA. Further dilutions were performed with sterile PBS.

11. Okadaic Acid

Okadaic acid, an inhibitor of serine phosphatase activity and, hence, a promoter of PKC actions, was used in cell culture experiments to determine its effect on cytokine production. One hundred micrograms of okadaic acid was reconstituted in 10 ml of 70% ethanol and further diluted with sterile PBS.

12. Cycloheximide

Cycloheximide, a non-specific inhibitor of protein synthesis, was utilized in tissue culture experiments involving the ANA1, RAW and peritoneal macrophages.

Cycloheximide was reconstituted and diluted to the required concentrations in PBS.

D. *In Vivo* Projects

1. The Effect of Exogenous Insulin Administration on Lethality and Glucose Homeostasis.

To investigate if insulin could alter dexamethasone protection against the lethal effects of endotoxicosis in vivo, the following experiments were designed.

Overnight, fasted male Holtzman rats were assigned to control and treatment groups as illustrated in table 4. The rats initially received either dexamethasone (DEX) at a dose of 0.33 mg/kg dexamethasone acetate , i.p., or an equal volume of saline. Three hours later, endotoxin (EXT) or saline, was administered at a dose of 20 mg/kg , i.p. One hour post-endotoxin, the rats were given either insulin or saline at 1.0 U, s.c. The animals were followed over a 12 hour time period and time of death was recorded. Prior to death, which was assessed to be the time the righting reflex was lost, a blood sample of 1 ml was taken and analyzed for glucose, lactate and insulin concentrations.

2. The Effect of Insulin Dose on Mortality

To determine the minimum effective dose of insulin needed to alter dexamethasone action in the endotoxic rat, male Holtzman rats were assigned to

Table 4-- Treatment groups for exogenous insulin experiments.

GROUP	DEXAMETHASONE	ENDOTOXIN	INSULIN
1.	-	-	-
2.	-	+	-
3.	-	+	+
4.	-	-	+
5.	+	-	-
6.	+	+	-
7.	+	+	+
8.	+	-	+

treatment groups illustrated above. The treatment protocol was identical to that used in the first experiment except three doses of insulin were tested, 1 U, 0.5 U and 0.25 U in order to determine the minimum effective dose. The animals were followed over a 12 hour time period and time of death was recorded.

3. The Effect of Endogenous Insulin Secretion on Lethality and Glucose Metabolism

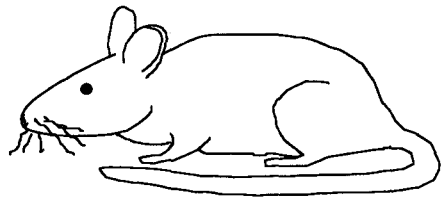
The ability of endogenous insulin to alter dexamethasone protection against endotoxicosis was tested by administering sodium tolbutamide, (Orinase, Upjohn, Kalamazoo, MI), a sulfonyl urea stimulator of insulin secretion. Male Holtzman rats were assigned to treatment groups as illustrated in table 5. The same treatment protocol as that illustrated in fig. 7 and discussed previously was followed, however instead of insulin, 66 mg/kg tolbutamide was administered s.c. 1 hour post-endotoxin. The animals were then followed over a 12 hour time period, and time of death was recorded. Prior to death, a blood sample was taken by cardiac puncture and analyzed for glucose, lactate and insulin concentrations.

4. The Effect of Restoring Euglycemia on Mortality

In order to determine if the hypoglycemia resulting from the treatment protocol was responsible for the mortality in these animals, 5% dextrose solution was administered i.p. to a each treatment group after insulin administration to determine if mortality could be altered. Plasma glucose was monitored initially

Table 5-- Treatment groups for endogenous insulin experiments.

GROUP	DEXAMETHASONE	ENDOTOXIN	TOLBUTAMIDE
1.	-	-	-
2.	-	+	-
3.	-	+	+
4.	-	-	+
5.	+	-	-
6.	+	+	-
7.	+	+	+
8.	+	-	+



DEXAMETHASONE ACETATE
(0.33 mg/kg)

3 hr.

ENDOTOXIN (*S. enteriditis*)
(20 ng/ kg)

1 hr. 1 hr.

INSULIN
(46 ug/kg)

TOLBUTAMIDE
(66 mg/kg)

analyses

- mortality
- plasma for:
glucose
lactate
insulin
TNF
IL-6

- mortality
- plasma for:
glucose
lactate
insulin

Fig. 7. Basic treatment protocol for the *in vivo* experiments.

every 15 minutes for two hour and then at 1 hour intervals during the treatment period. One ml of dextrose was administered if plasma glucose concentrations fell below 70 mg/dL . Mortality was assessed at the end of 12 hours.

E. *In Vivo* Cytokine Measurements

1. The Effect of Insulin on Cytokine Production in the Endotoxic Rat

In order to evaluate the effects of insulin on TNF and IL-6 production in a dexamethasone-protected endotoxic rat model, fasted male Holtzman rats (n=8/ group) were weighed and administered either DEX or saline 3 hours prior to endotoxin administration. At the end of the 3 hours, the rats were anesthetized with 50 mg/kg pentobarbital i.p. and placed supine on a warming pad heated to 37°C. A baseline tail-snip blood sample was obtained, and then 20 mg/kg endotoxin or an equal volume of 0.9% saline was injected, i.p. Blood samples were then taken every 15 minutes for a period of 2 hours. After 1 hour 1 U of either insulin, s.c., or saline was given. Blood samples were collected every 15 minutes for 2 hours post insulin. Blood was collected in heparinized microtubes and placed on ice until samples were centrifuged and the plasma was removed. Plasma was analyzed for glucose, lactate, TNF and IL-6.

2. The Effect of Insulin- Induced Hypoglycemia on Cytokine Production

To observe the effect of insulin-induced hypoglycemia on TNF and IL-6

production, one group of male Holtzman rats was anesthetized and given 5U of insulin s.c. Blood samples were taken every 15 minutes for 2 hours. Plasma was analyzed for TNF and IL-6.

F. *Ex Vivo* Liver Perfusion Studies

The liver is a biologically strategic organ in sepsis. The liver is necessary metabolically for the maintenance of euglycemia and the location as the first organ to process endotoxin from the bowel, may also make it a primary producer of cytokines from its resident population of macrophages, the Kupffer cells. The following experiment was designed to determine if the endotoxic liver was able to produce TNF and IL-6 and also to observe if the production of these cytokines could be modulated by dexamethasone and insulin.

1. Animal Preparation

Male Holtzman rats (500-600 g) were fasted overnight, but had ad libitum access to water prior to experimentation. The rat was injected with either 0.33 mg/kg dexamethasone acetate or saline, i.p.. Three hours post dexamethasone, the rat was injected with Salmonella enteritidis endotoxin (20 mg/kg) or saline, i.p.. Insulin (1 U) or saline was administered, s.c. one hour post endotoxin. The animal was anesthetized with 50 mg/kg sodium pentobarbital i.v. in preparation for excision of the liver.

2. Liver Perfusion Apparatus

Liver perfusion experiments were performed in a specially designed perfusion-aeration apparatus (Medical Research Apparatus; Clearwater FL) (fig. 8) as originally described by Miller et al., 1951 (225). Prior to the perfusions, all glassware was disinfected and autoclaved and all tubing was gas sterilized. Krebs bicarbonate buffer (KRB; 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$-$2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$-$7H_2O$ and 25 mM $NaHCO_3$; pH 7.4) containing 5 mg/ml bovine albumin (BSA) (RIA Grade; Sigma) was continuously recirculated through the system using a Masterflex peristaltic pump (Cole-Palmer Instruments Co., Chicago, IL). The KRB used in all experiments was filter- sterilized through a 0.2 μM filter and stored in sterilized 500 ml glass bottles at 4 °C. The endotoxin content of the buffer was determined to be < 0.01 ng/ml by Limulus Amoebocyte Lysate Assay (Whittaker, MA). For each experiment 100 ml of KRB was recirculated through the system. Temperature was maintained at 36-37 °C by a system of heating coils. The perfusate was oxygenated continuously with 95% O_2/ 5% CO_2 by passing over a gas exchange lung. To remove particulate or fibrous material produced during the perfusion, a sterile lucite filter lined with silk mesh was placed in the system. Between perfusions the apparatus was dismantled and washed with Micro disinfectant, rinsed and dried in a 300 °F oven. The apparatus was reassembled and rinsed twice with 150 ml of sterile irrigation normal saline.

Prior to initiation of experiments, the empirical determination of TNF and IL-6 stability during the perfusion period was required. Recombinant murine TNF

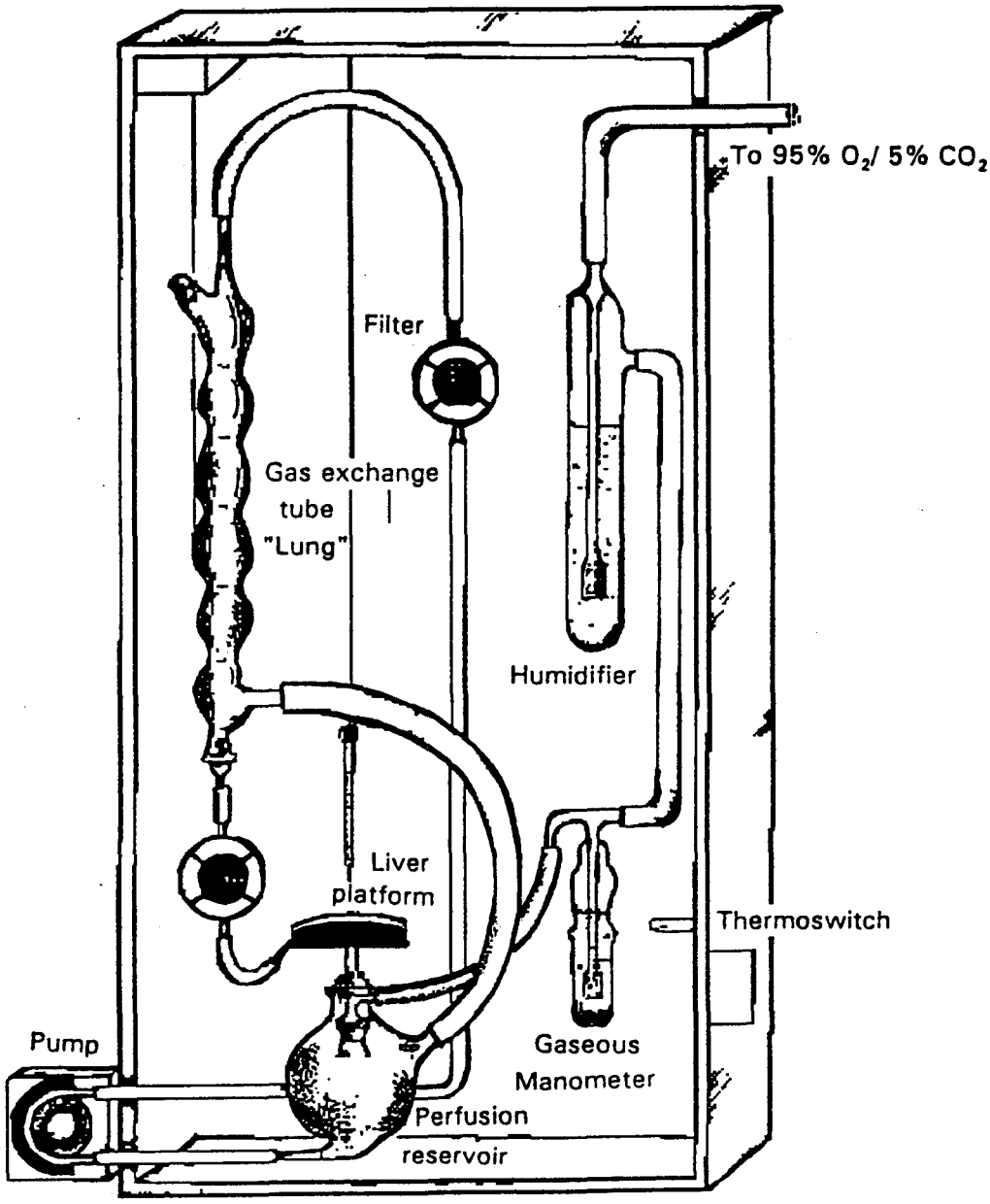


Fig. 8. Diagram of the liver perfusion apparatus

or recombinant murine IL-6 was added to KRB + 5% BSA at a concentration of 1 ng/ml was recirculated through the system under the normal perfusion conditions without the presence of a liver for one hour. 100 μl of perfusate was removed every 5 minutes and immediately frozen. The perfusate was then analyzed for TNF and IL-6.

3. Liver Removal

Removal of the liver was performed under sterile conditions. All instruments, gauze sponges, suture and cannulas were previously gas sterilized or autoclaved to reduce contamination.

Fasted, treated rats were injected i.p. with sodium pentobarbital to induce pain insensivitity. The level of anesthetic was assessed by unresponsiveness to a tail pinch. The rat was placed supine on a prewarmed metal tray and the abdomen was swabbed with betaine and 70% ethanol. A midline incision was made from the level of the pubic symphysis caudally to the xiphoid process just below the diaphragm. Two lateral incisions were made below the diaphragm left along the ribcage to the distal edge of the spleen and right along the ribcage to the distal edge of the liver. The intestines and mesentery were reflected to the animal's left and placed on sterile gauze soaked in sterile normal saline. This procedure exposed the portal vein. The connective tissue ligature attaching the posterior lobule of the right lobe of the liver to the fascia surrounding the abdominal vena cava was cut, and a 5 cm 3-0 suture was placed around the vena

cava above the renal veins but not tied. Another 5 cm suture was placed around the distal end of the portal vein but not tied. A final 5 cm suture was placed around the proximal end of the portal vein above the termination of the lineal vein. Heparin (Upjohn, Kalamazoo, MI) (500 U/rat) was injected into the dorsal penile vein and allowed to circulate for 6 minutes prior to continuation of the surgery. During this time period the anterior lobule of the right lobe and the Spigelian lobe were freed from the surrounding connective tissue. At the end of 6 minutes, the sutures surrounding the vena cava and the distal end of the portal vein were securely tied. A small incision was made in the portal vein, and a cannula (PE 260 tubing) was inserted into the portal vein and securely tied. The cannula was immediately flushed with warm KRB. The chest cavity was opened to expose the heart, and a suture was placed around the thoracic inferior vena cava. A 1 cm cannula (PE 280 tubing) was inserted through the right atrium into the inferior vena cava and tied with a suture. The liver was quickly excised from the abdominal cavity and continuously flushed with warm, KRB until the fluid from the liver was free of red blood cells. The liver was then placed into the perfusion-aeration apparatus chamber within 4 minutes of the tying of the first suture surrounding the vena cava.

4. Liver Perfusion

Prior to the introduction of the liver into the perfusion-aeration apparatus, the perfusion reservoir was filled with 100 ml of KRB + 5% BSA which circulated

through the system until the buffer reached a temperature of 37 °C. The excised liver was placed on moistened sterile gauze on a glass platform. The inflow cannula was connected to the perfusion tubing, and flow was established. The outflow was adjusted such that all lobes of the liver were perfused without undue distension. The temperature of the liver was maintained at 37 °C as measured by a thermistor placed between the lobes of the liver. Perfusion flow was kept constant between 30 - 35 ml/min. Flow did not fluctuate during the one hour perfusion period.

Once flow had been stabilized (< 2 minutes), a 200 μl sample of perfusate was removed from the reservoir at 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60 minutes after the initiation of flow. Flow was measured at 10 minute intervals during the perfusion. The perfusate samples were kept on ice and frozen at -20 °C until analysis. At the conclusion of the experiment, the liver was removed and gently dried with absorbent paper and weighed. Perfusate samples were analyzed for glucose, lactate, TNF and IL-6.

G. *In Vitro* Studies

The *in vivo* response to endotoxicosis is a complicated progression of metabolic, cardiovascular, neural and immunological events that blend into an infinite number of combinations and permutations. To determine the contribution of a particular cell population to the response and to study the cellular

mechanisms of these interactions *in vivo* often represents an insurmountable task. In an effort to elucidate the mechanisms of action of insulin and dexamethasone on TNF and IL-6 production by macrophages, the following series of experiments was designed and tested initially in two cell lines as well as in two primary macrophage populations.

1. Cell Preparation and Cell Lines

ANA1 or RAW 264.7 cells were maintained in 75 ml tissue culture stock flasks in RPMI 1640 with 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (1X antibiotics) and 2 mM glutamine. The cells were split at a 1:30 dilution upon reaching confluence, approximately once a week. For experiments, a stock flask was pipetted into a 50 ml conical centrifuge tube and centrifuged for 10 minutes at 3000 rpm. The medium was removed, and fresh medium was added to the cells. A 200 μl sample of cells was taken and diluted to 1 ml with 400 μl of RPMI 1640 and 400 μl 10% trypan blue and vortexed. A sample was placed on a hemocytometer, and viable cells were microscopically counted.

The cells were then diluted in medium to 10^6 cells per ml and plated in 24 well cell culture plates at 1 ml total volume /well. The cells were "rested" for 24 hours, the old media was aspirated, and new medium was added.

2. Experimental Design

The basic experimental design is illustrated in fig. 9. Cells were initially

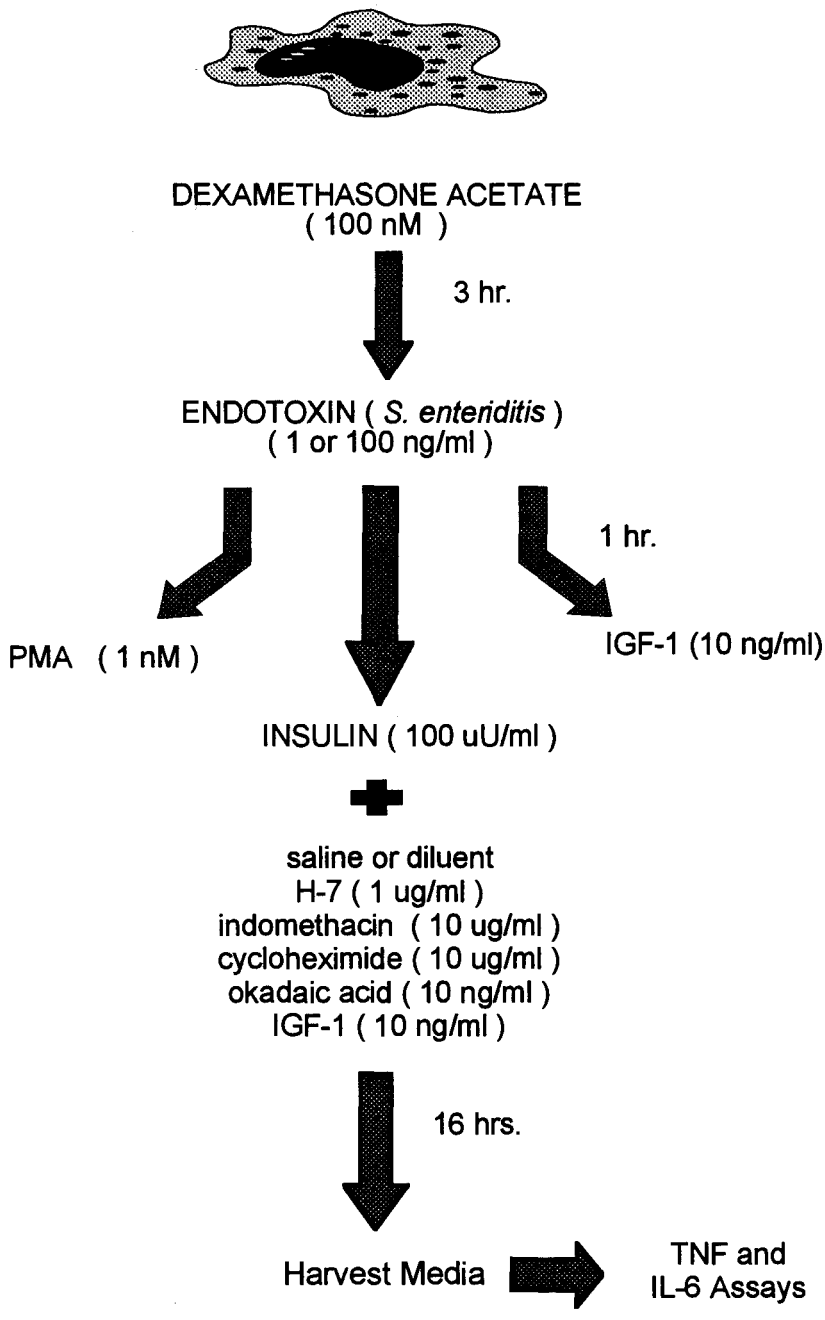


Fig. 9. Basic treatment protocol for the cell culture experiments.

treated with 100 nM dexamethasone phosphate or an equal volume of PBS. At 3 hours post-dexamethasone, 1 or 10 ng/ml S. enteritidis endotoxin or saline was administered to the cells. Finally, at one hour post-endotoxin, insulin (100 μU/ml) or saline was added to the cells. The medium in each well was then harvested at 16 hours after endotoxin addition. In the time course experiments, media was harvested at 2, 4, 8, 12, 16 and 24 hours post endotoxin. The medium samples were frozen at -20 °C until analysis for TNF, IL-6, glucose and lactate.

In a separate set of experiments, designed to elucidate the mechanisms of insulin action in these cells, several agents were employed in combination with the basic protocol illustrated in fig. 9. To examine the role of PGE_2 in this system, indomethacin (1 μM) was added with insulin. H-7 (1 μM) an inhibitor of protein kinase C was added with insulin. PMA was added to examine if insulin was acting as a phorbol-mimetic. Cycloheximide (1 μM) was added to examine if insulin was acting via synthesis of RNases. Okadaic acid was added to establish if the blocking of a phosphatase, which regulates PKC actions, was involved. IGF-1 was added to examine if insulin was acting like IGF-1 as a stimulator of cytokine production.

3. Primary Cultures

a) Peritoneal Macrophages

Peritoneal macrophages were obtained using a modified method described by Doherty et al., 1992, (84). Male Holtzman rats were injected i.p. with 10 ml of

10% thioglycollate broth (Difco, Detroit, MI). Four days post injection, the animals were killed by CO_2 narcosis and the abdomens were lavaged with 60 ml of sterile PBS. The lavage fluid was centrifuged for 10 minutes at 3000 rpm, and the fluid was aspirated leaving a cell pellet. To remove contaminant red blood cells, the pellet was washed with 2 ml of ACK Lysing Buffer (Quality Biological, Inc. Gaithersburg, MD) and resuspended in 20 ml RPMI 1640. Ten ml of Lymphocyte Separation Media (LSM) (Organon Teknika, Durham, NC) was carefully added to the cell suspension as an under layer. This mixture was then centrifuged at 3000 rpm for 30 minutes to remove dead cells and contaminating debris. The cells were aspirated from the interface between the LSM and the RPMI and resuspended in RPMI with 3 times normal concentration of antibiotics (3X), washed and centrifuged 3 times in RPMI with 3X antibiotics. After the third centrifugation the medium was aspirated and the cells were resuspended in RPMI with antibiotics. An aliquot of the cells was removed to determine total number of cells and also to determine cell types present in the peritoneal lavage fluid. The cells were diluted to 10^6 cells/ml, and 1 ml of cell suspension was added to each well of 24 well tissue culture plates. Two hours later, the medium was aspirated from each well to remove nonadherent cells and 1 ml of fresh RPMI was added to each well. Twenty-four hours later, the media was again removed from the wells and fresh medium was added. The cells were then allowed to incubate for 24 hours prior to experimentation.

The treatment protocol illustrated in figure 9 was utilized for experiments

on the peritoneal macrophages cells. To examine the roles of second messenger systems in these cells the compounds described above were utilized.

b) Kupffer Cells

Kupffer cells were obtained according to the procedure described by Pilaro et al., 1989, (261). Male Holtzman rats were anesthetized and the liver was isolated and excised according to the procedure described in the liver perfusion experiments. The liver was initially perfused for 5 minutes at 20 ml/min with 100 ml of Hanks Balanced saline (HBSS) (Ca^{2+}/Mg^{2+} free) buffered with 25mM HEPES and contained 0.5mM EGTA at pH 7.2 . The pump was then stopped; the tubing was attached to a second reservoir containing 100 ml of Leibovitz's (L-15) medium buffered with 25 mM HEPES, pH 7.2, which contained 300 mg pronase E, 20,000 U collagenase type IV, 10,000 IU penicillin and 10,000 μg streptomycin. The pump was restarted and perfusion of the liver was continued for 10 minutes or until the parenchymal cell mass separated away from the liver capsule. The perfusion was stopped, and the liver was transferred to a sterile petri dish containing 20 ml of L-15 media.

The liver capsule was cut with sterile scissors, and a sterile wide toothed metal comb was dragged through the liver to obtain a uniform cell suspension. The cell suspension was transferred to a sterile Erlenmeyer flask containing 20 ml L-15, pronase E and a small stir bar. The volume was brought to 100 ml with L-15 and incubated for 15 minutes in a 37 °C water bath. Five ml of 1 mg/ml

DNase (Sigma, St. Louis Mo) was added to the mixture and digestion continued until no visible tissue remained (45 minutes). The suspension was filtered through 60-70 μm nylon mesh into two 50 ml sterile centrifuge tubes and centrifuged at 50 x g for 1 minute at 4 °C to remove the hepatocytes. The supernant was transferred to sterile 50 ml centrifuge tubes and centrifuged at 300 x g for 5 minutes at 4 °C to pellet the nonparenchymal cells. The hepatocyte pellets were resuspended in 10 ml of L-15 and centrifuged at 50 x g for 1 minute. The supernant was removed and centrifuged to pellet the nonparenchymal cells. Each nonparenchymal pellet was resuspended in 10 ml of L-15 and combined. The suspension was centrifuged for 10 minutes to pellet the nonparenchymal cells. The cells were washed 4 times in total. After the last wash, the pellet was resuspended in 5 ml of L-15 in preparation for gradient centrifugation.

A two step gradient was used to separate the Kupffer cells from other nonparenchymal cells. A 30% metrizamide solution, pH 7.5, was made by adding 30 g metrizamide to 100 ml of NaCl-free Gey's balanced salt solution. In a 15 ml sterile tube, 3 ml of 30% metrizamide and 2 ml of L-15 was added to form the 18% bottom layer. The 13% top layer was formed by adding 5 ml of cells to 4 ml of 30% metrizamide and mixed well. This layer was gently layered on top of the 18% layer. The gradients were centrifuged at 1400 x g, 16 °C for 20 minutes. The Kupffer cells were collected from bands in the interface between the two gradients and reconstituted to 50 ml with L-15. The cells were centrifuged at 500 x g for 5 minutes to pellet the cells.

The cells were resupended in 10 ml and an aliquot was taken for differential staining and counting. The remaining suspension was diluted to 10^6 cells per ml in DMEM, 10% FBS, 4mM glutamine and 1% penicillin/streptomycin and plated into 96 well tissue culture plates. This isolation procedure resulted in a 83% yield of Kupffer cells as assessed by peroxidase staining.

The cells were allowed to attach for 4 hours, and the medium was removed and new medium was added. The cells incubated for 48 hours prior to experimentation. The cells were treated with the basic treatment protocol used for the ANA1 cells described previously in fig. 9.

H. Assays

1. Glucose and Lactate Measurements

A YSI model 2300A glucose/lactate monitor was utilized to analyze plasma, perfusate and cell culture media samples. The sensor technology is based on the principles conceived by Dr. Leland Clark, Children's Hospital Foundation, Cincinnati, OH. The instrument contains two probes fitted with a three layer membrane containing immobilized glucose oxidase or L-lactate oxidase in the middle layer. The sample (25 μl) is injected into a buffer filled chamber (600 μl) where the tip of the probe is situated. The substrate diffuses through the membrane and on contact with the enzymes is oxidized producing hydrogen peroxide (H_2O_2).

$$\text{D-glucose} + O_2 \xrightarrow[\textit{glucose oxidase}]{} \text{glucono-k-lactone} + H_2O_2$$

$$\text{L-lactate} + O_2 \xrightarrow[\textit{L-lactate oxidase}]{} \text{pyruvate} + H_2O_2$$

The hydrogen peroxide is in turn oxidized at a platinum electrode producing electrons.

$$H_2O_2 \xrightarrow[\textit{platinum electrode}]{} 2H^+ + O_2 + 2e^-$$

The electron flow is linearly proportional to the steady state H_2O_2 concentration and, therefore, proportional to the concentration of glucose or lactate. To prevent the oxidation of substances other than hydrogen peroxide from adding to the sensor current, the membrane contains an inner layer consisting of a very fine film of cellulose acetate. However, this film passes H_2O_2 readily, and excludes compounds with molecular weights greater than 200.

Prior to analysis of the samples, the instrument is calibrated with two standards. One standard contains 500 mg/dL glucose, lactate free; the other standard contains 15 mmol/L lactate, glucose free.

2. Insulin

Insulin was measured in plasma for the in vivo experiments. This procedure involved the use of a double antibody radioimmunoassay (Binax, South Portland, Maine) This assay had a sensitivity level of 2.0 μU/ml and an interassay coefficient of variability of less than 5%.

3. Tumor Necrosis Factor

a) Enzyme-Linked Immunoadsorbent Assay (ELISA)

Tumor necrosis factor was measured in plasma, perfusate and cell culture medium using a specific test kit for murine TNFα. The Factor-Test mTNF-α ELISA (Genzyme, Cambridge, MA) is a solid-phase enzyme immunoassay employing the multiple antibody sandwich principle. A hamster monoclonal antibody specific for murine TNFα is coated on microtiter wells in a 96-well plate. TNF present in standard samples and unknown specimens is captured by the solid-phase monoclonal antibody. A goat anti-murine TNF antibody, which binds to multiple epitopes on the TNF contained on the solid-phase is added. A third antibody, horseradish peroxidase-conjugated donkey anti-goat Ig is used to bind to the anti-murine TNF:murine TNF immune complexes. The peroxide enzyme reacts with peroxide substrate and a chromagen (OPD) to produce a yellow color proportional in intensity to the amount of TNF present. Color intensity is quantitated by measuring absorbance at 492 nm using an ELISA plate reader (Titertek, ICN, Irvine, CA). A reference standard curve is plotted using the mean absorbance of several concentrations of TNF ranging from 0.05 ng/ml to 3.2 ng/ml (fig. 10). The TNF levels in the experimental samples were determined from the linear regression equation generated from the standard curve.

At the time of these experiments, rat TNFα was unavailable for complete validation of the TNF ELISA. Since the monoclonal antibody against murine TNF is of hamster and not rat origin, and Estler et al.,1992, (98) have recently

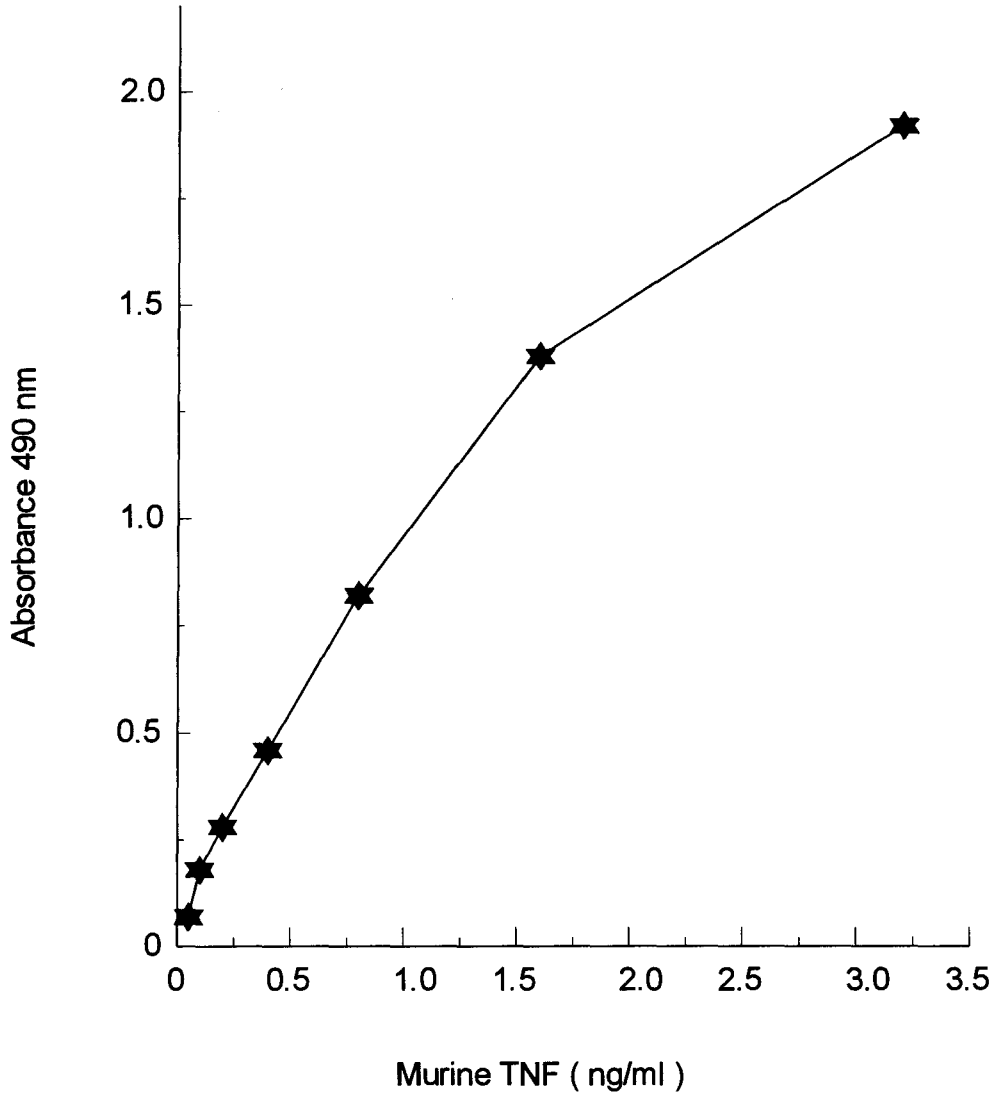


Fig. 10. Representative standard curve for TNF ELISA

demonstrated that a 92 % homology exists between the biologically active forms of mouse and rat TNF , we feel confident that rat TNF can be measured in this system.

b) Bioassay

Selected samples of rat perfusate, plasma and cell culture medium were analyzed using the L929 cytotoxicity assay (114, 258) and compared to the results obtained in the ELISA. The cytotoxicity assay measures the effect of TNFα on the L929 mouse myeloma cells as described previously by Flick et al., 1984, (114). Briefly, L929 cells are cultured in 96 well microtiter plates at a concentration of 150,000 cells/ml in DME + 5% FBS for 24 hours. 100 μl of medium is added to the first three wells. The rest of the plate received 50 μl of 4 μg/ml actinomycin D (Calbiochem, San Diego CA) to inhibit DNA synthesis. 50 μl of murine TNF standard protein was added at several dilutions to generate a standard curve, and 50 μl of test sample was added in triplicate to the rest of the wells. A typical standard curve is illustrated in fig. 11. The plate was incubated for 18 hours at 37 °C. The wells were washed twice with PBS and 100 μl of 0.2% crystal violet in 2% ethanol and stained for 10 minutes. The plate was rinsed with tap water and then air dried. The stained cells were solubilized with 200 μl 1% sodium dodecyl sulfate (SDS). Absorbance was determined at 550 nm using an ELISA plate reader (Titertek, ICN, Irvine, CA). The TNF concentration was determined from the standard curve from each assay.

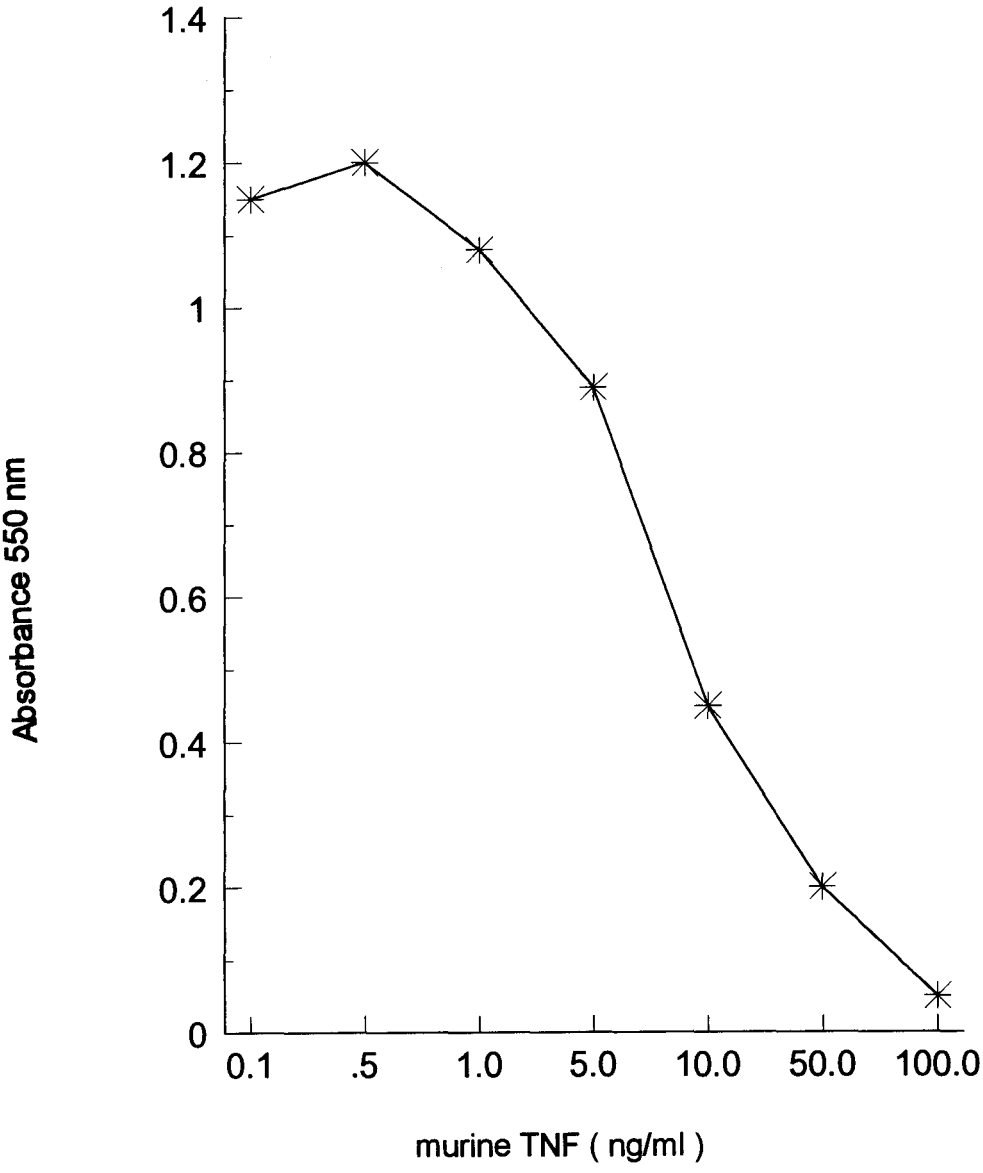


Fig. 11. Representative standard curve of the TNF bioassay.

4. Interleukin 6

a) ELISA

The interleukin 6 content of media from experiments involving ANA1 and RAW 264.7 cells was measured using an ELISA specific for murine interleukin 6 (Endogen, Boston, MA). The principle of this assay is similar to that described for the ELISA for murine TNFα. A representative standard curve for this assay is illustrated in fig. 12.

b) Bioassay

Samples of rat plasma, perfusate and cell culture medium from the peritoneal and Kupffer cells were analyzed for IL-6 using the B9 proliferation assay as described by Aarden et al., 1987 (1). B9 cells are murine hybridoma cells dependent on IL-6 for growth (194). B9 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 5% FBS, $5x10^{-5}$ M 2-mercaptoethanol and 1% penicillin-streptomycin. The medium was supplemented with IL-6 at 10-100 U/ml for maintaining the cells. For the assay, cells were washed 3 times to remove all residual IL-6 and 2500 cells in 50μl of IMDM were added to each well of 96 well microtiter plates. Samples and standards were diluted to a minimum dilution of 1:10 and 50 μl is added to each well. Rat interleukin 6 (courtesy of Dr. J. Gauldie) and recombinant human interleukin 6 were used to generate standard curves for this assay. (fig. 13). The plates were incubated for 72 hours at 37 °C in 5% CO_2. After 72 hours 10 μl of 3-[4,5-dimethylthiazol-2-yl] -2,5-

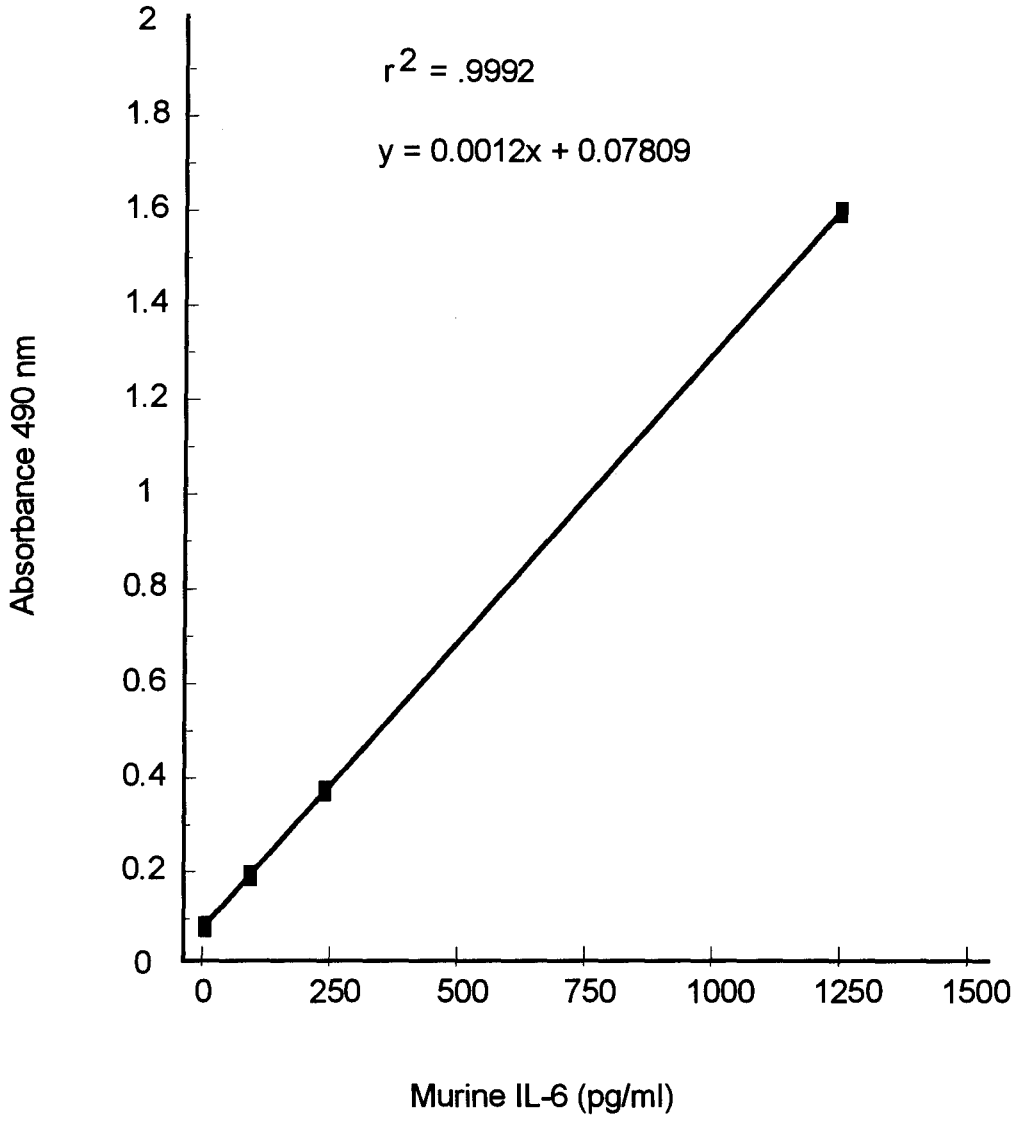


Fig. 12. Representative standard curve for IL-6 ELISA .

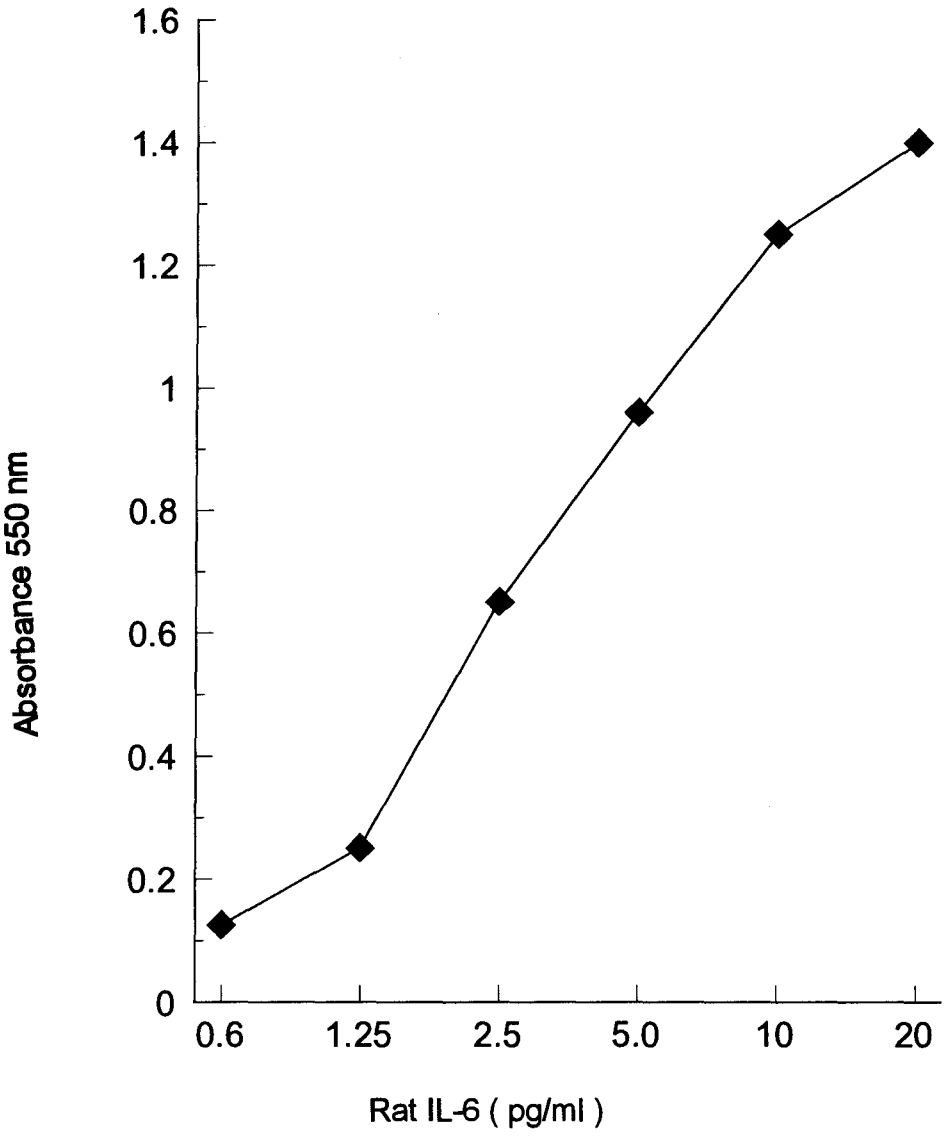


Fig. 13. Representative standard curve for the B9 bioassay

diphenyltetrazolium bromide (MTT) were added to each well and incubated for 4 hours. 50 μl Triton X was added to each well until color has fully developed (overnight). Absorbance was measured at 550 nm.

5. Endotoxin

Endotoxin content of perfusates and pharmacological reagents used in the experiments was determined using the Limulus Amebocyte Lysate Assay (LAL) (Whittaker, Walkersville,MD). The chromogenic LAL assay is a quantitative measure for gram-negative bacterial endotoxin. This assay is based on the principle developed by Levin and Bang,(1964), that endotoxin catalyses the activation of the proenzyme of LAL to the active form. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyzes the formation of p-nitroaniline (a yellow colored compound) from a colorless peptide substrate, Ac-Ile-Ala-Arg-pNA. The p-nitroanaline is measured photometrically at 405 nM. The assay is linear between the 0.02-0.10 ng/ml range. The concentration of endotoxin in the samples was calculated from the standard curve (fig.14).

I. Statistics

Results are reported as mean ± standard error of the mean. Chi squared analysis was performed on the lethality data. A one way Anova followed by a

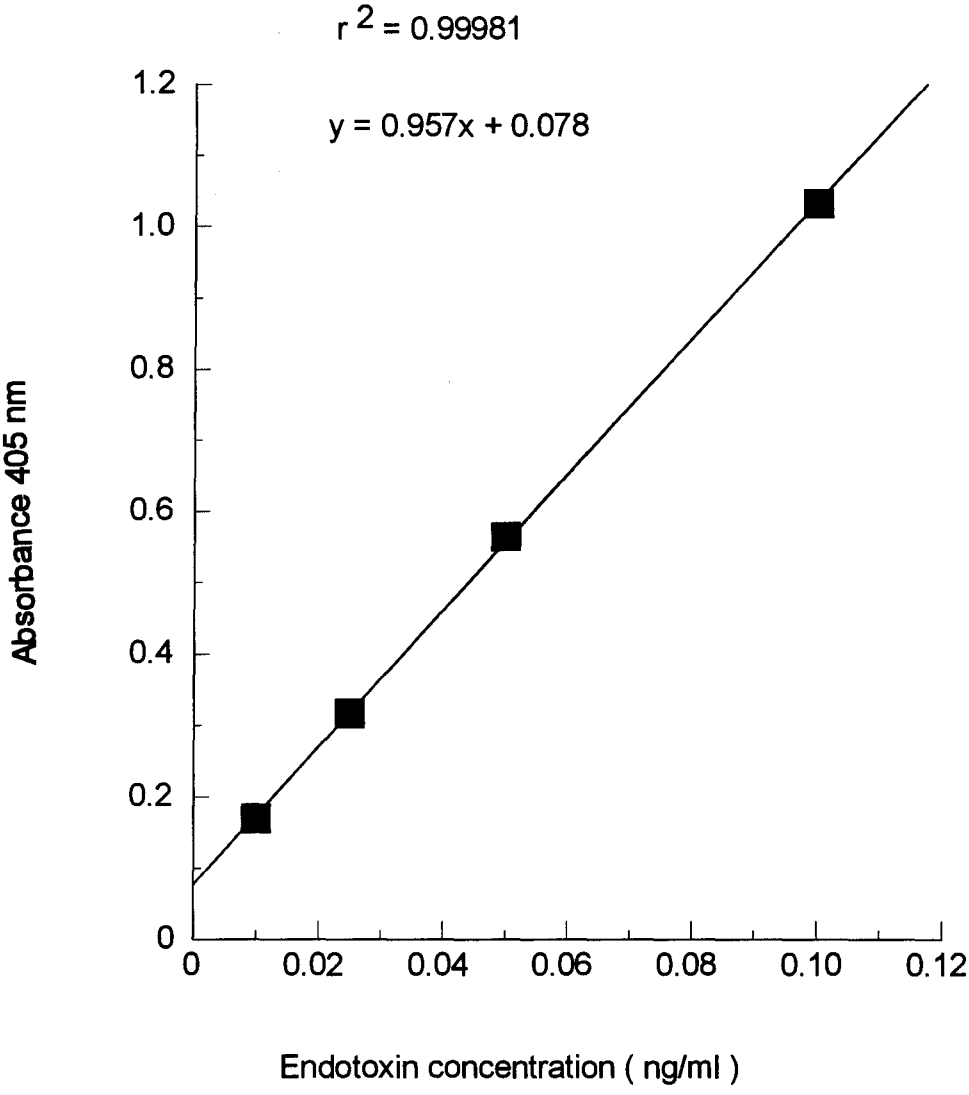


Fig. 14. Representative standard curve for the *Limulus* Amebocyte Lysate Assay used to measure endotoxin concentrations.

Tukey's post hoc test was performed on plasma glucose, lactate and insulin data from the in vivo experiments and on the data from the cell culture studies. A multiple variate analysis (treatment, time) with repeated measures was performed on the *in vivo* measurements and on the data from the liver perfusion experiments. In each case significance was accepted at $p<0.05$.

CHAPTER IV

RESULTS

A. *In Vivo* Projects

1. The Effect of Exogenous Insulin Administration and Endogenous Insulin Secretion on Lethality and Glucose Homeostasis.

The graphs used to illustrate the effects of exogenous and endogenous insulin on mortality and metabolic homeostasis are depicted in figs. 15, 16, 17 and 18. Each treatment group contained 8 to 15 animals. Mortality was determined after 12 hours, and the metabolic parameters, plasma glucose, lactate and insulin were measured approximately two hours after insulin administration as two of the treatment groups survival time was only within the two hour window after insulin. Dexamethasone, DEX, or saline was administered at 0.33 mg/kg, i.p., 3 hours prior to an injection of 20 mg/kg *S. enteritidis* endotoxin, ETX, or saline, i.p. One hour after ETX, insulin, INS, (1U) or saline was injected, s.c. To determine the effect of endogenously produced insulin in this system, tolbutamide, TOL, was administered (66 mg/kg) in substitution for insulin. Insulin, dexamethasone and tolbutamide were tested for endotoxin contamination using LAL tests. The endotoxin contamination for these substances was $\leq$ 0.1 ng/ml.

Fig. 15 depicts the mortality observed among the treatment groups. In this particular study 20 mg/kg of *S. enteritidis* endotoxin administered intraperitoneally resulted in a 66% mortality over 12 hours. Insulin (1U) administered one hour after endotoxin increased mortality to 100 %. All rats died within two hours after insulin administration. Laparotomy revealed that both the upper and lower intestinal tracts were dark purple and congested with petechiae and hemorrhages for both the ETX and the ETX/INS treatment groups. Tolbutamide treatment alone had no lethal effects on the rats. However , ETX/TOL treatment resulted in 100% mortality after 12 hours. These animals died within the 12 hours, however, later than the two hour period observed in the ETX/INS group. The examination of the intestinal tract in these animals revealed large regions of hemorrhage similar in degree to that observed in the ETX/INS group.

The administration of 0.33 mg/kg dexamethasone 3 hours prior to endotoxin administration significantly reduced mortality to 6.25%. When insulin was administered to a dexamethasone protected group, DEX/ETX/INS, mortality was significantly reversed and increased to 75%. The appearance of the bowel on autopsy also exhibited increased areas of petechiae and hemorrhaging in comparison to the DEX/ETX group which demonstrated only minimal and isolated areas of petechiae. However, the bowel pathology observed in the DEX/ETX/INS group was not of a comparable degree of intensity as that observed in the ETX/INS group. Administration of tolbutamide to dexamethasone protected rats,

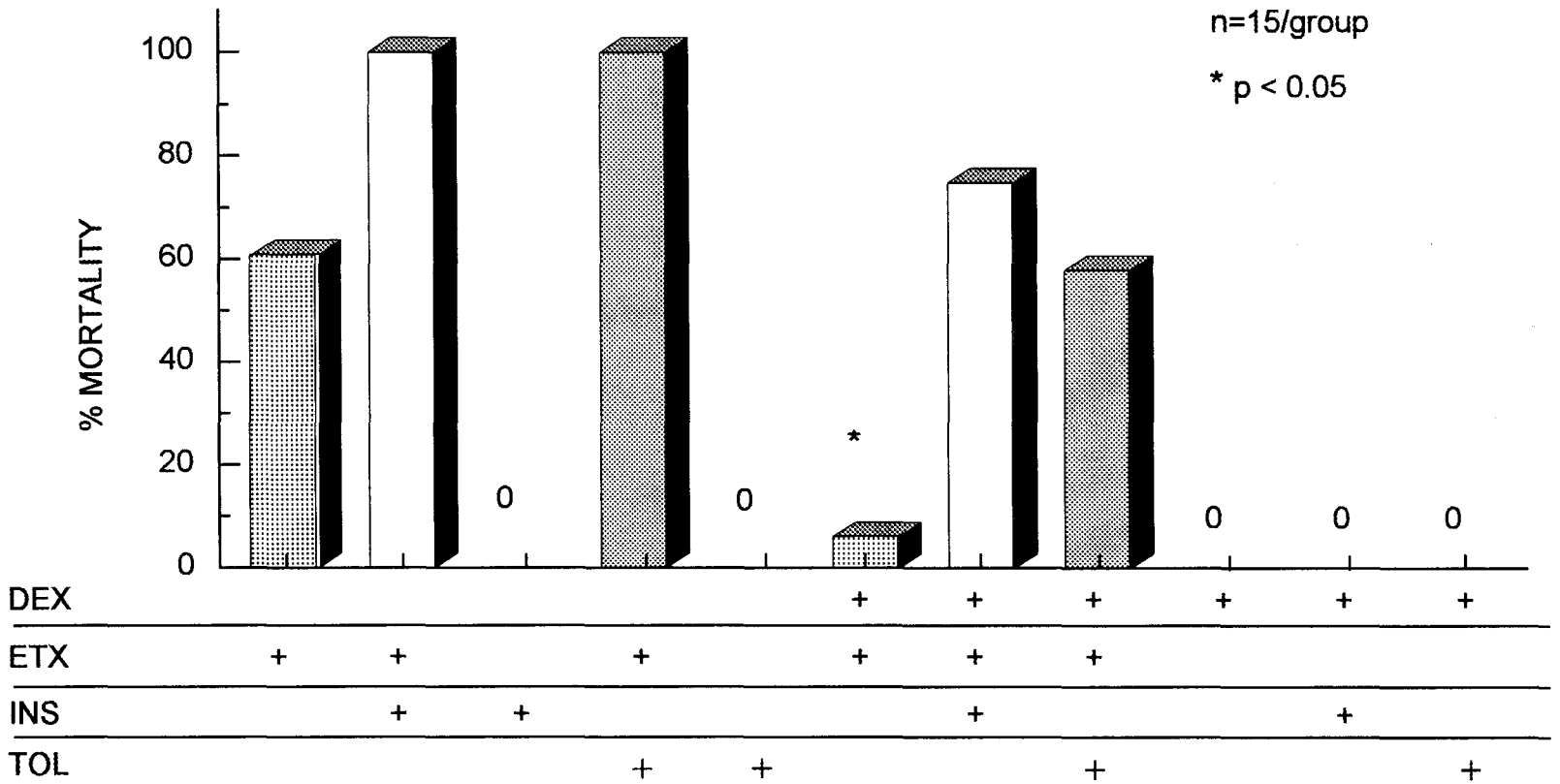


Fig. 15. The effect of exogenous and tolbutamide stimulated insulin secretion on mortality in endotoxic and dexamethasone protected endotoxic rats. Mortality was calculated at 12 hours post endotoxin administration. DEX = dexamethasone (0.33 mg/kg); ETX = endotoxin (20 mg/kg); INS = insulin (1 U); TOL = tolbutamide (66 mg/kg). * $p < 0.05$ compares DEX/ETX to DEX/ETX/INS and DEX/ETX/TOL

DEX/ETX/TOL, significantly increased mortality from 6.25% to 58%. These animals exhibited only limited areas of petechiae on pathological examination of the intestinal tract. The control groups illustrated in fig.15, insulin, INS, dexamethasone, DEX, and the combination, DEX/INS did not die as a result of the treatments. These groups did not have any gross evidence of bowel pathology upon autopsy.

The plasma glucose concentration of the saline control group was 101.7 ± 6.2 mg/dL. As illustrated in fig. 16 insulin treatment alone caused a mild hypoglycemia and resulted in a plasma glucose of 77.8 ± 6.5 mg/dL. This value was not significantly different from the plasma glucose concentration (81.8 ± 6.9 mg/dL) in the ETX treated animals. Endotoxin and insulin in combination significantly decreased plasma glucose concentrations (8.1 ± 3.3 mg/dL) and a profound hypoglycemia was observed . TOL administration caused a decrease in glucose (83.8 ± 5.8 dL) that was not significantly different from ETX or INS alone. Plasma glucose concentration of the ETX/TOL group was significantly reduced to 5.9 ± 2.2 mg/dL.

The DEX/ETX animals exhibited a mild hyperglycemia (135.6 ± 8.1 mg/dL) which was not significantly different from the DEX alone treatment group (129.2 ± 5.1 mg/dL). Insulin treatment resulted in a significantly reduced the plasma glucose level in the DEX/ETX/INS group (16.7 ± 6.6 mg/dL). Tolbutamide substituted for insulin resulted in a similar decline in plasma glucose in the DEX/ETX/TOL group (31.4 ± 3.8 mg/dL) however, not to the same intensity as

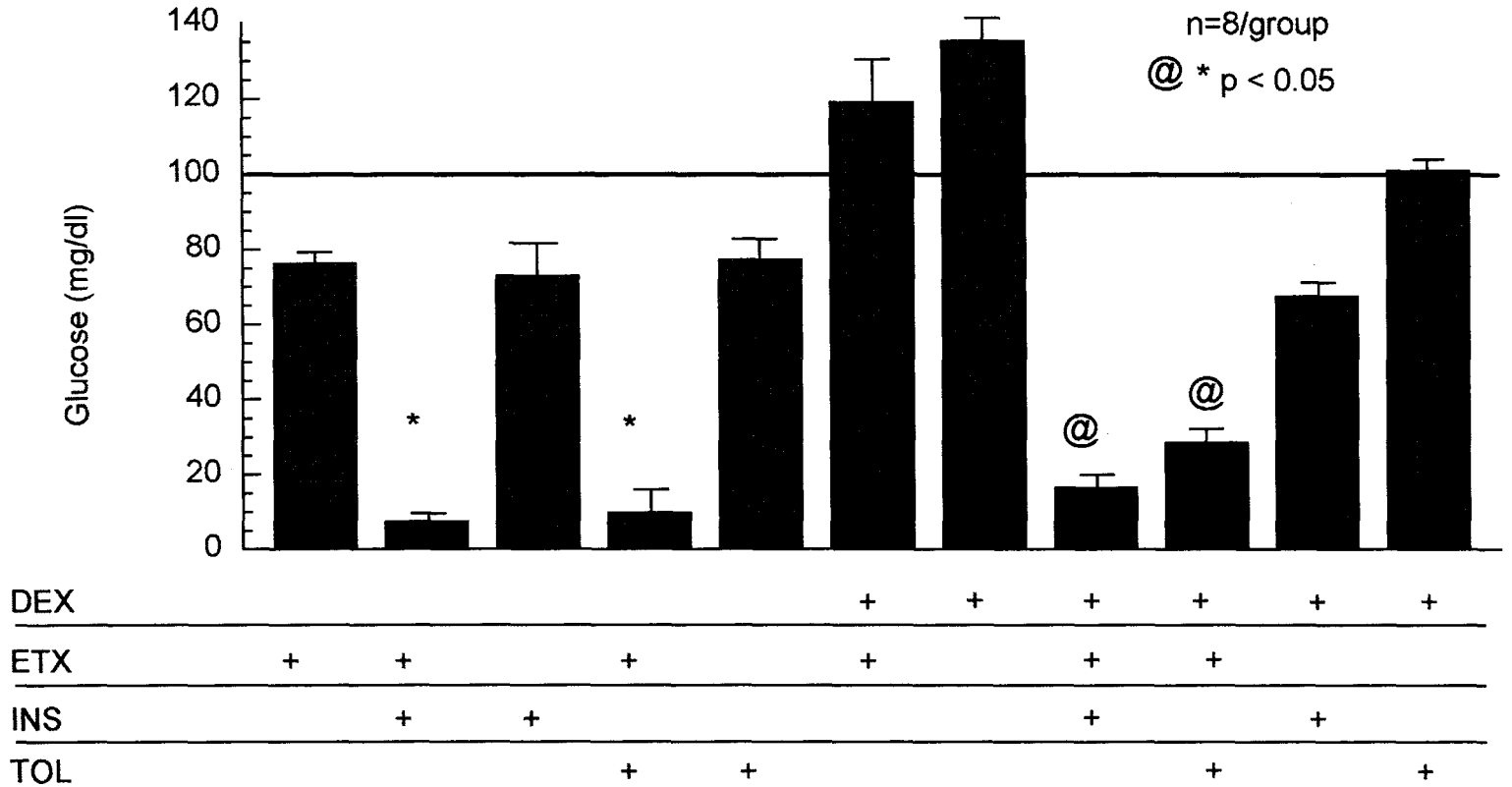


Fig. 16. The effect of exogenous and tolbutamide stimulated insulin secretion on plasma glucose in endotoxic and dexamethasone protected endotoxic rats. Plasma was collected at 3 hours post endotoxin administration. DEX = dexamethasone (0.33 mg/kg); ETX = endotoxin (20 mg/kg); INS = insulin (1 U); TOL = tolbutamide (66 mg/kg). The bars represent the means + SEM for each group. The solid line indicates plasma glucose concentration of saline treated rats. * $p < 0.05$ compares ETX to ETX/INS and ETX/TOL.
@ $p < 0.05$ compares DEX/ETX to DEX/ETX/INS and DEX/ETX/TOL

that observed in the DEX/ETX/INS group.

The mean plasma lactate concentration of the saline treated animals was 1.8 ± 0.06 mmol/L. Endotoxin treatment significantly increased plasma lactate values to 2.75 ± 0.35 mmol/L as depicted in fig. 17. ETX/INS treatment and ETX/TOL treatments profoundly elevated plasma lactate concentrations to 7.9 ± 1.19 mmol/L and 6.8 ± 1.34 mmol/L respectively. Insulin or tolbutamide without endotoxin did not alter plasma lactate concentrations.

Dexamethasone pretreatment of the endotoxic group did not significantly alter lactate values above those of ETX alone (2.5 ± 0.28 mmol/L). Insulin posttreatment of the DEX/ETX group significantly increased plasma lactates to 7.4 ± 1.5 mmol/L. However, TOL, posttreatment did not alter plasma lactates (2.28 ± .41 mmol/L) above those of the ETX group.

Plasma insulin concentrations illustrated in fig. 18 indicated that saline treated animals had a fasting insulin level of 18.5 ± 8.1 μU. Endotoxin elevated plasma insulin concentrations to 40.2 ± 5.4 μU. All treatment groups administered insulin exogenously had significantly elevated levels of plasma insulin. INS alone increased plasma insulin to 85 μU. Plasma insulin concentration of the ETX/INS group was 165 ± 27.6 μU. Tolbutamide did increase plasma insulin in the ETX/TOL group ,(68.3 ± 7.0 μU) however, not statistically above that of endotoxin alone.

The DEX/ETX group had elevated plasma insulin concentrations (65.8 ± 5.6 μU) however, these were not different from the ETX group. DEX/ETX/INS

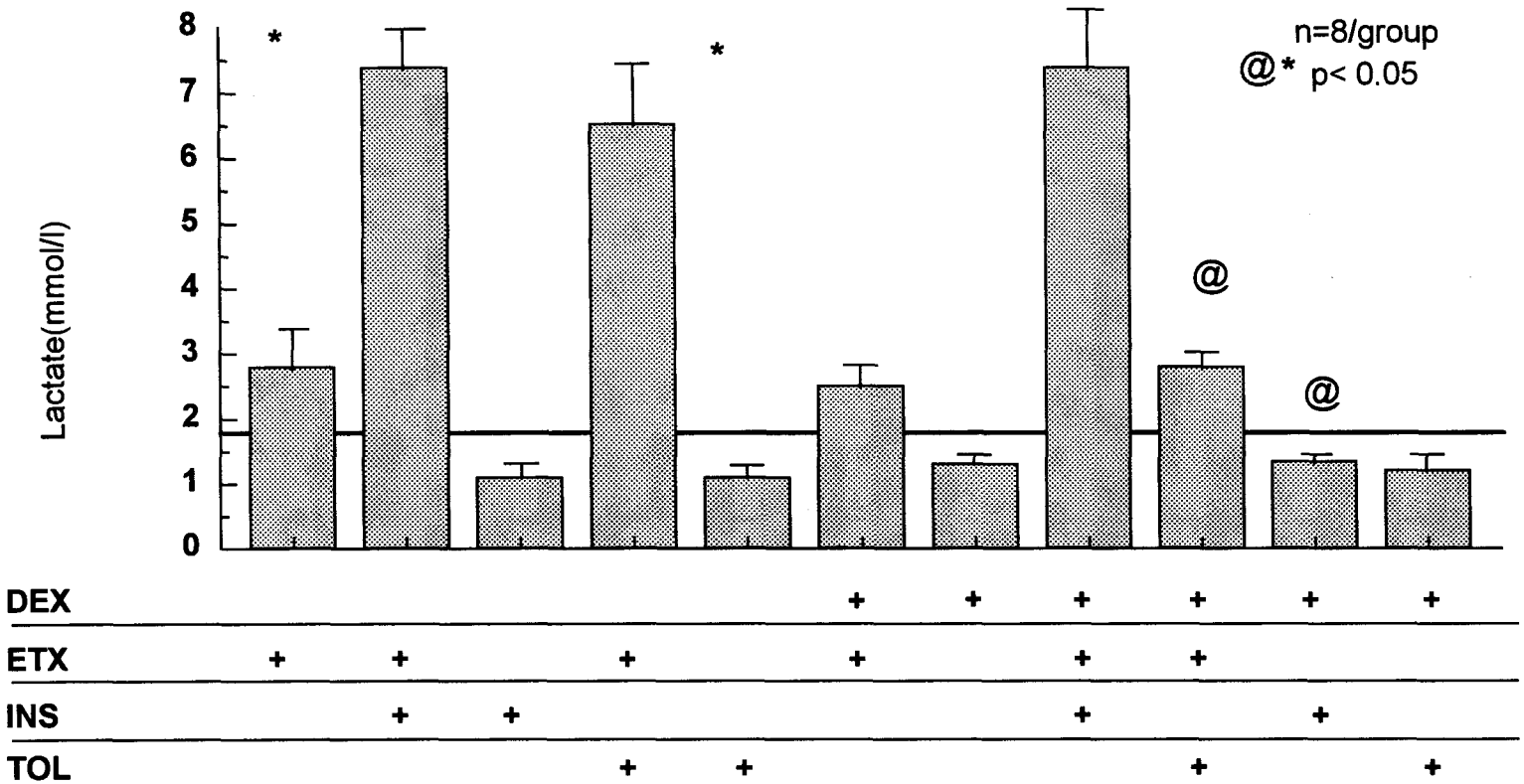


Fig. 17. The effect of exogenous and tolbutamide stimulated insulin secretion on plasma lactate in endotoxic and dexamethasone protected endotoxic rats. Plasma was collected at 3 hours post endotoxin administration. DEX = dexamethasone (0.33 mg/kg); ETX = endotoxin (20 mg/kg); INS = insulin (1 U); TOL = tolbutamide (66 mg/kg). The bars represent the means + SEM for each group. The solid line indicates plasma lactate concentration of saline treated rats. * $p < 0.05$ compares ETX to ETX/INS and ETX/TOL.
@ $p < 0.05$ compares DEX/ETX to DEX/ETX/INS and DEX/ETX/TOL

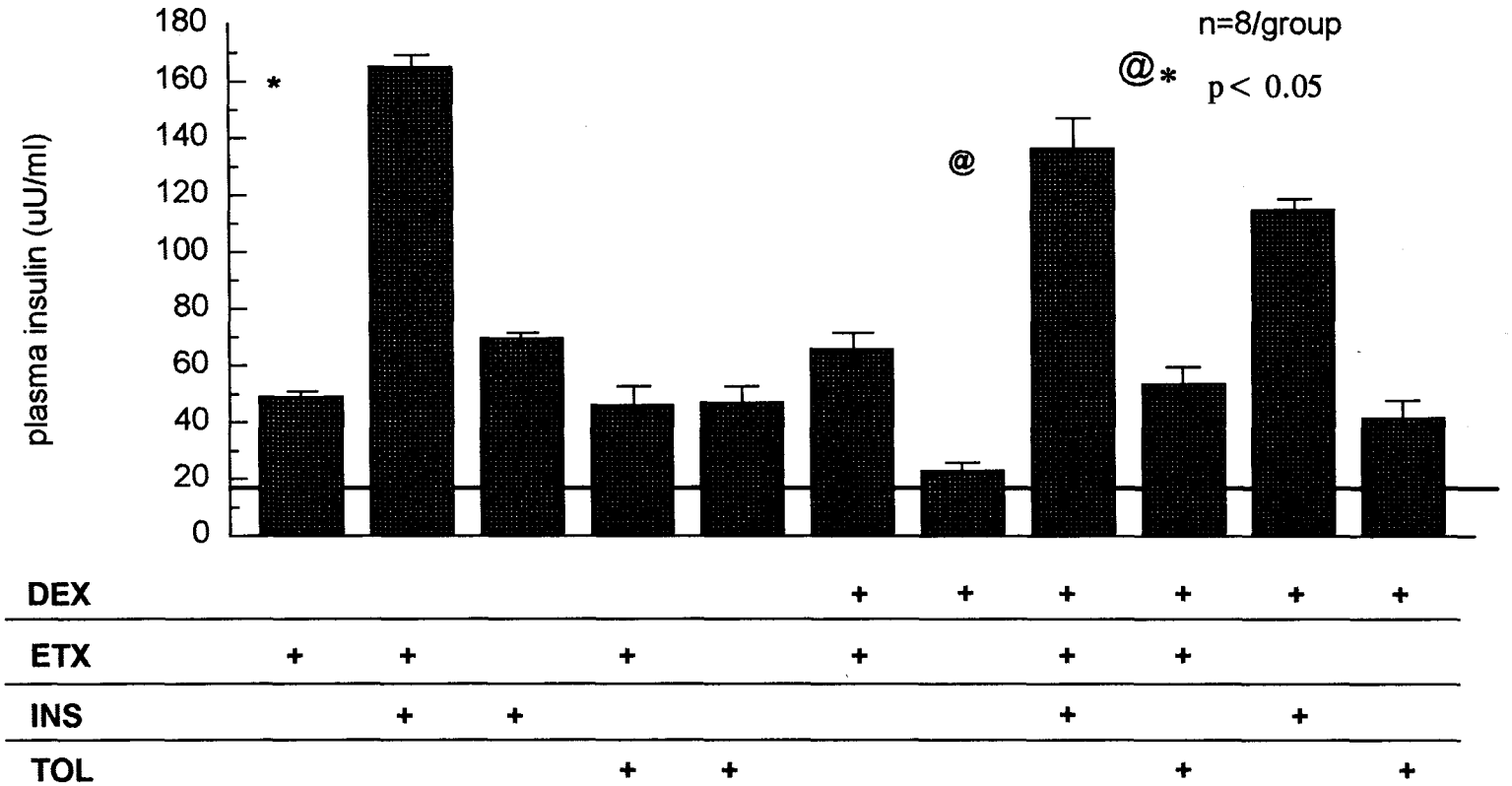


Fig. 18. The effect of exogenous and tolbutamide stimulated insulin secretion on plasma insulin in endotoxic and dexamethasone protected endotoxic rats. Plasma was collected at 3 hours post endotoxin administration. DEX = dexamethasone (0.33 mg/kg); ETX = endotoxin (20 mg/kg); INS = insulin (1 U); TOL = tolbutamide (66 mg/kg). The bars represent the means + SEM for each group. The solid line indicates plasma insulin concentration of saline treated rats. * $p < 0.05$ compares ETX to ETX/INS ; @ $p < 0.05$ compares DEX/ETX to DEX/ETX/INS .

treatment elevated plasma insulin to 136 ± 27.7 μU. DEX/ETX/TOL treatment however, only mildly increased plasma insulin (54.8 ± 4.3 μU). DEX alone suppressed insulin secretion and DEX/TOL treatment caused plasma insulin concentrations to equal that observed by ETX treatment (44.4 ± 4.4 μU vs. 40.2 ± 5.4 μU) .

2. The Effect of Insulin Dose on Mortality

Table 6 summarizes the results of administering three different doses of insulin, 1 U, 0.5 U or 0.25 U of insulin on mortality 12 hours after insulin injection.

Insulin administered without endotoxin was not lethal to the rats at any of the three doses. Dexamethasone and insulin in combination was also obsereved to be not lethal. Endotoxin mortality as shown in figure 15 was 61%. With the addition of 1 U or 0.5 U of insulin to endotoxic rats mortality was increased to 100%. The addition of 0.25 U resulted in mortality that was not different from endotoxin treatment alone. Dexamethasone pretreatment of endotoxic rats reduced mortality from 61% to 6.25%. The addition of 1 U of insulin to the dexamethasone protected group increased mortality to 75%. This increase in mortality (75%) was also observed with the addition of 0.5 U of insulin to the dexamethasone-protected group. However, the mortality observed when 0.25 U of insulin was administered to dexamethasone-protected rats was not significantly altered from that observed with endotoxin treatment alone.

Table 6 --The effect of insulin dose on mortality in endotoxic and control rats

Insulin dose	1 U	0.5 U	0.25 U
INS	0 % (0/15)	0 % (0/8)	0% (0/8)
ETX/INS	100%15/15)	100 %(8/8)	63% (5/8)
DEX/ETX/INS	75% (11/15)	75 % (6/8)	63% (5/8)
D/INS	0 % (0/15)	0 % (0/8)	0% (0/8)

ETX = endotoxin; INS = insulin; DEX = dexamethasone.
( ) = mortality per group

3. The Effect of Restoring Euglycemia on Mortality

Profound hypoglycemia was observed in the ETX/INS and the DEX/ETX/INS treatment groups. To determine if restoring euglycemia would be effective in countering the lethal effects of these treatment regimes, 5% dextrose solution was injected intraperitoneally and plasma glucose was monitored for 2 hours post insulin treatment.

Table 7 compares mortality rates in the treatments administered dextrose during the experiment and the plasma glucose concentrations immediately prior to death. It was almost impossible to maintain euglycemia over the two hour period and plasma glucose concentrations fluctuated dramatically in the ETX/INS and the DEX/ETX/INS groups. As shown in table 7 administration of dextrose did not alter mortality in the E/I treated and D/E/I treated groups even though plasma glucose concentrations were 71.8 ± 9.4 mg/dL and 86.5 ± 5.3 mg/dL respectively. Dextrose treatment also significantly increased plasma glucose (122.6 ± 8.2 mg/dL) and mortality in the ETX treatment group.

4. The Effect of PMA and H-7 on Mortality

To determine if the effect of insulin on mortality was via the activation of the protein kinase C pathway, two experiments were designed. First, phorbol myristate acetate, PMA, a PKC activator, was substituted for insulin in the protocol described above. Secondly, H-7, a blocker of protein kinase C activity, was coadministered with insulin to examine if insulin action could be altered.

Table 7-- Effect of dextrose (D5) treatment on plasma glucose concentrations and mortality .

	Plasma Glucose (mg/dL)		Mortality	Mortality
Treatment	- D5	+ D5	- D5	+ D5
ETX	81.8 ± 12.9	122.6 ± 8.2 *	11/15	6/6 ns
ETX/INS	8.1 ± 3.2	71.8 ± 9.4 *	15/15	6/6 ns
DEX/ETX/INS	16.6 ± 6.5	86.5 ± 5.3 *	12/15	5/6 ns

ETX = endotoxin; INS = insulin; DEX = dexamethasone.
* $p < 0.05$ compares no dextrose (-D5) to dextrose (+ D5).
ns no significant difference.

Table 8 depicts the effect of this treatment regime on mortality over 12 hours. PMA treatment did not cause mortality nor did the DMSO based diluent used to dissolve the PMA. ETX/PMA treatment produced 100% mortality. DEX/ETX/PMA treatment increased mortality from 6.25% in the DEX/ETX group to 80 %.

When H-7 was administered with insulin, H-7 was unable to reduce or alter mortality in any of the test groups. H-7 also had no effect on the survival of the groups that did not receive insulin.

B. *In Vivo* Cytokine Measurements

1. The Effect of Insulin on Cytokine Production in the Endotoxic Rat.

In this study the effect of insulin on TNF and IL-6 production in an *in vivo* setting was evaluated. Initially, however, plasma glucose and lactate concentrations were obtained. Fig. 19 illustrates the plasma glucose responses of the various treatments groups over 180 minutes. Endotoxin treatment stimulated a rapid increase in plasma glucose to a maximum value of 161 ± 5.8 mg/dL, approximately 90 minutes post endotoxin. This hyperglycemia was then observed to decrease over time to within normal range by the end of the 180 minute sampling period. Insulin post-treatment (ETX/INS) incited a profound hypoglycemia (10.3 ± 3.79 mg/Ll at 120 minutes) such that the rats died prior to the 150 minute sampling point. Dexamethasone pretreatment of endotoxic rats

Table 8--The effect of PMA and H-7 on mortality in endotoxic and dexamethasone protected endotoxic rats.

Treatment	Mortality
PMA	0% (0/6)
ETX/PMA	93% (13/14)
DEX/PMA	0% (0/6)
DEX/ETX/PMA	80% (12/15)
ETX/H-7	63% (5/8)
ETX/INS/H-7	100% (8/8)
DEX/ETX/H-7	12.5% (1/8)
DEX/ETX/INS/H-7	75% (6/8)

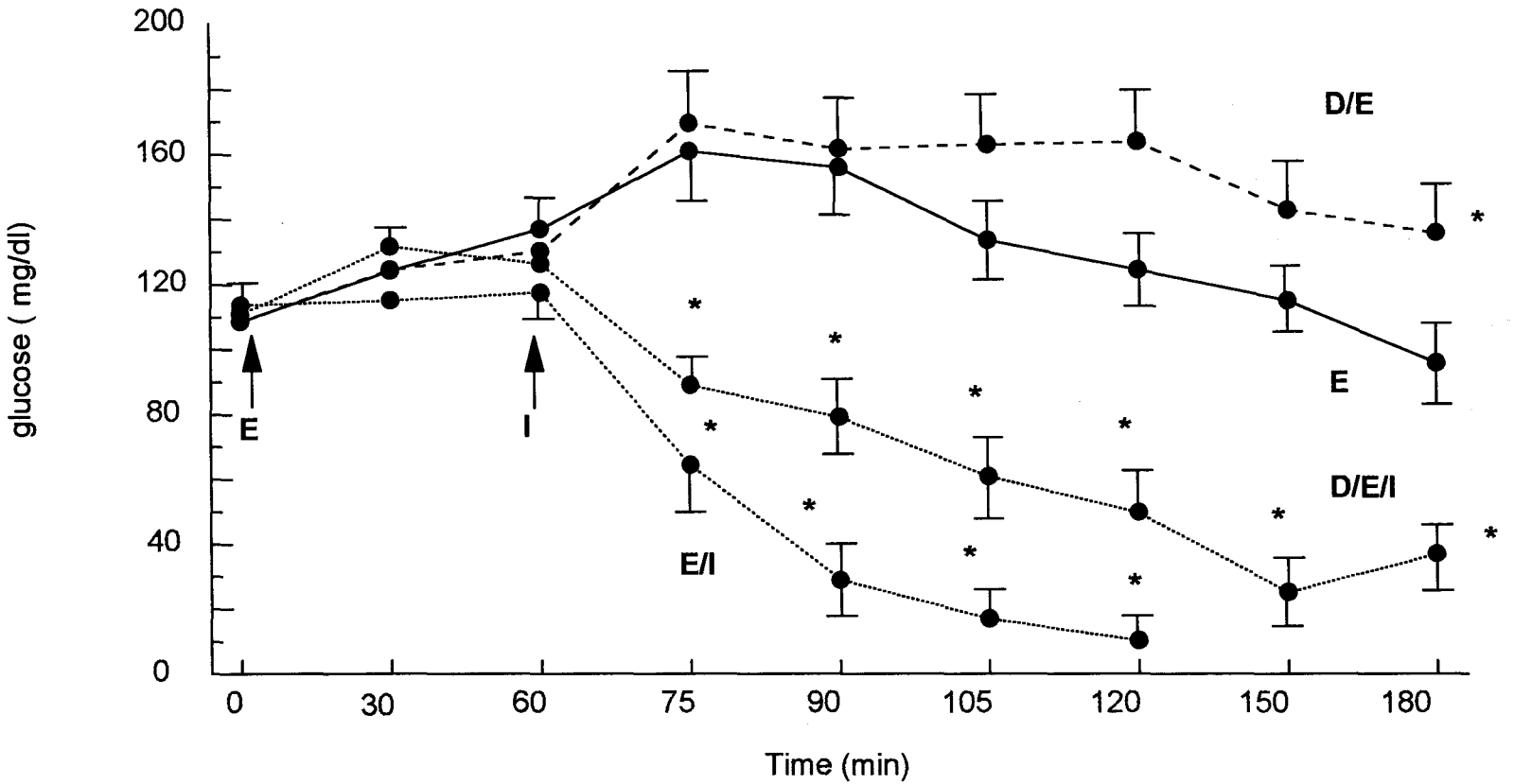


Fig. 19. Plasma glucose response to an insulin challenge in endotoxic and dexamethasone protected endotoxic rats over a 3 hour time period. Endotoxin was administered at t = 0 and insulin was injected at t = 60. Values represent the means + SEM for each group (n=8/group). E = endotoxin; I = insulin; D = dexamethasone. * $p < 0.05$ compares D/E to D/E/I and E/I to E.

(DEX/ETX) did not alter glucose above that of endotoxin treatment (169.5 ± 16.3 mg/dL), however, the dexamethasone induced hyperglycemia persisted until 180 minutes even though the plasma glucose concentration of the ETX group was declining. Insulin post-treatment of dexamethasone-protected endotoxic rats (DEX/ETX/INS) markedly decreased plasma glucose concentrations over time. The decline in plasma glucose was less dramatic than without dexamethasone pretreatment and the rats in this group survived to the end of the 180 minute sampling period. Insulin ,INS, alone initiated a mild hypoglycemia (76.9 ± 3.71 mg/dL) that remained constant over the time course. Dexamethasone, DEX, and in combination with insulin, DEX/INS treatments did not alter plasma glucose significantly from saline controls over the experimental time course.

Plasma lactate values as illustrated in fig. 20 were significantly increased by endotoxin over time. The maximum lactate concentrations were observed at 105 minutes after endotoxin to be 4.75 ± 0.75 mmol/L. Although not statistically different, increased plasma lactate concentrations above that of endotoxin treatment were observed in the ETX/INS group. The maximum plasma lactate value observed was 5.1 ± 1.8 mmol/L at 120 minutes post endotoxin. DEX/ETX treatment reduced plasma lactate concentrations significantly below those of ETX and ETX/INS over the entire time course. The maximum plasma lactate values observed were 3.2 ± 0.48 mmol/L at 180 minutes. DEX/ETX/INS treatment did not significantly alter plasma lactate concentrations from those in the DEX/ETX treatment group.

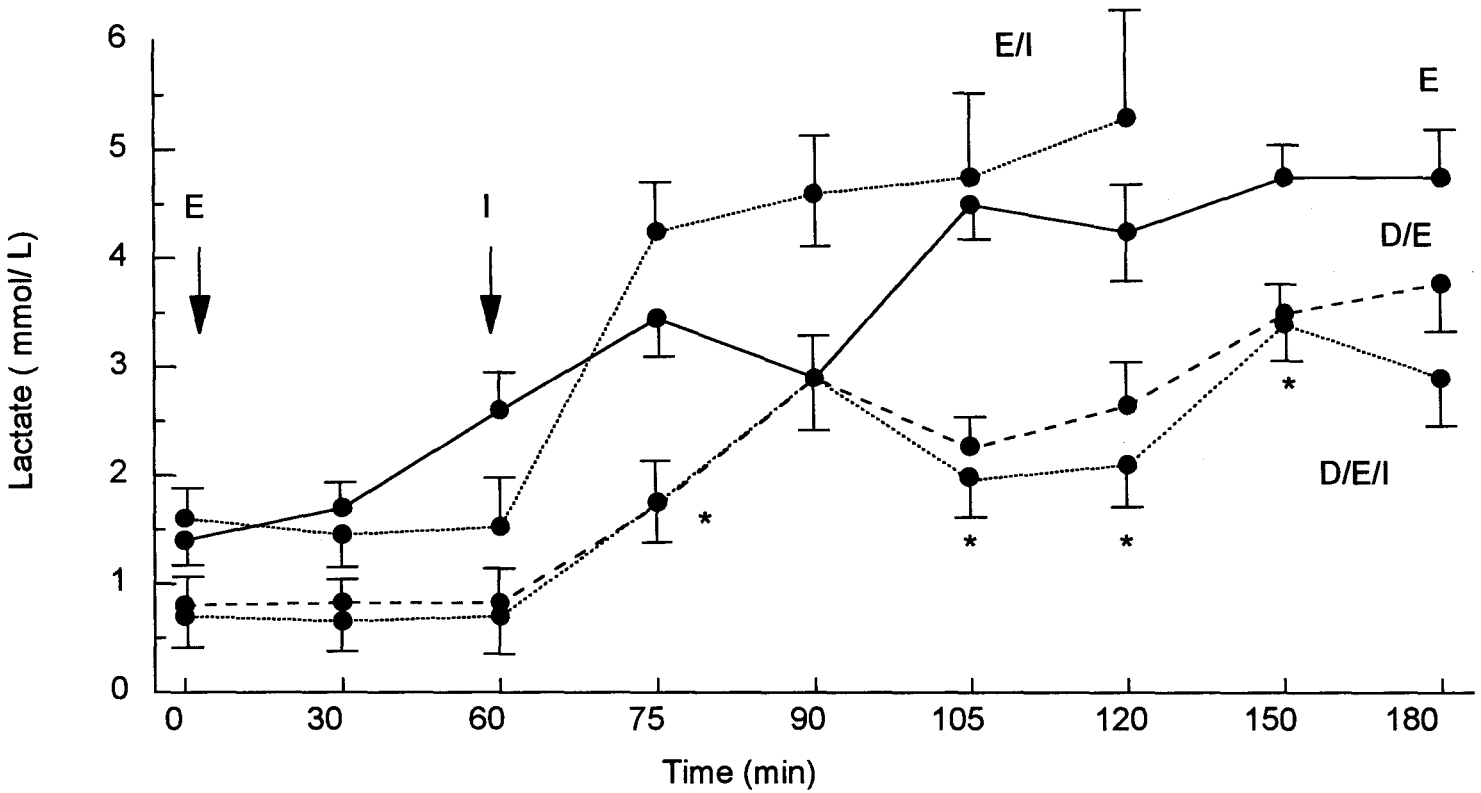


Fig. 20. Plasma lactate response to an insulin challenge in endotoxic and dexamethasone protected endotoxic rats over a 3 hour time period. Endotoxin was administered at t = 0 and insulin was injected at t = 60. Values represent the means + SEM for each group (n=8/group). E = endotoxin; I = insulin; D = dexamethasone.

* $p < 0.05$ compares D/E to D/E/I and E/I to E.

Administration of endotoxin resulted in an almost typical response in plasma TNF as illustrated in fig. 21 . TNF appeared in the plasma 60 minutes post -endotoxin, peaked maximally (3.72 ± 0.845 ng/ml) at 90 minutes and was almost absent from the plasma (0.375 ± 0. 412 ng/ml) at 180 minutes. ETX/INS treatment did not alter TNF concentrations significantly from the ETX group until 120 minutes post endotoxin. The plasma TNF in this group at this time point was sustained at 3.32 ± 1.0 ng/ml and appeared to be increasing whereas the ETX group TNF response (2.2 ± 0.768 ng/ml) was in the initial phase of decline. Dexamethasone pretreatment had a significant effect on the production of TNF in the DEX/ETX group. TNF was completely suppressed and did not appear until 90 minutes post endotoxin. Peak values were significantly reduced in comparison to the ETX group (0.558 ng/ml vs. 3.72 ng/ml). Insulin post treatment of the DEX/ETX/INS group elevated TNF at 75 minutes post endotoxin and maximum levels were observed at 90 minutes post endotoxin (1.35 ± 0.986 ng/ml). This elevation in TNF was significantly different , $p < 0.05$, from the DEX/ETX group. INS, DEX or DEX/INS without endotoxin did not ellicit a response in plasma TNF.

Plasma IL-6 was not observed to be present in significant amounts until 120 minutes post endotoxin (table 9). Endotoxin treatment caused a significant increase in IL-6. This increase was maximal at 180 minutes (2.964 ± 0.369 ng/ml). ETX/INS treatment resulted in a significant increase in IL-6 at 120 minutes (638.1 ± 187.3 pg/ml). Values at 150 and 180 minutes were not obtained due to the death of the animals. Dexamethasone pretreatment suppressed IL-6 until 150

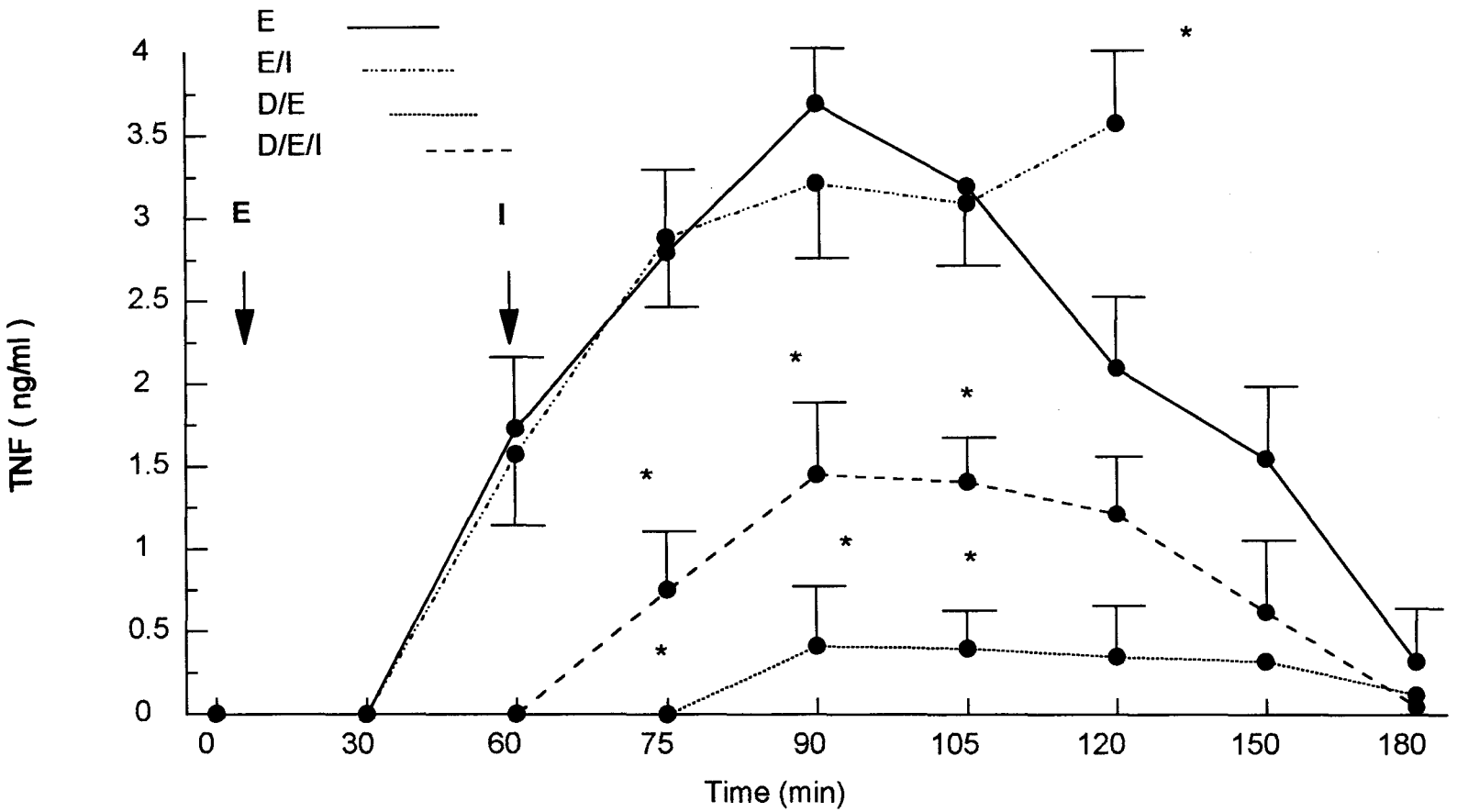


Fig. 21. Plasma TNF response to an insulin challenge in endotoxic and dexamethasone protected endotoxic rats over a 3 hour time period. Endotoxin was administered at t = 0 and insulin was injected at t = 60. Values represent the means + SEM for each group (n=8/group). E = endotoxin; I = insulin; D = dexamethasone.
* $p < 0.05$ compares D/E to D/E/I and E/I to E.

Table 9-- Plasma IL-6 production by endotoxic and dexamethasone protected endotoxic rats in response to an insulin injection.

	0 minutes	120 minutes	150 minutes	180 minutes
E	20.9 ± 2.1	152.1 ± 43.1	1621.3 ± 59.1	2964.5 ± 369.5
E/I	23.2 ± 3.3	638.1 ± 87.3*	N/D	N/D
D/E	18.9 ± 1.4*	23.9 ± 4.3*	322.0 ± 159.3*	77.1 ± 46.04*
D/E/I	19.1 ± 1.3	22.0 ± 1.2	142.1 ± 91.4	295.2 ± 70.1

Values represent the mean ± SEM for each treatment group (n = 6/group) in pg/ml at 0, 120, 150 and 180 minutes post endotoxin.
E = endotoxin; I = insulin; and D = dexamethasone.
N/D = not determined due to lethality.
* $p < 0.05$ compares E to E/I and E to D/E at the same time point.

minutes post endotoxin although maximal values were significantly below those of endotoxin alone. DEX/ETX/INS treatment increased IL-6 above that of DEX/ETX treatment alone at 180 minutes, although not statistically significant.

Fig. 22 compares plasma glucose and TNF concentrations in a model of insulin induced hypoglycemia. Plasma glucose concentrations fell dramatically in response to a 5 U challenge of insulin, s.c., plasma TNF concentrations over the time period of the experiment did not change. Plasma IL-6 levels were not detected in this experiment.

C. *Ex Vivo* Liver Perfusion Studies

1. Cytokine Stability

Prior to examining the production of TNF or IL-6 by the isolated perfused liver in the endotoxic rat, the ability to measure TNF and IL-6 concentrations in the perfusate and the stability of both peptides in the perfusion apparatus under normal perfusion conditions had to be determined.

Fig. 23 demonstrates the stability of TNF in the perfusion apparatus over a 120 minute perfusion without a liver present in the circuit. 75 ng and 20 ng of murine TNF were added to 100 ml of perfusate. As illustrated, both amounts remained stable over the 120 minute perfusion period.

Fifty nanograms of murine IL-6 was also tested in order to establish IL-6 stability in the perfusion. Fig. 24 depicts the IL-6 concentration in the perfusate

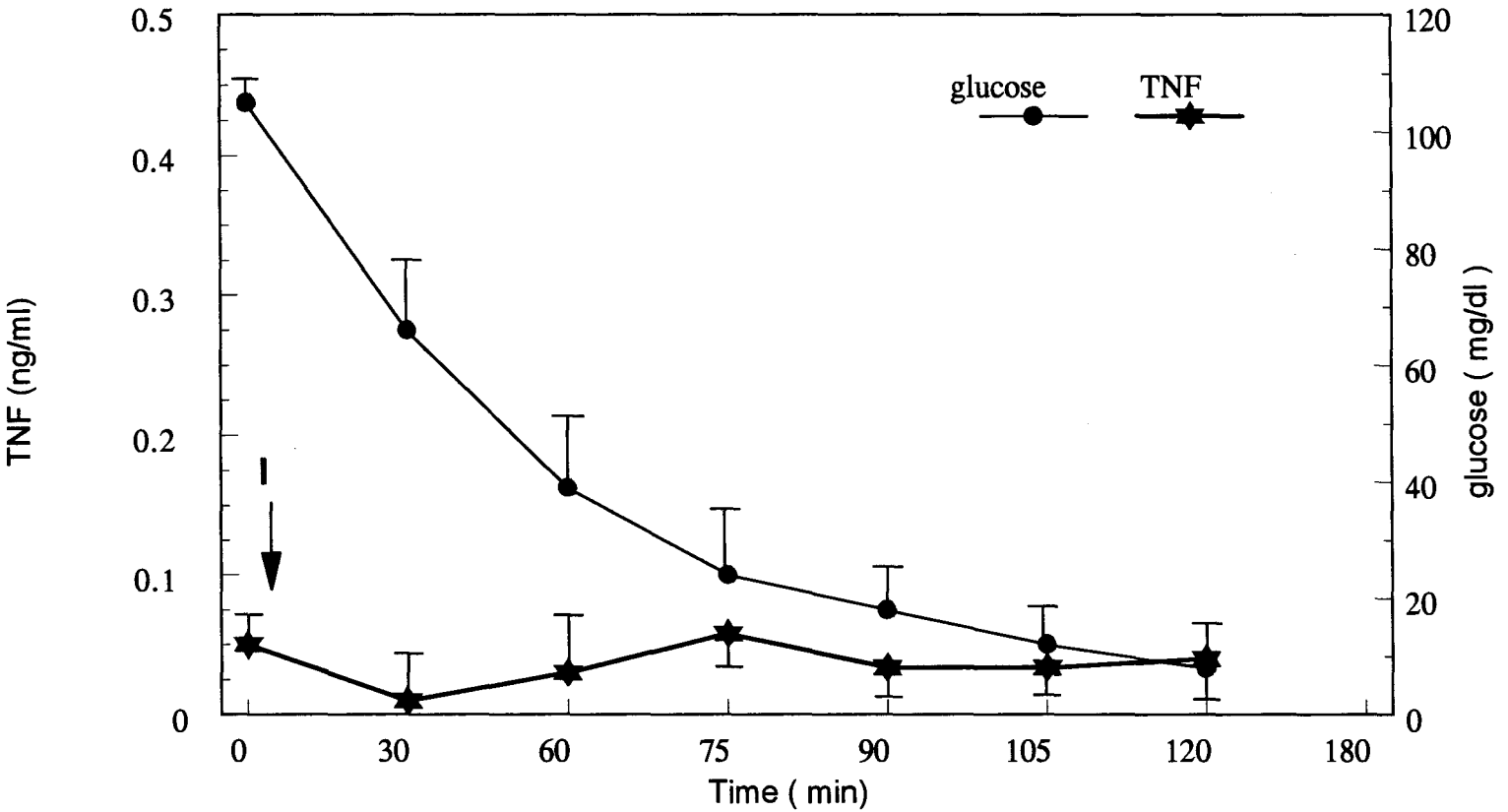


Fig. 22. The effect of insulin induced hypoglycemia on plasma glucose concentrations and plasma TNF over 180 minutes in anathetised normal rats. Insulin , I, (5 U) was injected s.c. immediately after the t=0 blood sample and blood samples were taken at the indicated time points. Values represent the means + SEM for the group (n = 6) at each time point.

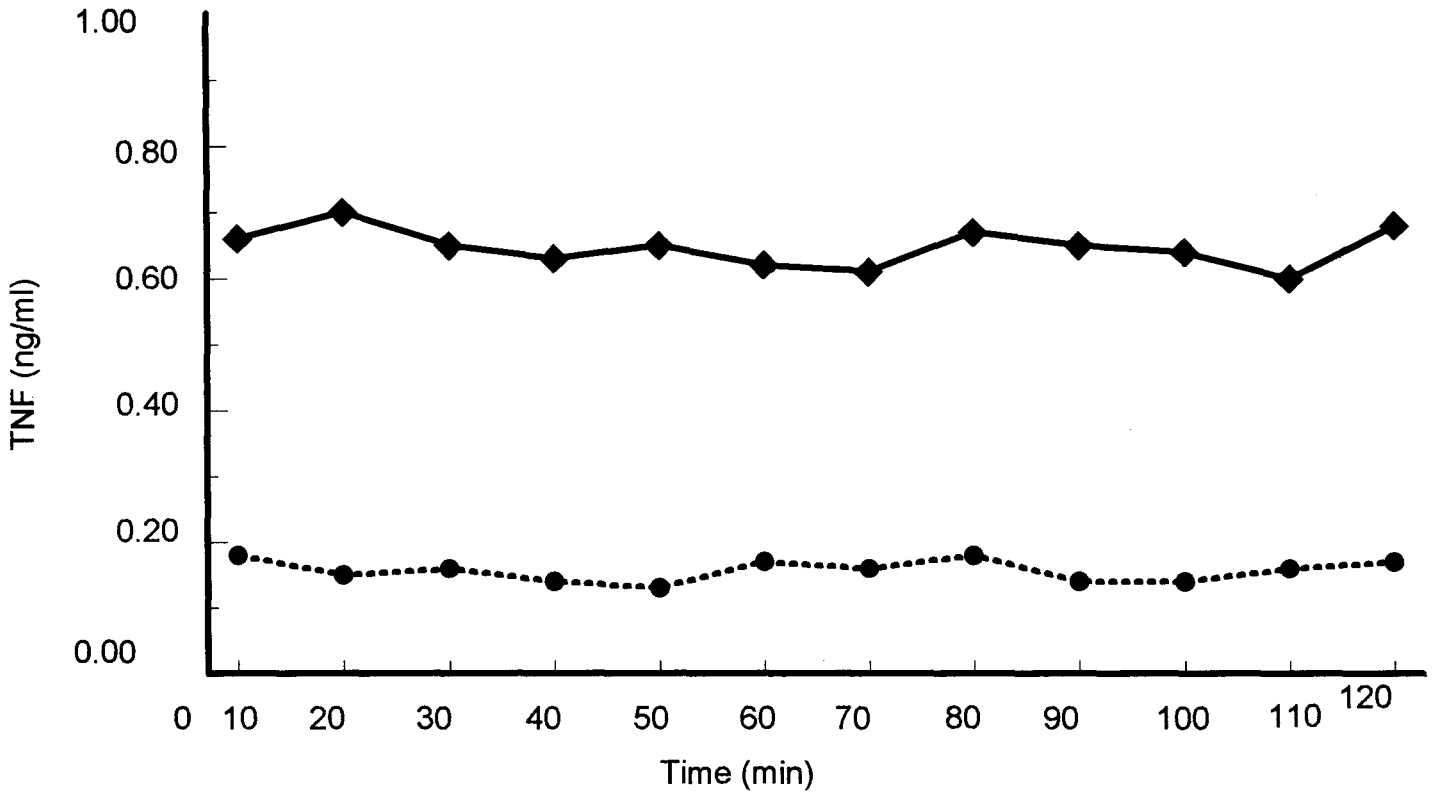


Fig. 23. Stability of recombinant murine TNF in the isolated perfusion apparatus over 120 minutes of perfusion at 37 ^{o} C without the presence of a liver in the system. Two doses of TNF, 20 ng and 75 ng were initially disolved in 100 mls of Krebs Ringers Buffer with 5% bovine serum albumin and then perfused through the system under standard operating conditions for two hours. Perfusate samples were removed at 10 minute intervals and analysed for TNF.

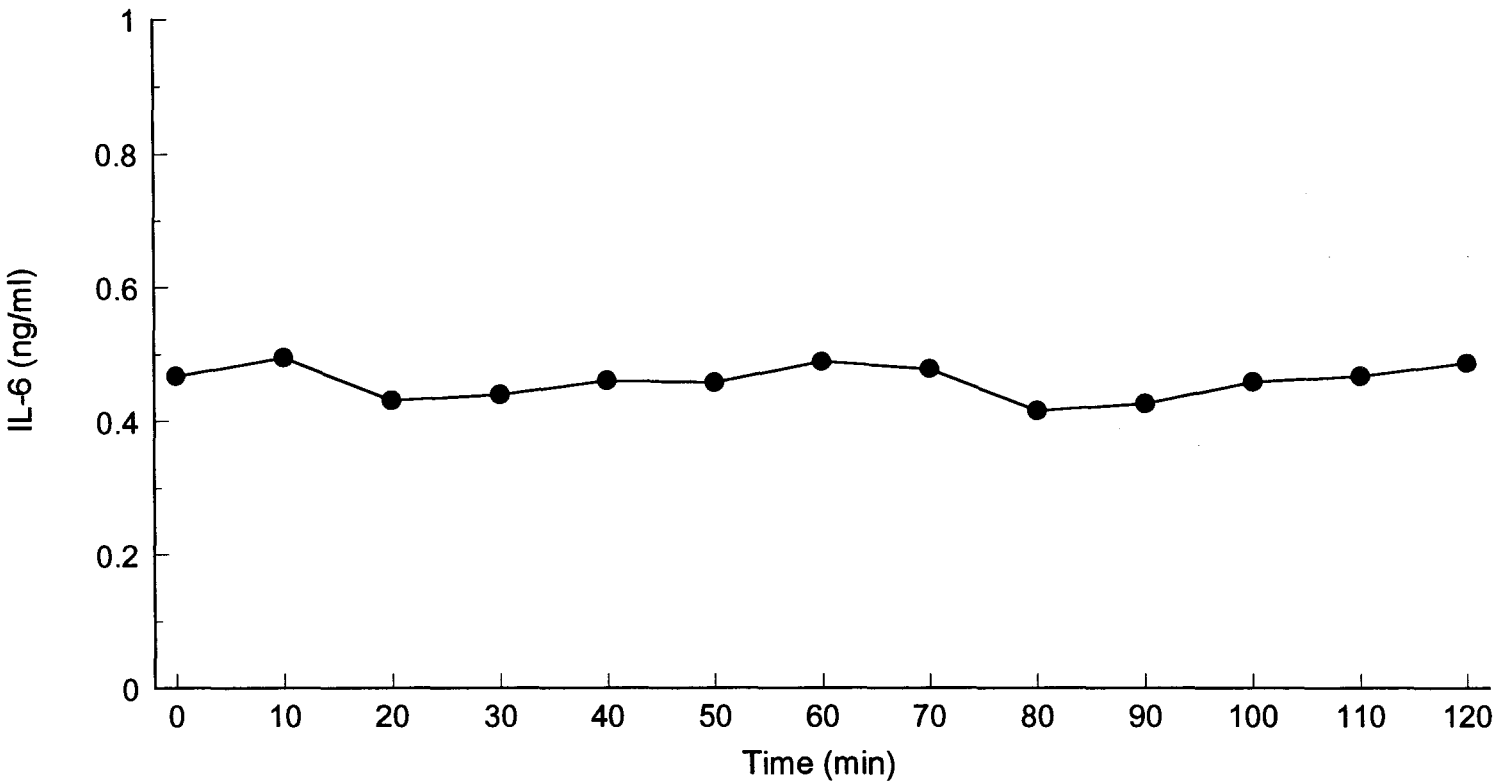


Fig. 24. Stability of recombinant murine IL-6 in the isolated perfusion apparatus over 120 minutes of perfusion at 37 ^{o} C without the presence of a liver in the system. Fifty nanograms of IL-6 were initially disolved in 100 mls of Krebs Ringers Buffer with 5% bovine serum albumin and perfused through the system under standard operating conditions for two hours. Perfusate samples were removed at 10 minute intervals and analysed for IL-6 by ELISA.

samples over 120 minutes of a "liverless" perfusion. Interleukin 6 concentrations remained constant over the 120 minute perfusion period. The maximal fluctuation was 12%.

The concentration of endotoxin in the perfusate prior to the introduction of a liver into the circuit was determined. Perfusate endotoxin concentrations were found to be less than 0.1 ng/ml initially.

2. The Effect of Dexamethasone and Insulin on Cytokine Production in the Isolated Perfused Liver.

The perfusion studies were performed with livers from rats treated *in vivo* with the experimental protocol described above in section A. Fig. 25 illustrates the TNF concentration in the perfusate over a 60 minute perfusion period. In the control, saline treated group TNF was not present in the perfusate until 50 minutes after the initiation of the perfusion. At 60 minutes TNF was also increased (0.233 ± 0.04 ng/ml) and appeared to be further increasing. DEX alone in the perfusion was able to significantly suppress this increase in TNF observed at the 50 and 60 minute time points. There was no measurable TNF in the perfusate of INS treated livers until 60 minutes at which time TNF was determined to be (0.18 ± 0.06 ng/ml). DEX/INS treatment completely suppressed TNF production at all time points.

ETX treatment significantly increased perfusate TNF concentration above the saline treated rats. TNF increased in the perfusate at a steady rate over the

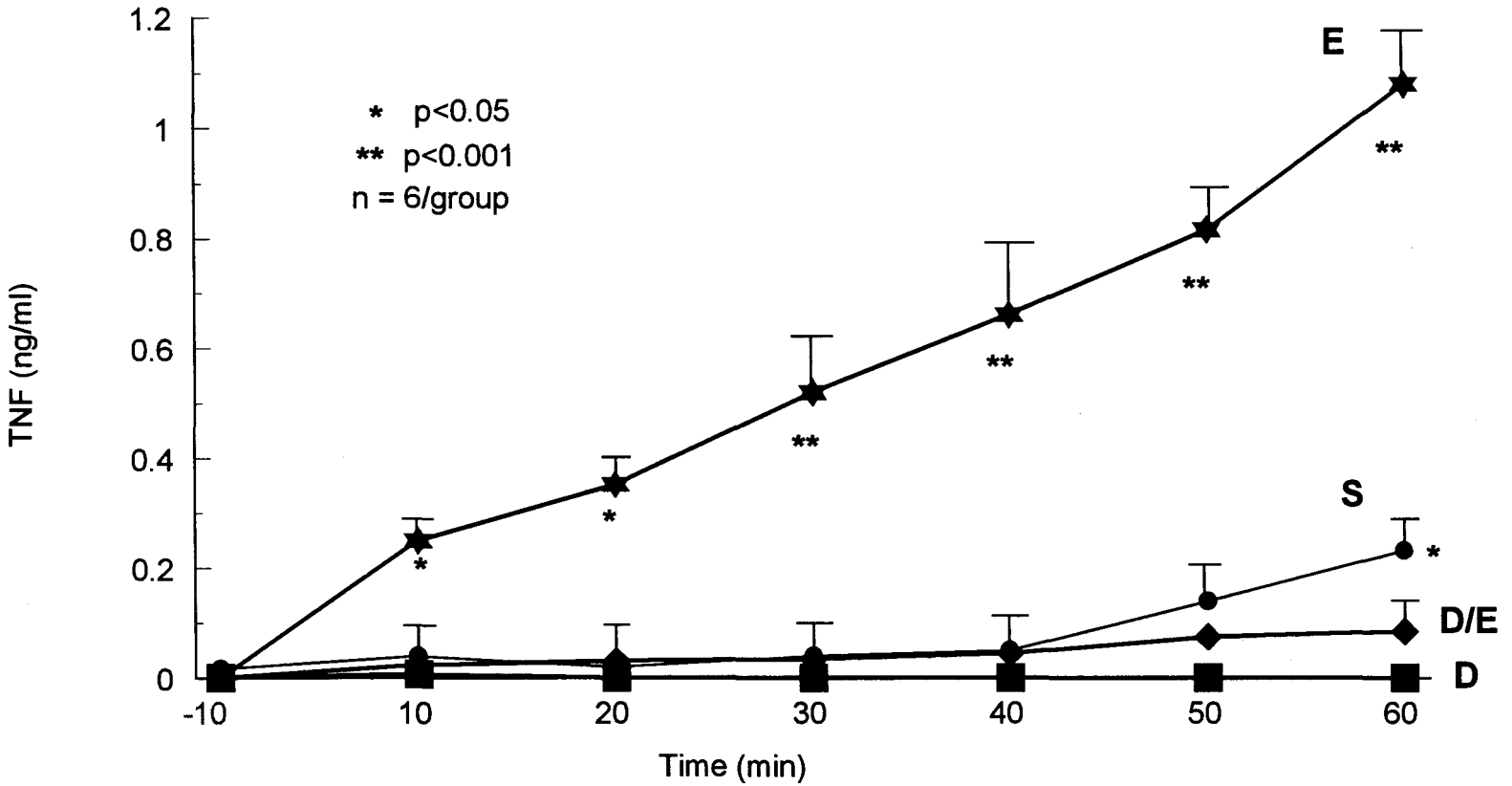


Fig. 25. TNF production by the isolated perfused liver over 60 minutes. The rats were injected with dexamethasone, D, (0.33 mg/kg) 3 hours prior to the administration of endotoxin, E, (20 mg/kg) or saline, S, . The liver was then removed and perfused for 1 hour. Values represent the mean + SEM for each group. * $p < 0.05$ compares E with D/E and S with D and D/E. ** $p < 0.001$ compares E with S, D, and D/E.

60 minute perfusion period. Maximal TNF production was observed at the end of the perfusion period (1.08 ± 0.22 ng/ml). DEX/ETX pretreatment *in vivo* significantly suppressed TNF production by the perfused liver . The maximum concentration of TNF observed was 85.8 ± 36.4 pg/ml.

Fig. 26 examines the effect of insulin addition on TNF production by the perfused liver over 60 minutes. ETX/INS treatment resulted in a significantly increased initial TNF concentration in the perfusate (0.429 ± 0.062 ng/ml) in comparison to ETX treatment (0.25 ± 0.05 ng/ml). The initial increase in TNF production continued until approximately 40 minutes after the initiation of the perfusion, when TNF production almost ceased. DEX/ETX treatment significantly decreased TNF production in comparison with ETX and ETX/INS. Insulin post-treatment *in vivo* in the DEX/ETX/INS group could not restore TNF production. TNF perfusate concentrations of the DEX/ETX group and the DEX/ETX/INS group were not significantly different over the entire time course of the experiment.

Fig. 27 graphically illustrates the IL-6 levels in the perfusate of the treatment groups during the 60 minute perfusion period. The saline treated group had undetectable levels of IL-6 until 40 minutes of the perfusion. IL-6 concentration in the perfusate was 53.1 ± 3.9 pg/ml. This amount did not significantly change at the 50 and 60 minute sampling periods (54.1 ± 4.0 pg/ml and 52.8 ± 3.7 pg/ml). INS treatment initiated a similar response as measurable concentrations of IL-6 were detected to be 26.7 ± 2.87 and 41.3 ± 3.1 pg/ml at 50 and 60 minutes respectively. DEX and DEX/INS treatment were found to

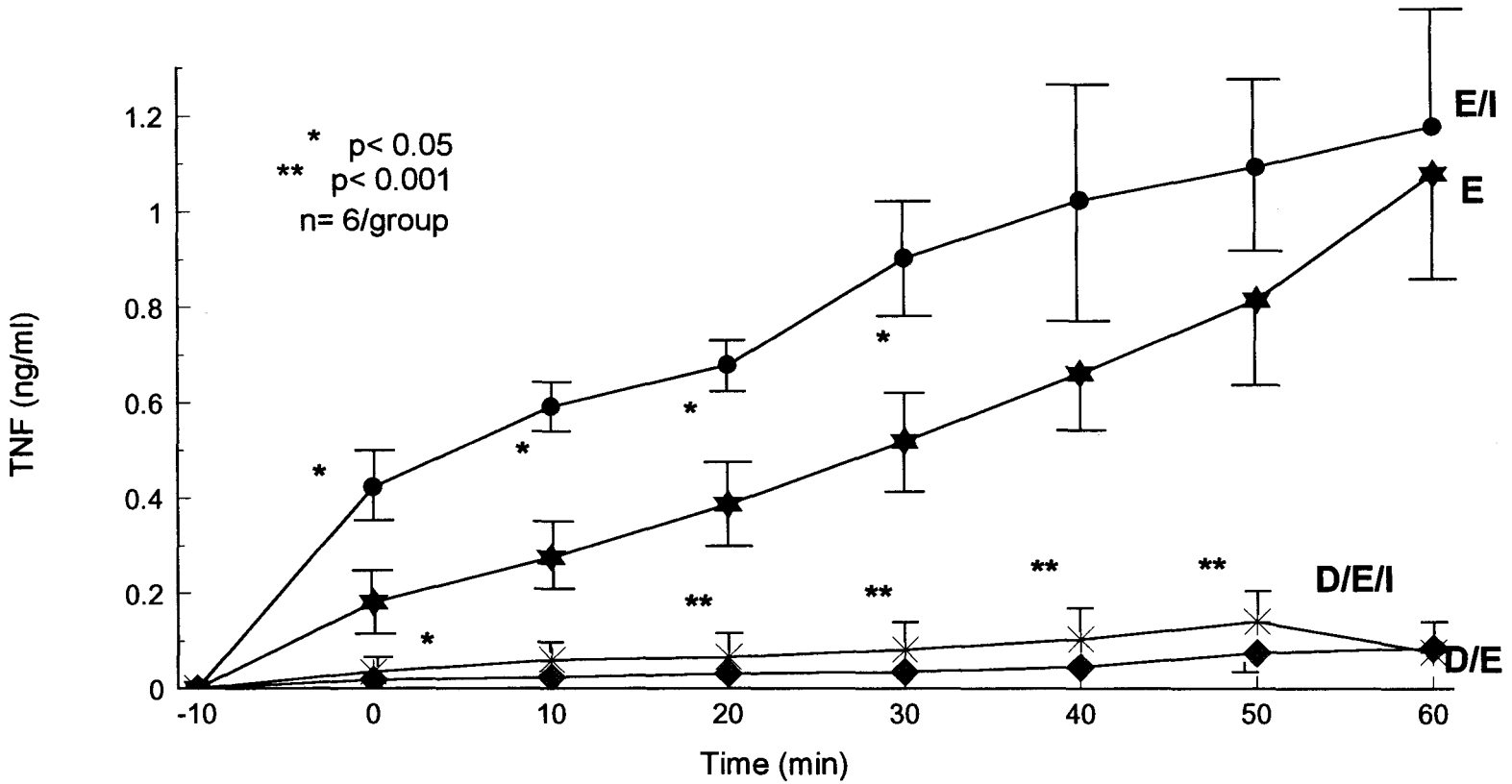


Fig. 26. TNF production by the isolated perfused liver over 60 minutes. The rats were injected with dexamethasone, D, (0.33 mg/kg) 3 hours prior to the administration of endotoxin, E, (20 mg/kg) or saline, S, . Insulin , I , (1 U) or saline was administered 1 hour post-endotoxin. The liver was then removed and perfused for 1 hour. Values represent the mean + SEM for each group. * $p < 0.05$ compares E with E/I.
** $p < 0.001$ compares E and E/I with D/E and D/E/I.

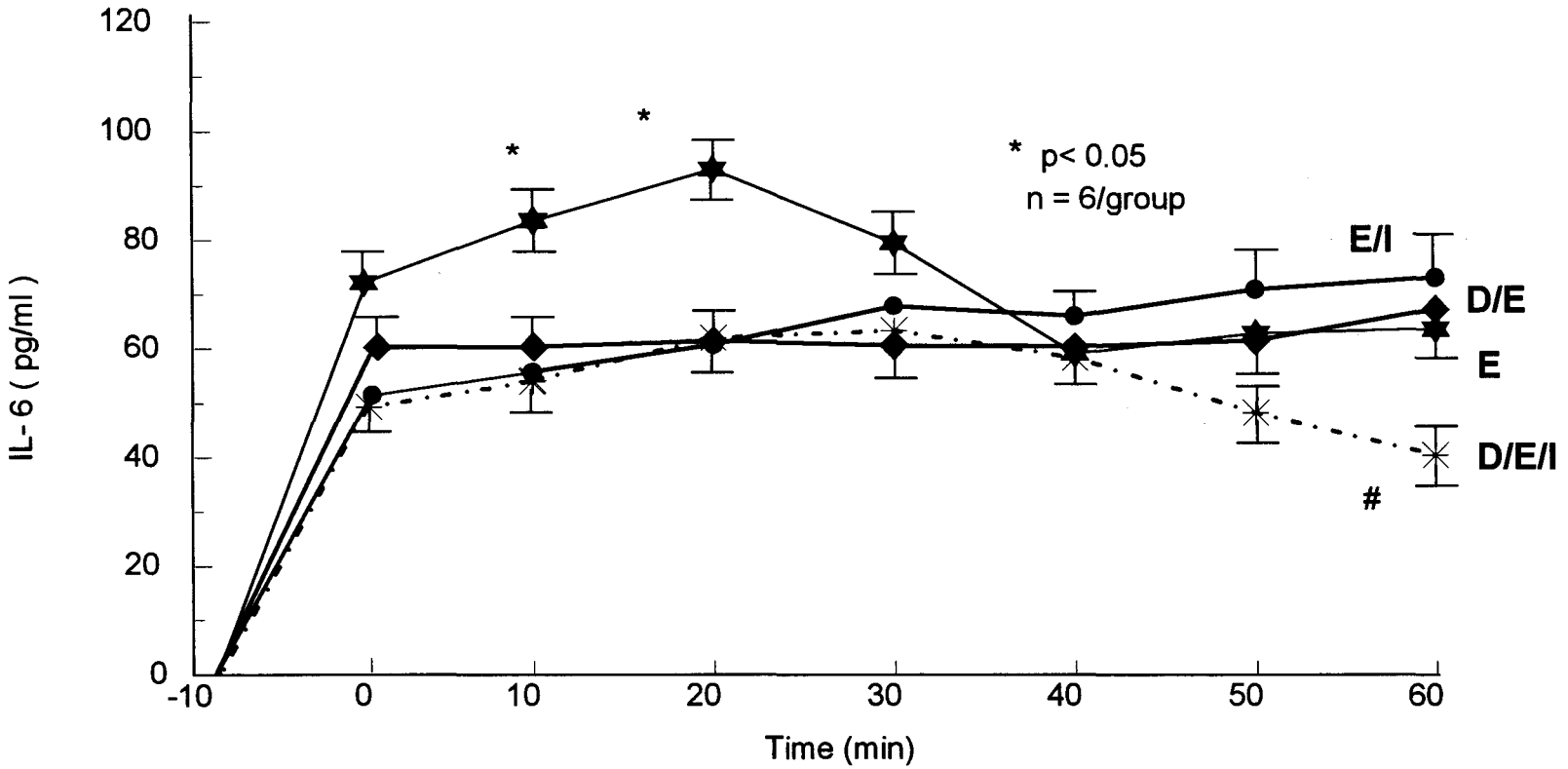


Fig. 27. IL-6 production by the isolated perfused liver over 60 minutes. The rats were injected with dexamethasone, D, (0.33 mg/kg) 3 hours prior to the administration of endotoxin, E, (20 mg/kg) or saline, S, . Insulin , I , (1 U) or saline was administered 1 hour post-endotoxin. The liver was then removed and perfused for 1 hour. Values represent the mean + SEM for each group. * $p < 0.05$ compares E with E/I , D/E and D/E/I at 10 and 20 minutes. # $p < 0.05$ compares D/E/I with E , E/I and D/E at 60 minutes.

suppress the increases in IL-6 at the 50 and 60 time points as no detectable levels of IL-6 were found in these groups at all time points.

Initial IL-6 production by the ETX perfused liver was approximately 77.8 ± 5.72 pg/ml. The peak concentration (93.1 ± 5.2 pg/ml) was observed at 20 minutes. The IL-6 concentration in this group then declined over the remaining perfusion time period such that at 40 minutes it appeared that IL-6 production by the perfused liver had ceased. DEX/ETX treatment significantly suppressed IL-6 production (60.4 ± 4.8 pg/ml) below that of the ETX treatment group. There was no change in IL-6 production observed over the time course of the perfusion. The ETX/INS treatment group had a significantly lower initial IL-6 concentration (55.7 ± 7.08 pg/ml) than the ETX group. This concentration did increase slowly until a peak concentration of 73.1 ± 1.48 ng/ml was observed at 60 minutes. DEX/ETX/INS treatment caused no significant change in IL-6 over time until 50 minutes, where IL-6 concentrations began to decline. The concentration (0.45 ± 6.3 pg/ml) was significantly different from all the other experimental groups at 60 minutes.

D. *In Vitro* Studies

1. Cell Lines

In order to investigate the effects of hormonal modulation on TNF and IL-6 production in isolated cell systems, two macrophage cell lines, ANA1 and RAW

264.7 cell lines were initially examined. The experimental design employed was similar to that used in the *in vivo* and *ex vivo* studies.

The cells were cultured at density of $1x10^6$ cells/ml. Dexamethasone phosphate (100 nM) was added to the cells three hours prior to treatment with *S. enteritidis* endotoxin (1 ng/ml or 100 ng/ml). Insulin (100 μU) was added 1 hour post endotoxin. The medium was harvested 16 hours post endotoxin and analyzed for TNF and IL-6 concentrations. These treatments did not alter cell viability or the rate of growth over the test period. Endotoxin concentrations of the dexamethasone and insulin was determined to be less than 0.1 ng/ml.

Fig. 28 and Fig. 29 illustrate the TNF and IL-6 levels in the medium of ANA1 macrophages 16 hours after endotoxin. In response to 1 ng/ml endotoxin, ANA1 cells produced 3.4 ± 0.42 ng/ml of TNF and 6.5 ± 0.81 ng/ml of IL-6. Contrary to the response observed in the *in vivo* experiments, insulin post-treatment of endotoxic macrophages significantly suppressed TNF (1.7 ± 0.036 ng/ml) and IL-6 production (2.5 ± 0.91 ng/ml). Dexamethasone pretreatment of endotoxin-stimulated ANA1 cells (DEX/ETX) did not significantly suppress TNF production, however, the IL-6 concentration in the media was completely abolished (< 1 pg/ml). The addition of insulin to the DEX/ETX/INS group did not statistically increase TNF concentrations. The addition of insulin did, however, restore IL-6 production (5.0 ± 0.85 ng/ml).

The macrophage cell line, RAW 264.7, was also tested using the same protocol. The response of these cells to hormonal modulation is graphically

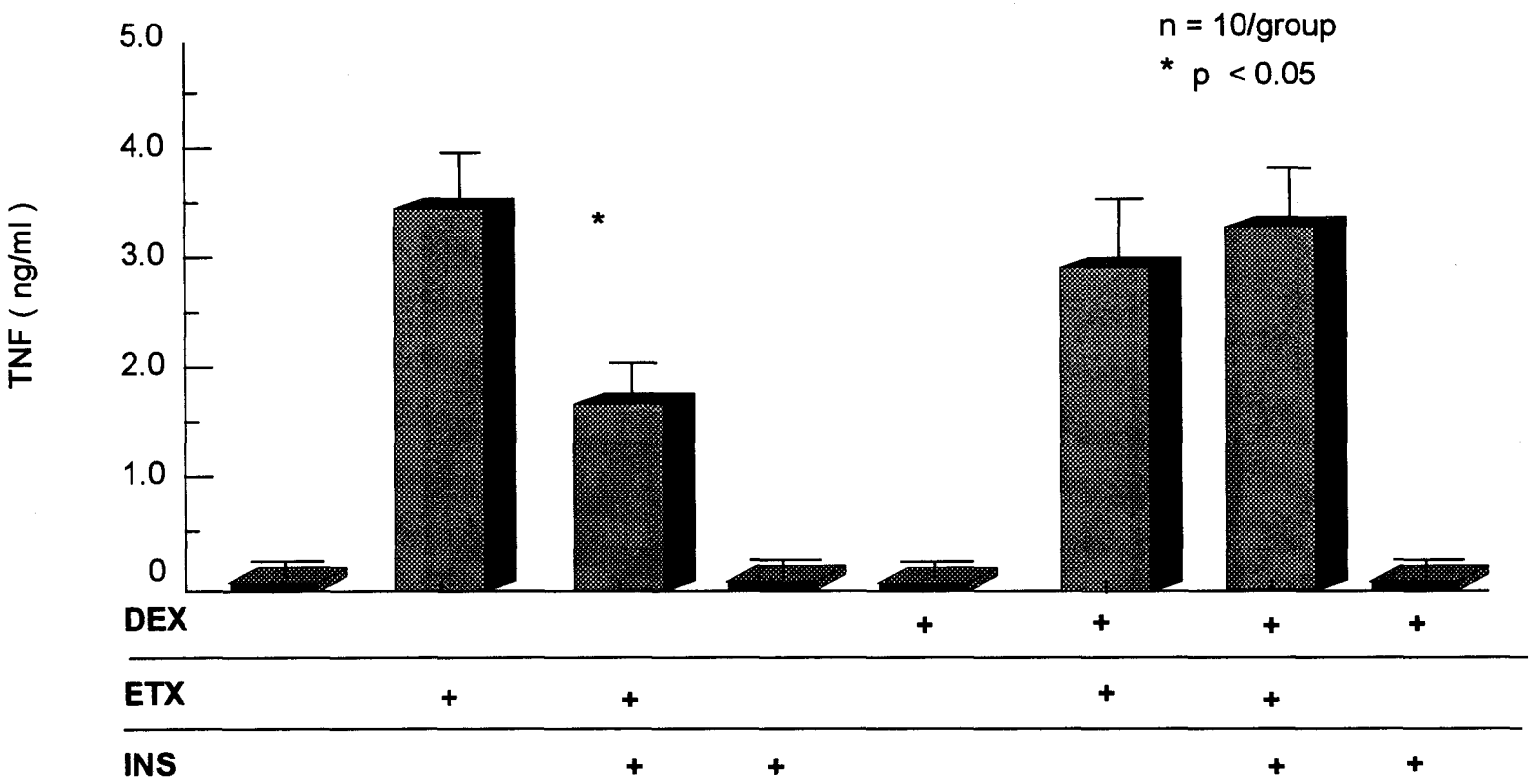


Fig. 28. TNF production by ANA1 macrophages . ANA1 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for TNF by ELISA. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX/INS with the other groups.

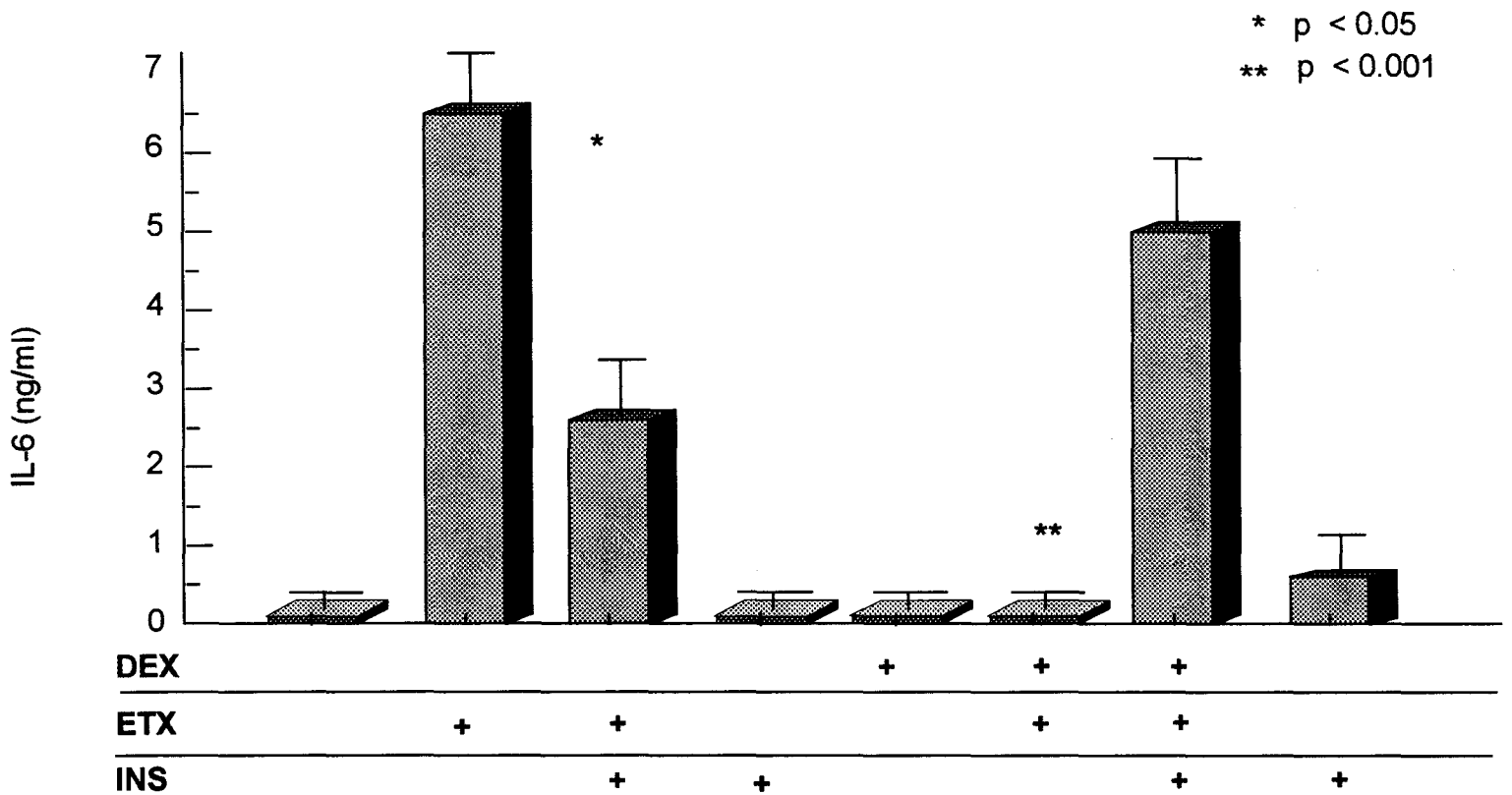


Fig. 29. IL-6 production by ANA1 macrophages . ANA1 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by ELISA. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX/INS with the other groups. ** $p < 0.001$ compares DEX/ETX with DEX/ETX/INS.

illustrated in Figs. 30 and 31. In response to 1 ng/ml of endotoxin, these cells produced impressive quantities of TNF (9.3 ± 0.59 ng/ml) and IL-6 (3.6 ± 0.26 ng/ml). Insulin post- treatment of the endotoxin-treated group, ETX/INS, did not significantly alter the TNF or IL-6 concentrations in the medium.

DEX did not stimulate TNF or IL-6 production. DEX/ETX treatment significantly diminished TNF concentrations in comparison to ETX treatment (6.4 ± 0.21 ng/ml vs. 9.3 ± 0.59 respectively). The IL-6 concentration of the medium was also significantly reduced to 1.5 ± 0.12 ng/ml. Insulin post treatment, DEX/ETX/INS, did not alter either TNF or IL-6 production in these cells.

2. Primary Macrophage Cells

Two primary macrophage cell types (peritoneal macrophages and Kupffer cells) were tested with the above described protocol to determine the effects on isolated cells that are approaching the *in vivo* setting. The timing and dosages administered are the same as described above except the dose of endotoxin was increased to 100 ng/ml as 1 ng/ml did not induce the production of TNF or IL-6 in the peritoneal macrophages.

The TNF and IL-6 production by rat peritoneal macrophages is described in Figs. 32 and 33, respectively. Saline treatment did not provoke TNF production in the peritoneal macrophages. There was however, basal production of IL-6 in the medium of 138.5 ± 29.5 pg/ml by these cells. ETX stimulated cells produced 1.6 ± 0.22 ng/ml TNF and 467 ± 30.9 pg/ml of IL-6. The addition of insulin,

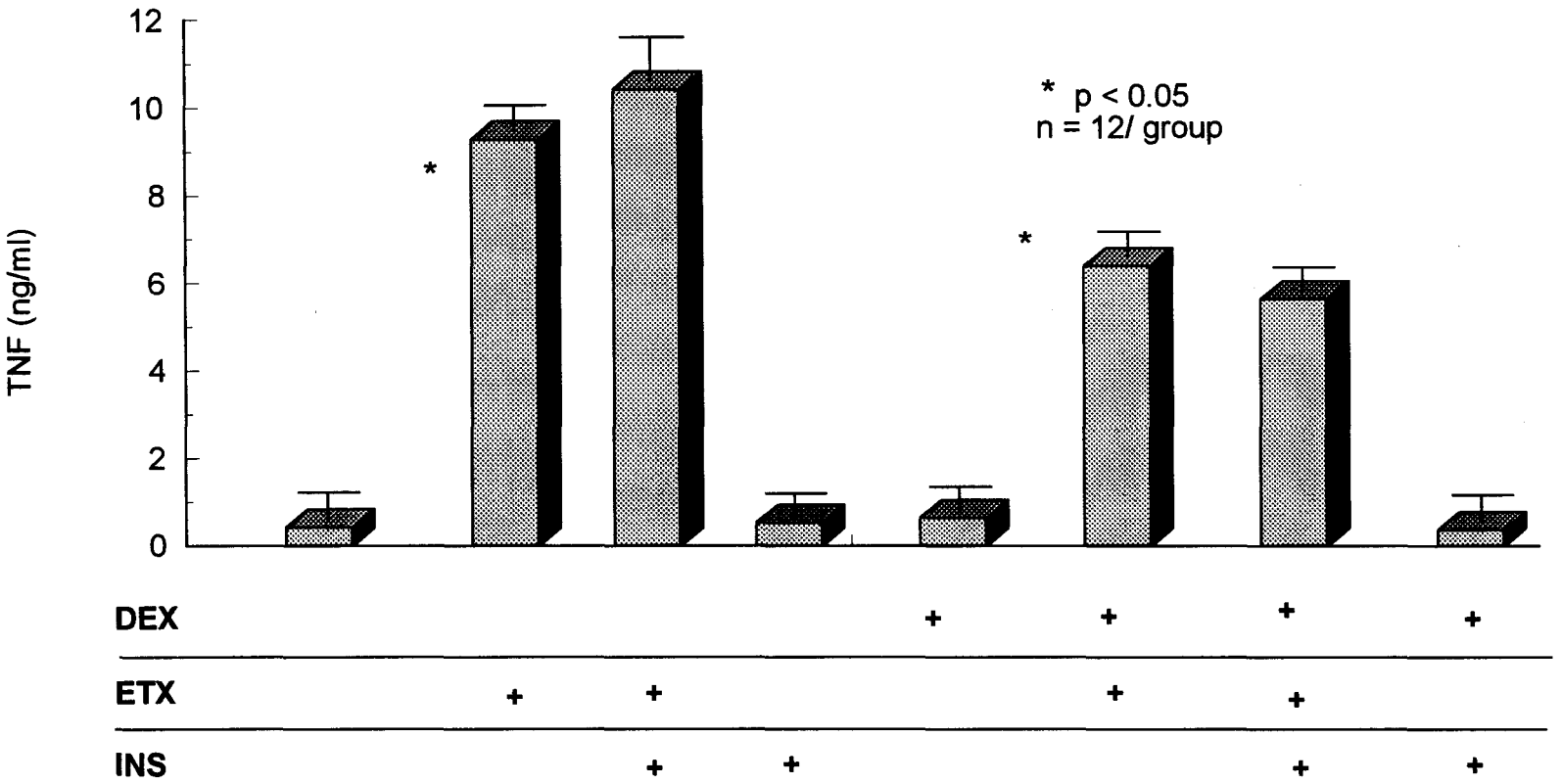


Fig. 30. TNF production by RAW 264.7 macrophages . RAW 264.7 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for TNF by ELISA.. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX with saline and with DEX/ETX.

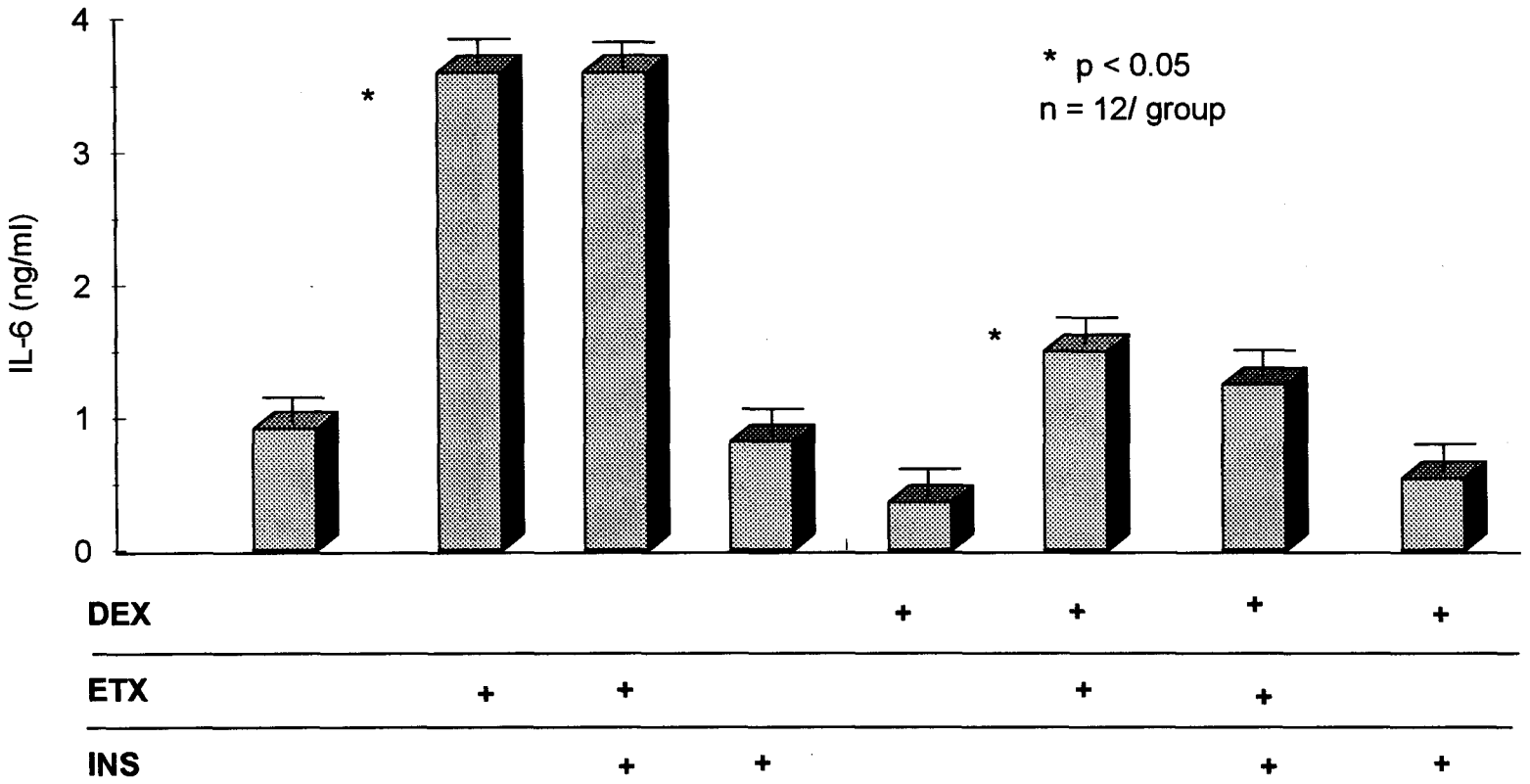


Fig. 31. IL-6 production by RAW 264.7 macrophages . RAW 264.7 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by ELISA. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX saline and with DEX/ETX.

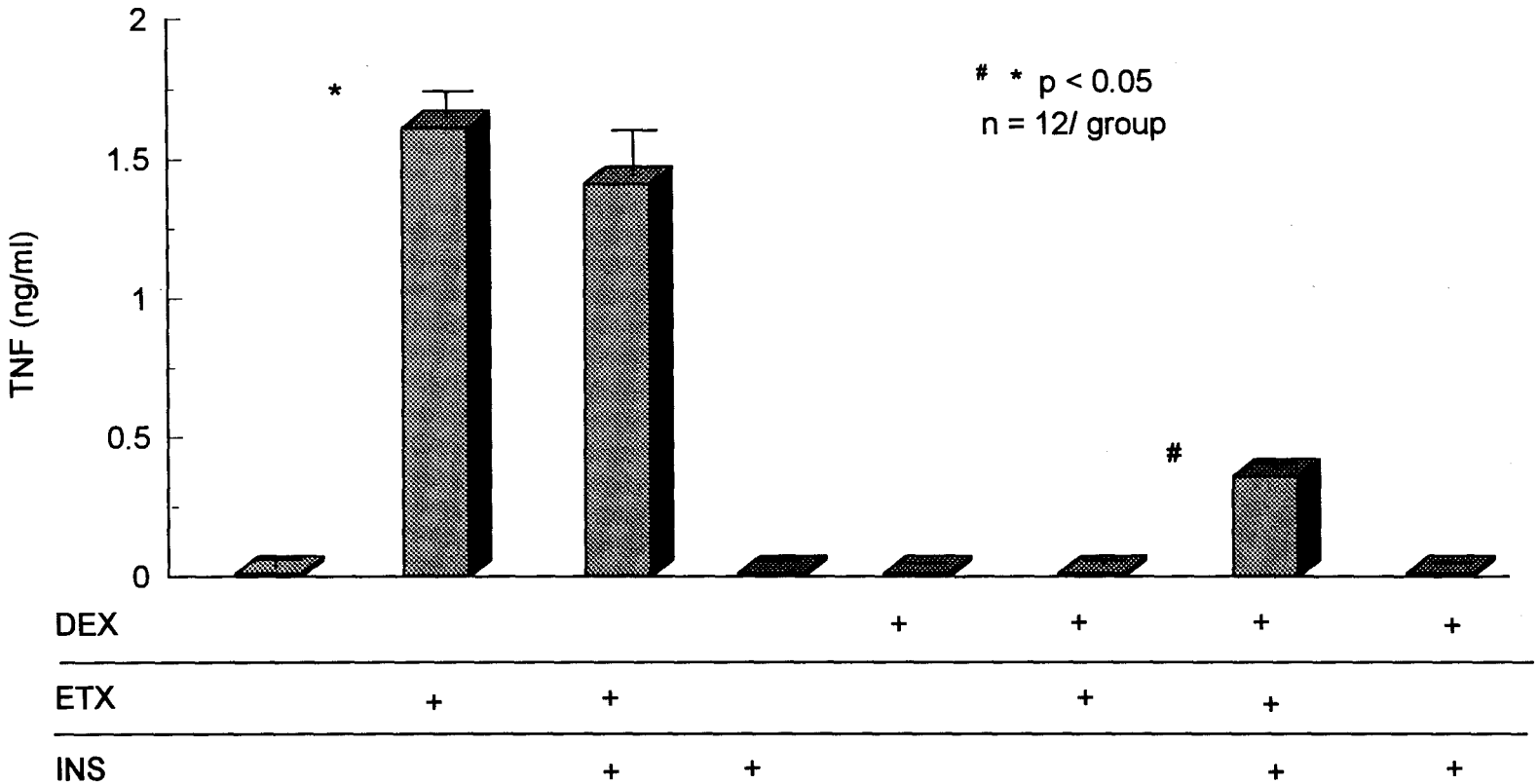


Fig. 32. TNF production by peritoneal macrophages. The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (100 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for TNF by ELISA.
+ designates the agents given. The absence of + designates saline was given .
* $p < 0.05$ compares ETX with saline . # $p < 0.05$ compares DEX/ETX with DEX/ETX/INS.

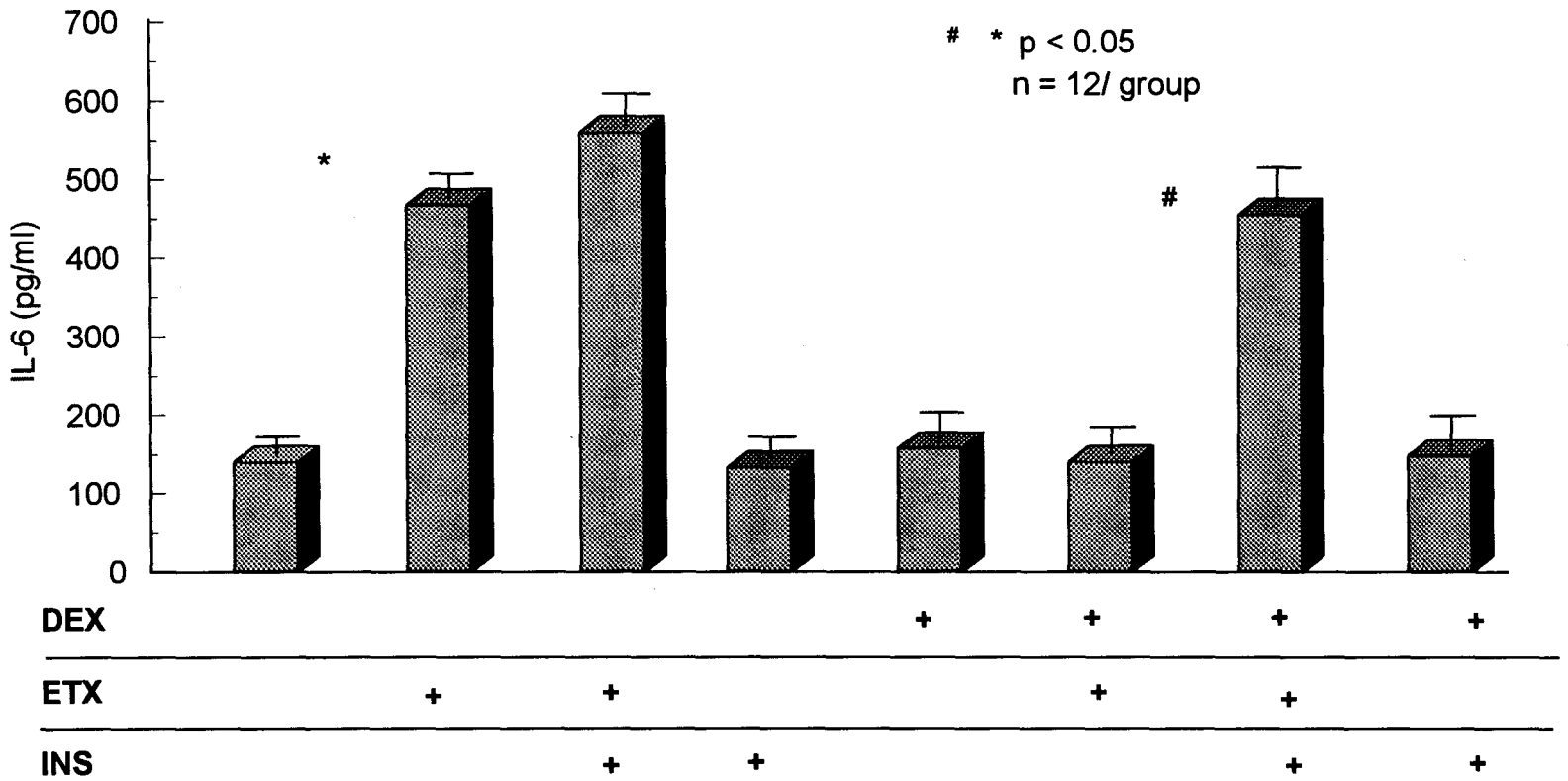


Fig. 33. IL-6 production by peritoneal macrophages. The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (100 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by B9 bioassay. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX with saline. # $p < 0.05$ compares DEX/ETX with DEX/ETX/INS.

ETX/INS, did not significantly alter TNF or IL-6 production in these cells (1.41 ± 0.12 ng/ml and 561.6 ± 42.3 pg/ml). DEX/ETX treatment completely suppressed both TNF and IL-6 production. Insulin administration to a DEX/ETX group stimulated a modest but significant increase in TNF concentration (0.354 ± ng/ml). DEX/ETX/INS treatment in these cells completely restored IL-6 production (455.4 ± 33.9 pg/ml) such that there was no statistical difference from the ETX group. INS, DEX, or DEX/INS treatment did not alter TNF or IL-6 production.

Fig. 34 and fig. 35 illustrate the response of Kupffer cells to hormonal modulation. Kupffer cells in this system did not constitutively produce TNF, however, IL-6 was constitutively produced at a level of 176.6 ± 22.15 pg/ml. Endotoxin stimulation (100 ng/ml) prompted a significant increase in TNF concentration (2.43 ± 0.326 ng/ml). Interleukin 6 was also significantly increased by endotoxin treatment (428.45 ± 19.4 pg/ml). As observed with the ANA1 cells, insulin post-treatment of endotoxin stimulated Kupffer cells resulted in a significant decrease, $p<0.001$ in TNF and IL-6 production, $p<0.01$, in comparison with ETX treatment. Dexamethasone pretreatment, DEX/ETX, significantly suppressed both TNF and IL-6 production to control values. Insulin post-treatment, DEX/ETX/INS resulted in a small increase in TNF production (0.675 ± 0.29 ng/ml) and a modest increase in IL-6 production (302.5 ± 21.3 pg/ml) in comparison to DEX/ETX treatment. DEX, DEX/INS and INS treatment groups were found to have TNF and IL-6 concentrations that were not significantly different from saline treated cells.

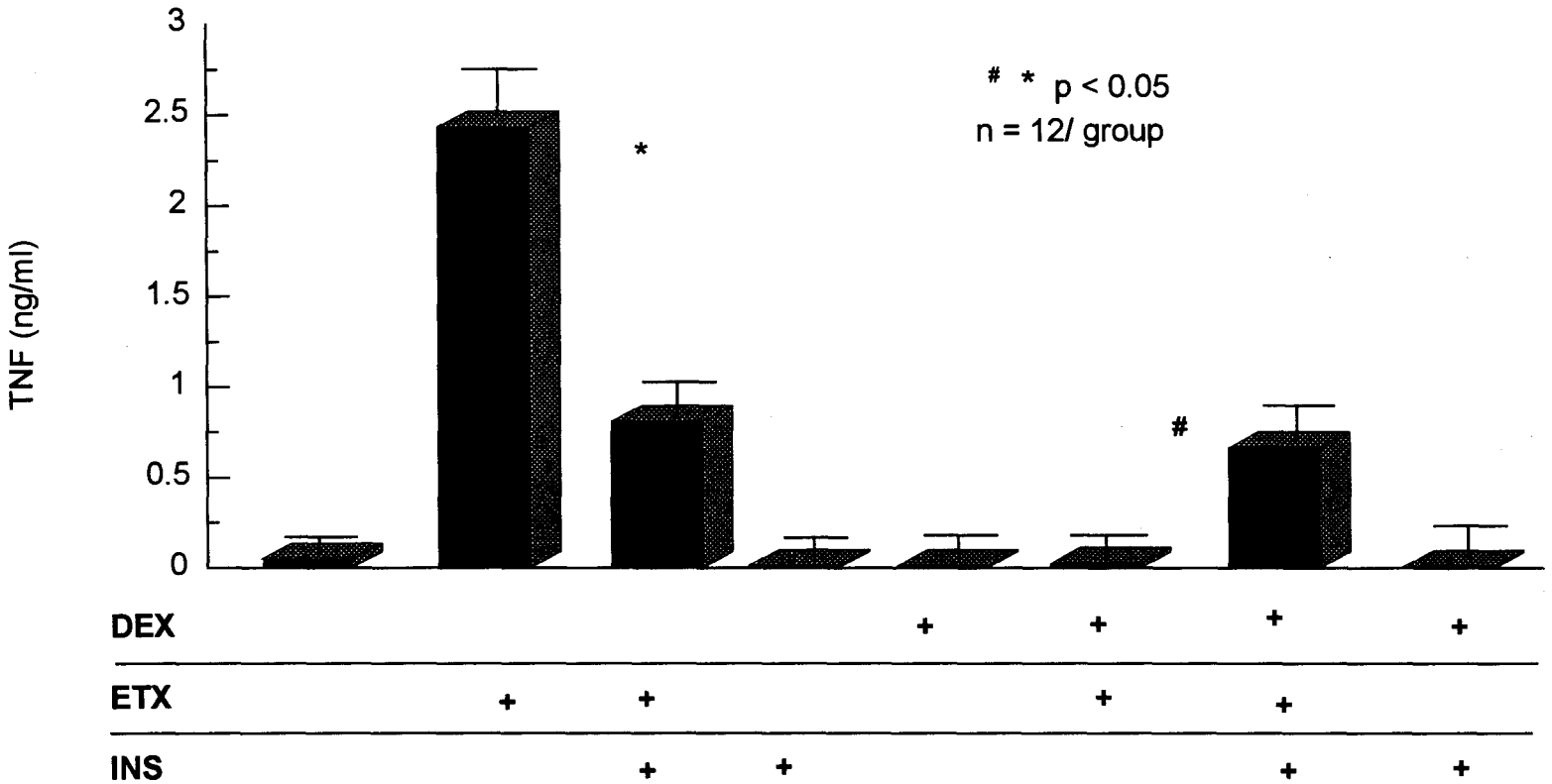


Fig. 34. TNF production by Kupffer cells. Kupffer cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (100 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for TNF by ELISA. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX with saline and with ETX/INS. # $p < 0.05$ compares DEX/ETX with DEX/ETX/INS.

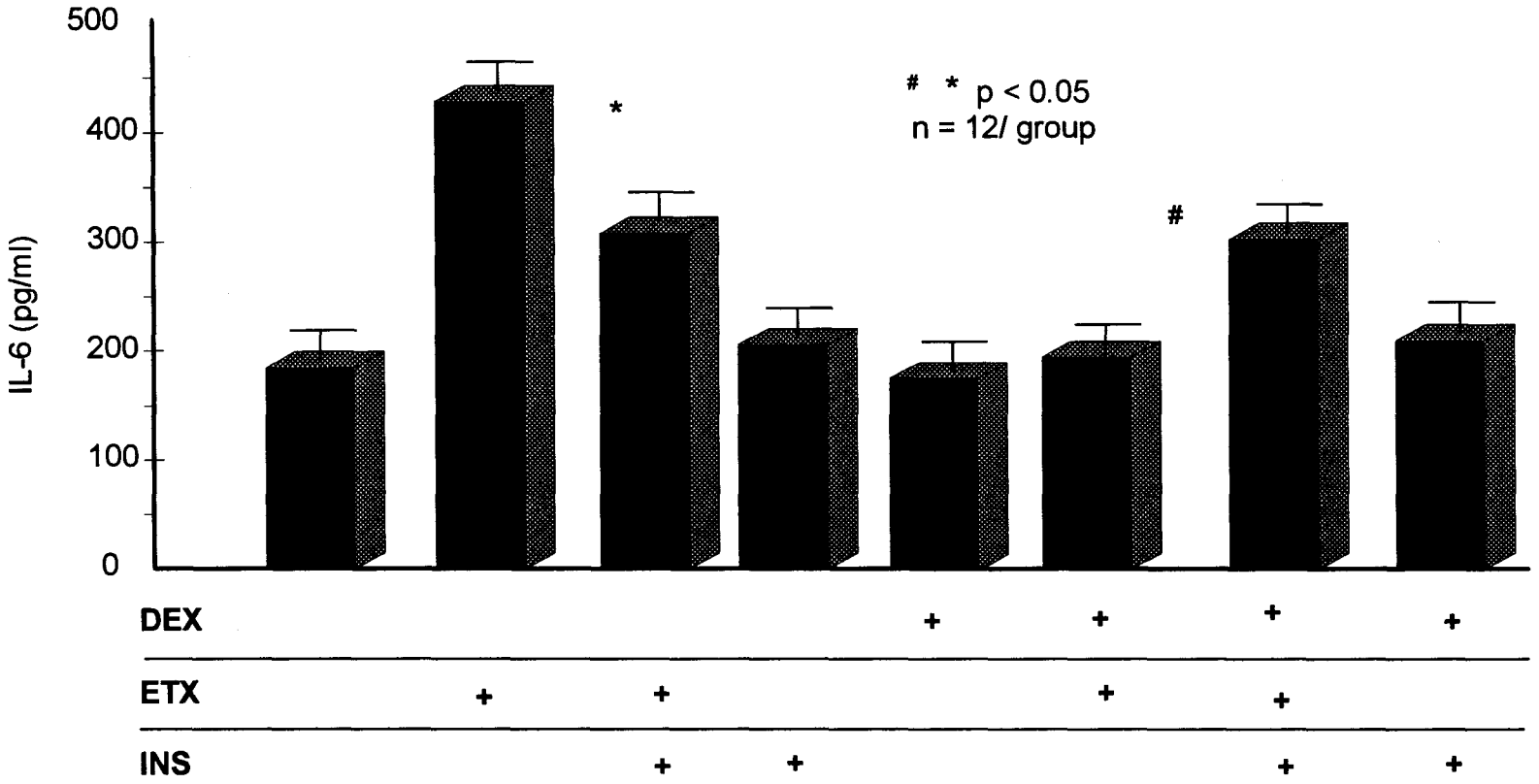


Fig. 35. IL-6 production by Kupffer cells. Kupffer cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (100 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) was added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by B9 bioassay. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX with ETX/INS. # $p < 0.05$ compares DEX/ETX with DEX/ETX/INS.

3. Mechanisms of Insulin Action on TNF and IL-6 *In Vitro*

To investigate the possible mechanisms of insulin action on TNF and IL-6 production several pharmacological agonists and antagonists of known second messenger pathways were added to the *in vitro* protocol described above. The ANA1 cells and the peritoneal macrophages were chosen as the cell types to be tested because of their initial positive responses to the various treatment groups. Briefly, dexamethasone phosphate (100 nM) was added to the cells three hours prior to treatment with *S. enteritidis* endotoxin at 1 ng/ml for the ANA1 cells or 100 ng/ml for the peritoneal macrophages. Insulin (100 μU) and/or a pharmacological agent was added 1 hour post endotoxin. The medium was harvested 16 hours post endotoxin and analyzed for TNF and IL-6 concentrations. These treatments, with the exception of cycloheximide, did not alter cell viability or the rate of growth over the test period. The endotoxin contamination of all the pharmacological substances used was determined to be less than 0.1 ng/ml.

a) The Role of Protein Kinase C

Table 10 depicts the effect of PMA on ANA1 macrophages. PMA stimulated ANA1 cells to produce 1.20 ± 0.34 ng/ml of IL-6. ETX stimulated IL-6 significant increases in IL-6 medium concentration (6.58 ± 0.72 ng/ml). ETX/PMA treatment did not cause a significant augmentation in IL-6 (5.62 ± 1.72 ng/ml). Dexamethasone pretreatment, DEX/ETX, severely depressed IL-6 production and the addition of PMA to the media, DEX/ETX/PMA, overcame the depression (3.28

Table 10--The Effect of PMA on IL-6 production in ANA1 macrophages.

Groups	IL-6 (ng/ml)
saline	≤ 0.001
ETX	6.58 ± 0.72**
PMA	1.20 ± 0.34*
ETX/PMA	5.62 ± 1.7
DEX/ETX	≤ 0.001
DEX	≤ 0.001
DEX/ETX/PMA.	3.28 ± 0.84**
DEX/PMA	≤ 0.001

Cells were treated with dexamethasone , DEX, (100 nM) 3 hours prior to the addition of *S. enteritidis* endotoxin, ETX, (1 ng/ml). One hour later PMA (1 nM) was added to the cells. Media was harvested 16 hours after ETX stimulation and analysed for IL-6 by ELISA.

*$p<0.01$ compares saline with PMA.

**$p<0.001$ compares saline with ETX and also DEX/ETX with DEX/ETX/PMA.

± 0.84 ng/ml). Dexamethasone was also shown to depress PMA induced IL-6 secretion.

Since the data in table 10 demonstrated that PMA and insulin were acting in a similar manner with respect to IL-6 production in ANA1 macrophages, H-7, an inhibitor of protein kinase C was added to the original protocol as a cotreatment with insulin to observe the effects. Table 11 illustrates the results from this experiment. H-7 cotreatment had no effect on the initial observations demonstrated in fig. 29.

The effect PMA (1 nM) was also tested on peritoneal macrophages. Table 12 summarizes the results of PMA treatment on TNF and IL-6 production. PMA alone was able to stimulate both TNF (0.98 ± 0.36 ng/ml) and IL-6 (362 ± 87 pg/ml) secretion. ETX/PMA treatment significantly increased TNF but not IL-6 secretion in comparison to ETX treatment. DEX/ETX/PMA was shown to restore TNF secretion in comparison to DEX/ETX treatment, however, IL-6 was not statistically increased. As demonstrated with the ANA1 cells dexamethasone treatment was able to suppress PMA induced TNF secretion.

Table 13 depicts the effect of H-7 cotreatment on the insulin-induced cytokine production of peritoneal macrophages. As previously shown in the ANA1 cells H-7 did not alter either TNF or IL-6 production.

b) The Role of Prostaglandins

To determine if the modulation of prostaglandin production was involved

Table 11--The effect of H-7 on IL-6 production in ANA1 macrophages.

Groups	IL-6 (ng/ml)
saline / H-7	≤ 0.001
ETX/H-7	5.31 ± 0.731
ETX/INS/H-7	5.12 ± 0.92
INS/H-7	≤ 0.001
DEX/ETX/H-7	≤ 0.001
DEX/H-7	≤ 0.001
DEX/ETX/INS/H-7	4.82 ± 0.631
DEX/INS/H-7	≤ 0.001

Cells were treated with dexamethasone , DEX, (100 nM) 3 hours prior to the addition of *S. enteritidis* endotoxin, ETX, (1 ng/ml). One hour later insulin (100 μU) and H-7 (1 μg/ml) was added to the cells. Media was harvested 16 hours after ETX stimulation and analysed for IL-6 by ELISA.

Table 12--The effect of PMA on TNF and IL-6 production in peritoneal macrophages.

Groups	TNF (ng/ml)	IL-6 (ng/ml)
saline	≤ 0.001	0.153 ± 0.076
ETX	1.84 ± 0.713	0.643 ± 0.049
PMA	0.98 ± 0.365	0.362 ± 0.087
ETX/PMA	3.10 ± 0.75 *	0.740 ± 0.140
DEX/ETX	≤ 0.001	0.142 ± 0.023
DEX	≤ 0.001	0.137 ± 0.011
DEX/ETX/PMA	0.741 ± 0.12*	0.321 ± 0.13
DEX/PMA	≤ 0.001	0.254 ± 0.085

Cells were treated with dexamethasone , DEX, (100 nM) 3 hours prior to the addition of *S. enteritidis* endotoxin, ETX, (100 ng/ml). One hour later PMA (1 nM) was added to the cells.
*$p<0.05$ compares ETX with ETX/PMA and DEX/ETX with DEX/ETX/PMA

Table 13-- The effect of H-7 on TNF and IL-6 production in peritoneal macrophages.

Groups	TNF (ng/ml)	IL-6 (ng/ml)
saline / H-7	≤ 0.001	0.122 ± 0.036
ETX/H-7	1.63 ± 0.486	0.564 ± 0.031
ETX/INS/H-7	1.30 ± 0.871	0.497 ± 0.92
INS/H-7	≤ 0.001	≤ 0.001
DEX/ETX/H-7	≤ 0.001	≤ 0.001
DEX/H-7	≤ 0.001	≤ 0.001
DEX/ETX/INS/H-7	0.410 ± 0.176	0.534 ± 0.043
DEX/INS/H-7	≤ 0.001	≤ 0.001

Cells were treated with dexamethasone , DEX, (100 nM) 3 hours prior to the addition of *S. enteritidis* endotoxin, ETX, (100 ng/ml). One hour later insulin (100 μU) and H-7 (1 μg/ml) was added to the cells. Media was harvested 16 hours after ETX stimulation and analysed for IL-6 by ELISA.

in the insulin-induced IL-6 synthesis by ANA1 cells, INDO (1 μM) was added to the cells with insulin. Fig. 36 portrays the effect of INDO in this system. INDO treatment stimulated the production of 4.43 ± 0.40 ng/ml of IL-6. ETX/INDO and ETX/INS/INDO treatment was not different from INDO alone. Dexamethasone suppressed INDO stimulated IL-6 production as well as the IL-6 concentration of the DEX/ETX/INDO group. The treatment combination DEX/ETX/INS/INDO was observed to increase IL-6 concentration (2.19 ± 0.235 ng/ml).

Figs. 37 and 38 examine the effect of INDO on TNF and IL-6 production in the peritoneal macrophages. INDO treatment did not significantly alter TNF or IL-6 production in comparison to the original treatment groups that are depicted in fig. 32 and 33.

c) The Effect of IGF-I on Cytokine Production

The growth factor IGF-I and its receptor are structurally homologous to insulin and the insulin receptor. Insulin at elevated physiological concentrations is able to bind to IGF-I receptors. To determine if IGF-I can induce similar responses in cytokine production as those observed with insulin treatment, 10 ng/ml IGF-I was substituted for insulin in the original treatment protocol.

Fig. 39 summarizes the effect of IGF-I treatment on TNF and IL-6 production in ANA1 cells. IGF-I alone did not stimulate secretion of either IL-6 or TNF. ETX/IGF-1 treatment dramatically increased both TNF and IL-6 production. This increase was significantly above that observed for ETX alone.

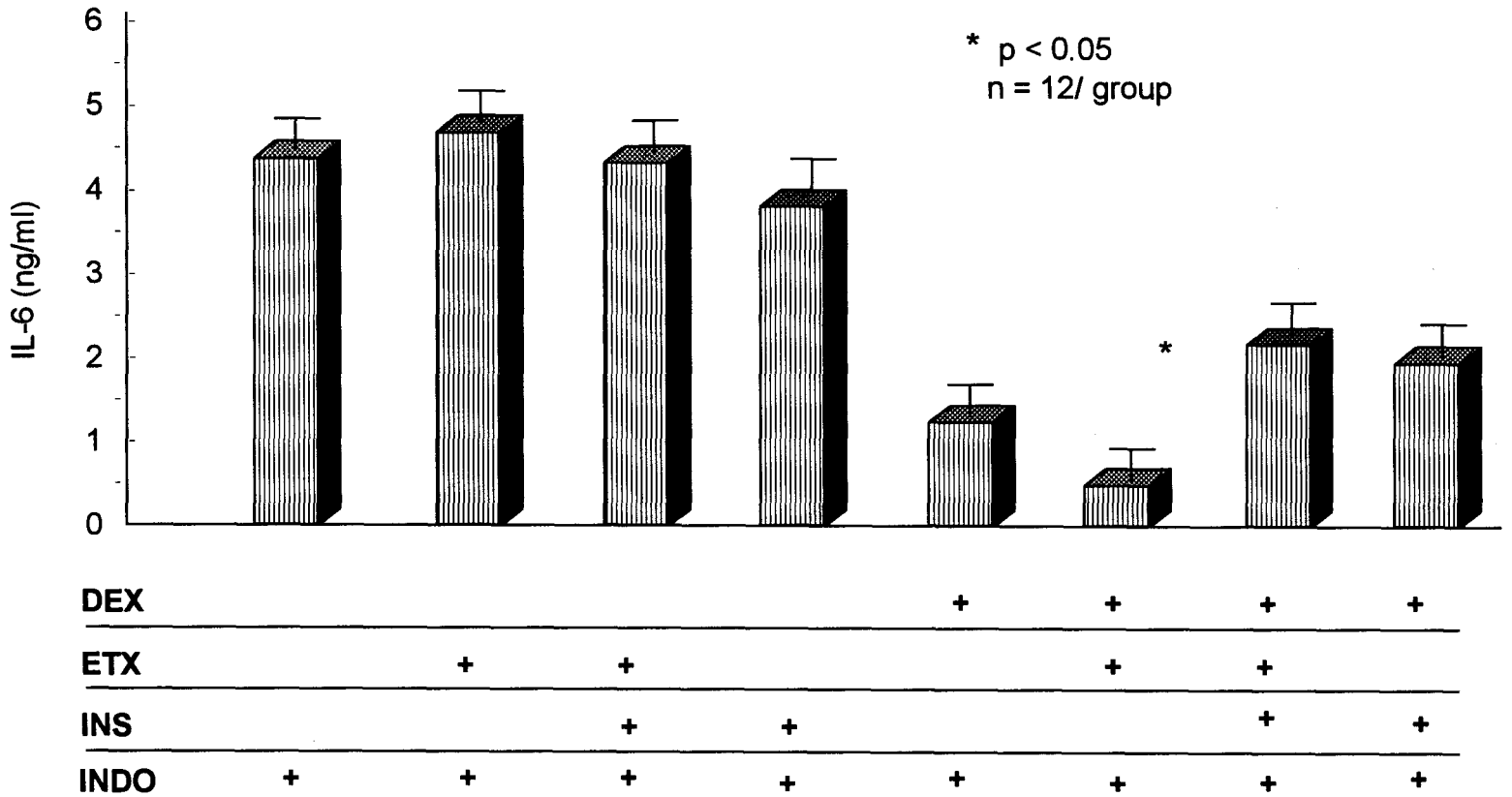


Fig. 36. The effect of indomethacin, INDO, on IL-6 production by ANA1 macrophages . ANA1 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and INDO (1 uM) were added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by ELISA. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares DEX/ETX/INDO with DEX/ETX/INS/INDO.

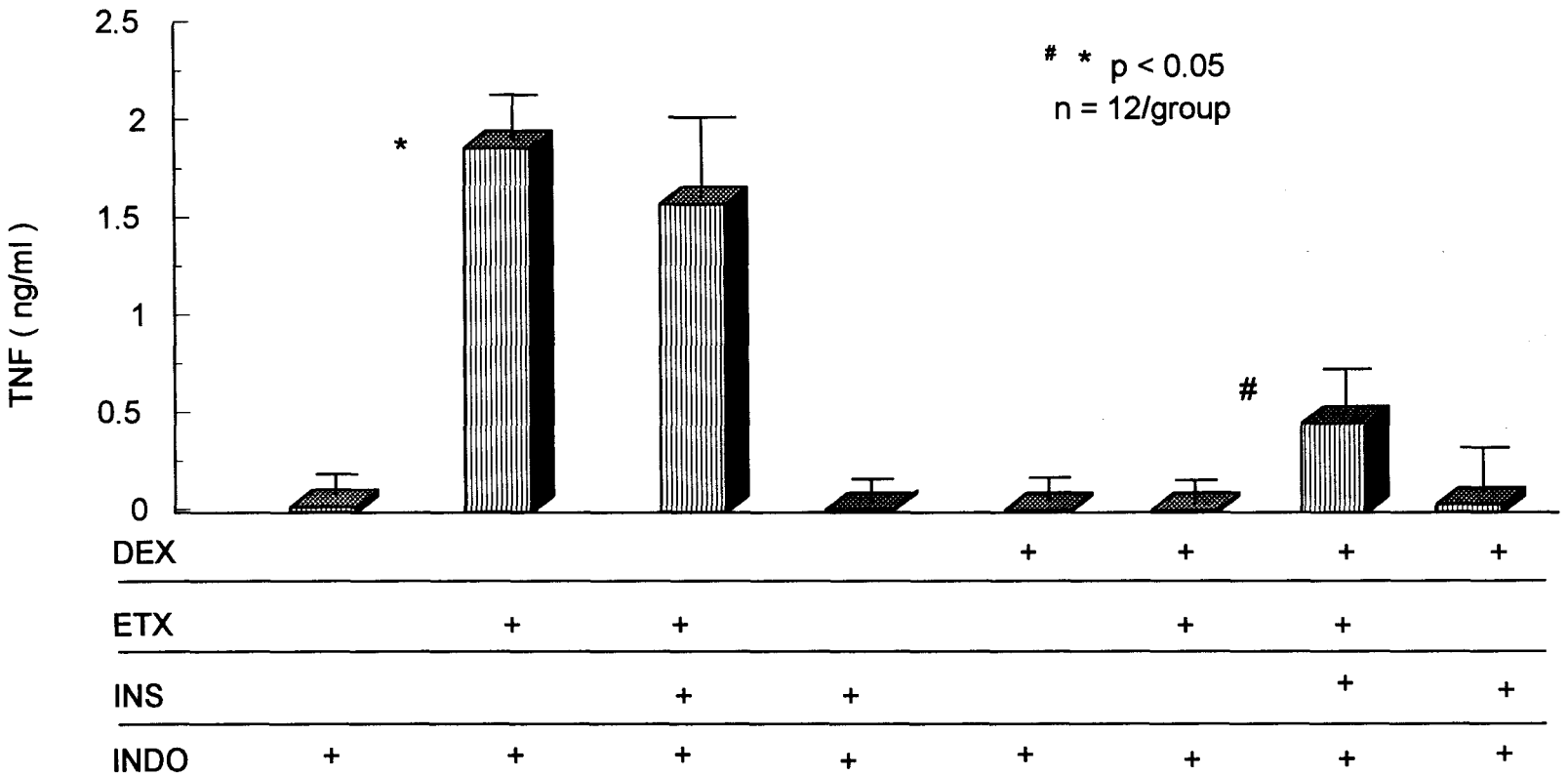


Fig. 37. The effect of indomethacin, INDO , on TNF production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and INDO (1 uM) were added to the cells. Media was harvested 16 hours after ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares INDO with ETX/INDO. # $p < 0.05$ compares DEX/ETX/INDO with DEX/ETX/INS/INDO

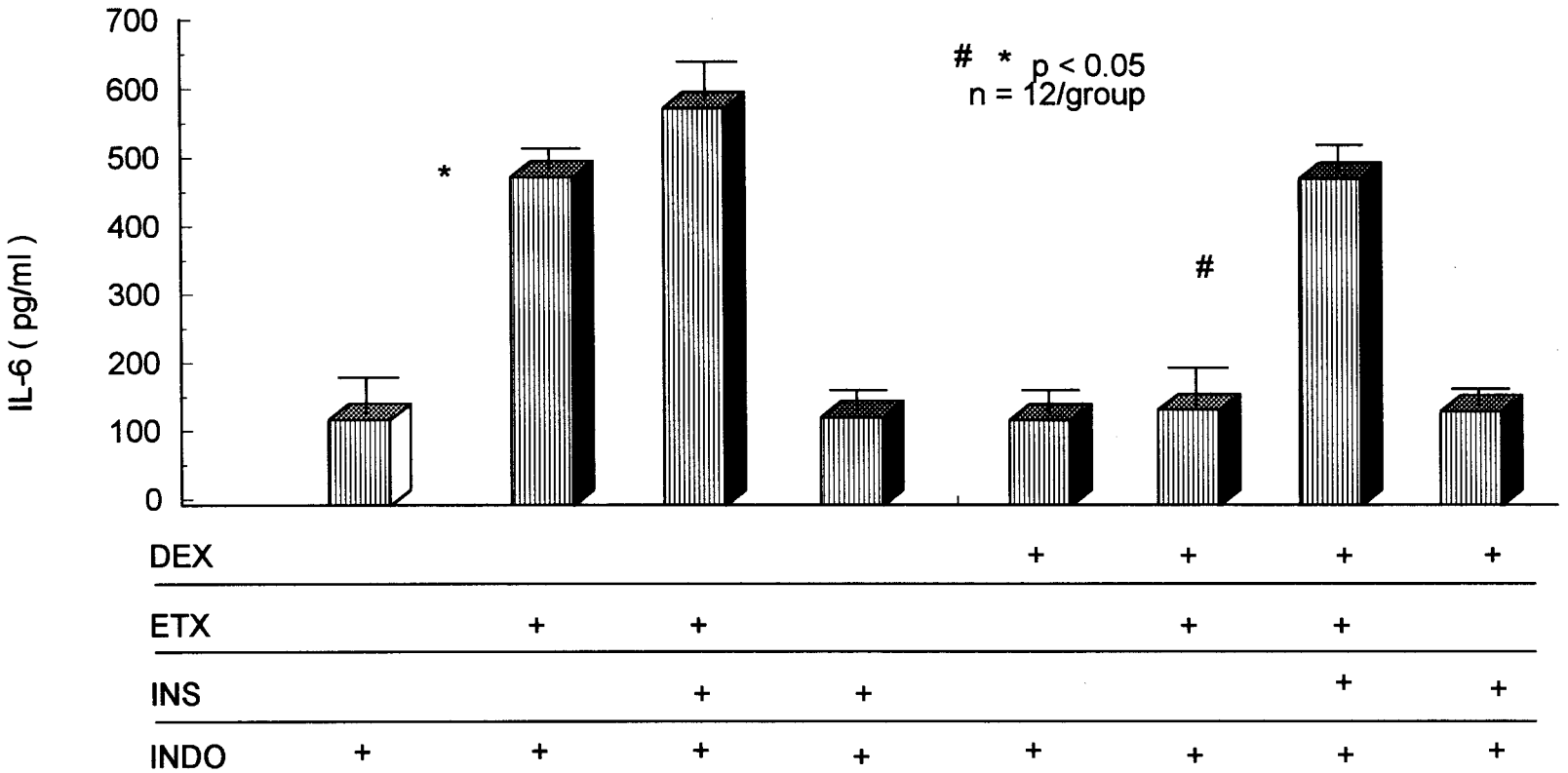


Fig. 38. The effect of indomethacin, INDO , on IL-6 production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and INDO (1 uM) were added to the cells. Media was harvested 16 hours after ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares INDO with ETX/INDO. # $p < 0.05$ compares DEX/ETX/INDO with DEX/ETX/INS/INDO

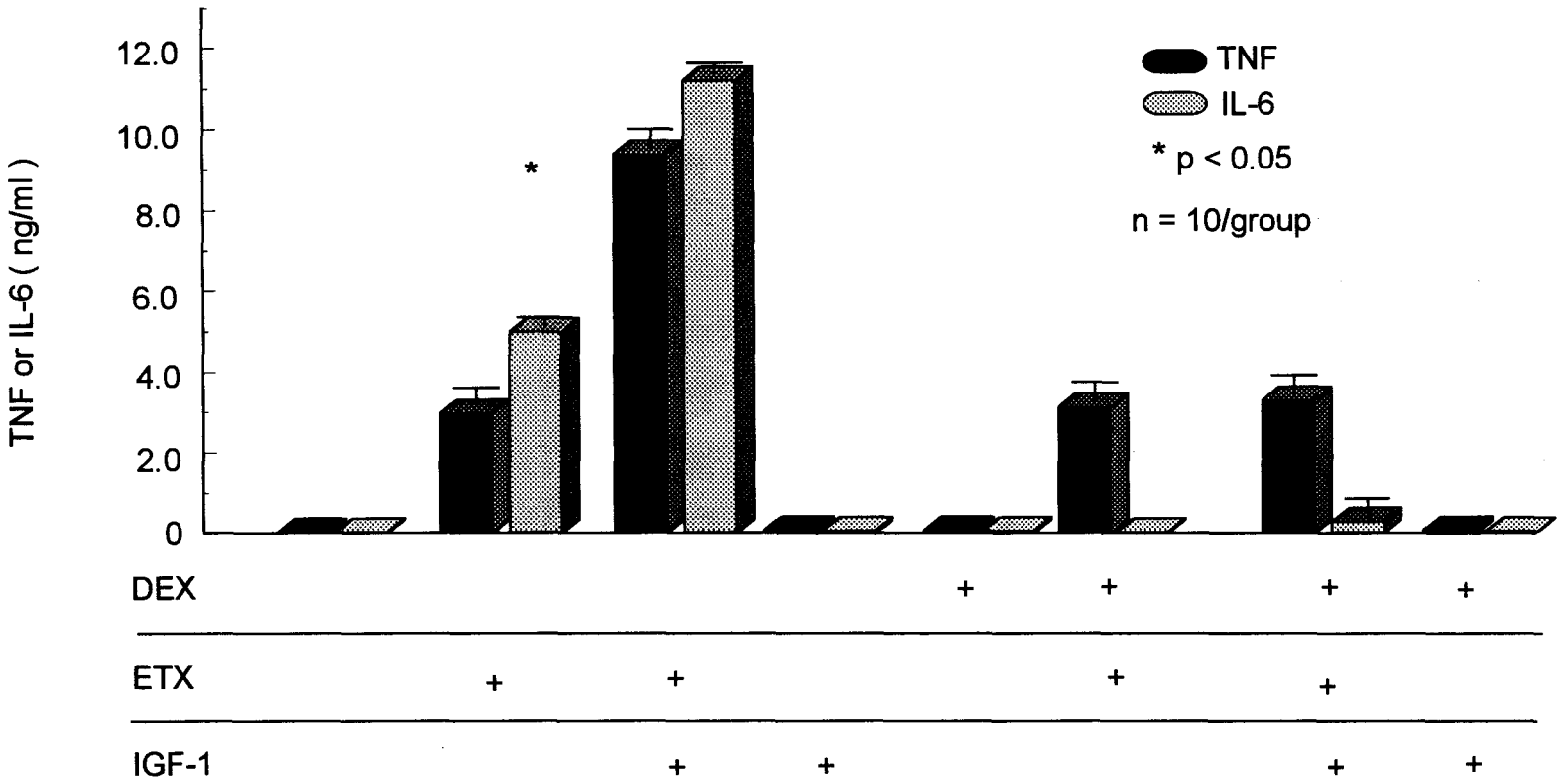


Fig 39. TNF and IL-6 production by ANA1 macrophages . ANA1 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, IGF-1, (10 ng/ml) was added to the cells. Media was harvested 16 hours after ETX and analysed for TNF and IL-6 by ELISA. + designates the agents given. The absence of + designates saline was given . * $p < 0.05$ compares ETX/IGF-1 with the other groups.

Dexamethasone pretreatment of ANA1 cells abolished IL-6 concentrations. Insulin post treatment, DEX/ETX/INS, was able to re-establish IL-6 production. This was not observed for DEX/ETX/IGF-I treatment. Il-6 was not significantly increased above baseline values.

IGF-I was able to stimulate TNF and IL-6 production in peritoneal macrophages as shown in Fig. 40 and 41. The amounts were 0.613 ± 0.14 ng/ml and 345 ± 25.1 pg/ml respectively. The addition of ETX, in the ETX/IGF-I group, significantly increased TNF concentration to 2.43 ± 0.353 ng/ml in comparison to ETX treatment which was 1.6 ± 0.224 ng/ml (data not shown). ETX/IGF-I treatment also dramatically elevated the medium IL-6 content (625 ± 45 pg/ml). The combination of ETX/IGF-I/INS resulted in a trend towards a decrease in TNF and IL-6 that was not statistically significant. Dexamethasone suppressed IGF-I-induced TNF and IL-6 secretion. DEX/ETX/IGF-I treatment re-established both TNF and IL-6 secretion. Both the TNF and IL-6 media concentrations of the DEX/ETX/INS/IGF-I group exhibited a decrease, however, this was not statistically different from the DEX/ETX/IGF-I group.

d) The Role of Serine Phosphatases

Serine phosphatases have been implicated in the signal transduction pathways necessary for cytokine synthesis and secretion. The effect of the inhibitor of serine phosphatase activity, okadaic acid, OKA, on IL-6 production by ANA1 cells and TNF and IL-6 production by peritoneal macrophages is displayed

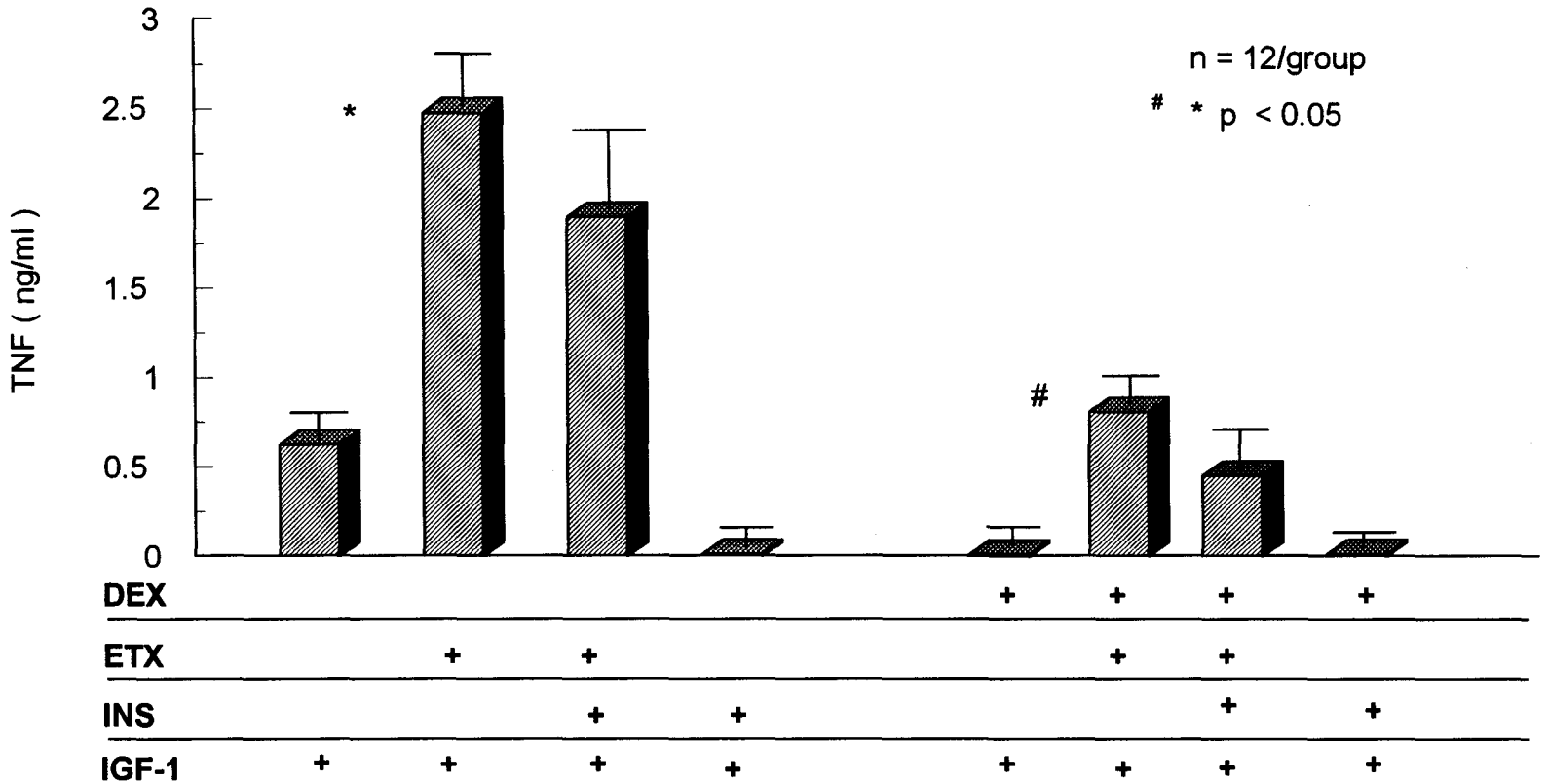


Fig. 40. The effect of IGF-I , on TNF production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and IGF-1 (10 ng/ml) were added to the cells. Media was harvested 16 hours after ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares IGF-1 with ETX/IGF-1.
# $p < 0.05$ compares ETX/IGF-1 with DEX/ETX/IGF-1.

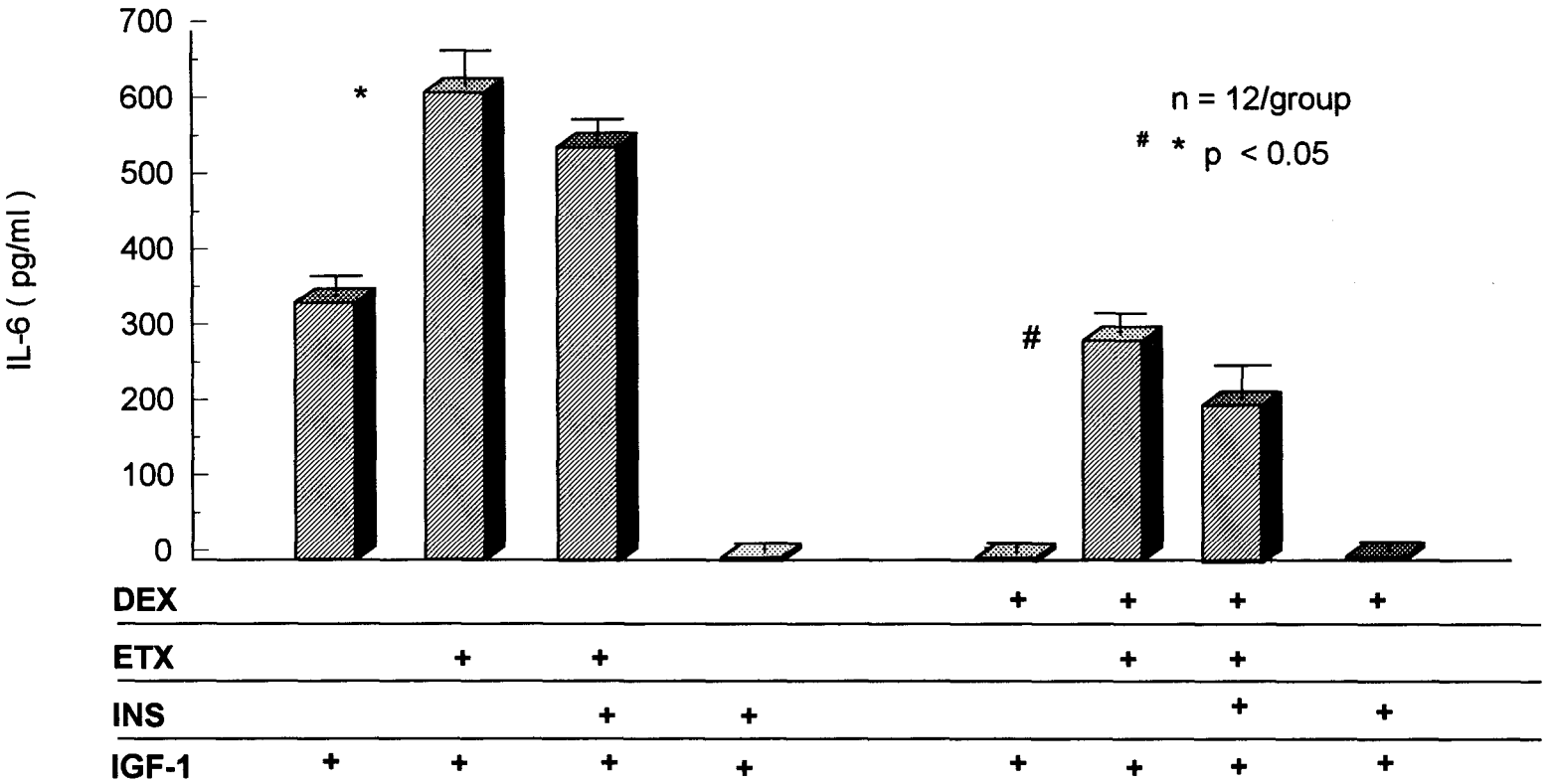


Fig. 41. The effect of IGF-I , on IL-6 production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and IGF-1 (10 ng/ml) were added to the cells. Media was harvested 16 hoursafter ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares IGF-1 with ETX/IGF-1.
# $p < 0.05$ compares ETX/IGF-1 with DEX/ETX/IGF-1.

in fig. 42, fig. 43 and fig. 44.

OKA incited secretion of IL-6 by ANA1 cells. The secretion was not further increased by the addition of ETX, in fact, IL-6 production was decreased. ETX/INS/OKA stimulated an increase in IL-6 production. Dexamethasone pretreatment suppressed OKA-induced IL-6 secretion. The treatment groups DEX/ETX/OKA and DEX/ETX/INS/OKA were not significantly different from DEX/OKA treatment.

OKA treatment resulted in both TNF (1.22 ± 0.56 ng/ml) and IL-6 (391.8 ± 28.5 pg/ml) production by peritoneal macrophages (fig. 43 and 44). ETX/OKA stimulated a markedly enhanced secretion of TNF (4.32 ± 0.53 ng/ml). The IL-6 content of the medium was also increased (587.3 ± 44.4 pg/ml) by ETX/OKA treatment. Dexamethasone was able to suppress OKA-induced TNF but not IL-6 secretion. DEX/ETX/OKA treatment resulted in a mild increase in TNF and no increase in IL-6. DEX/ETX/INS/OKA had opposite effects on TNF and IL-6 production. TNF was severely suppressed whereas IL-6 was dramatically increased (492.5 ± 640 pg/ml).

e) The Role of RNAase I

RNAse I has been implicated as being responsible for the degradation of mRNA of cytokines with the UAUAU rich regions. These regions confer message instability and thus result in rapid message turnover. Cycloheximide has been shown to "superinduce" cytokine production by inhibiting the synthesis of RNAses

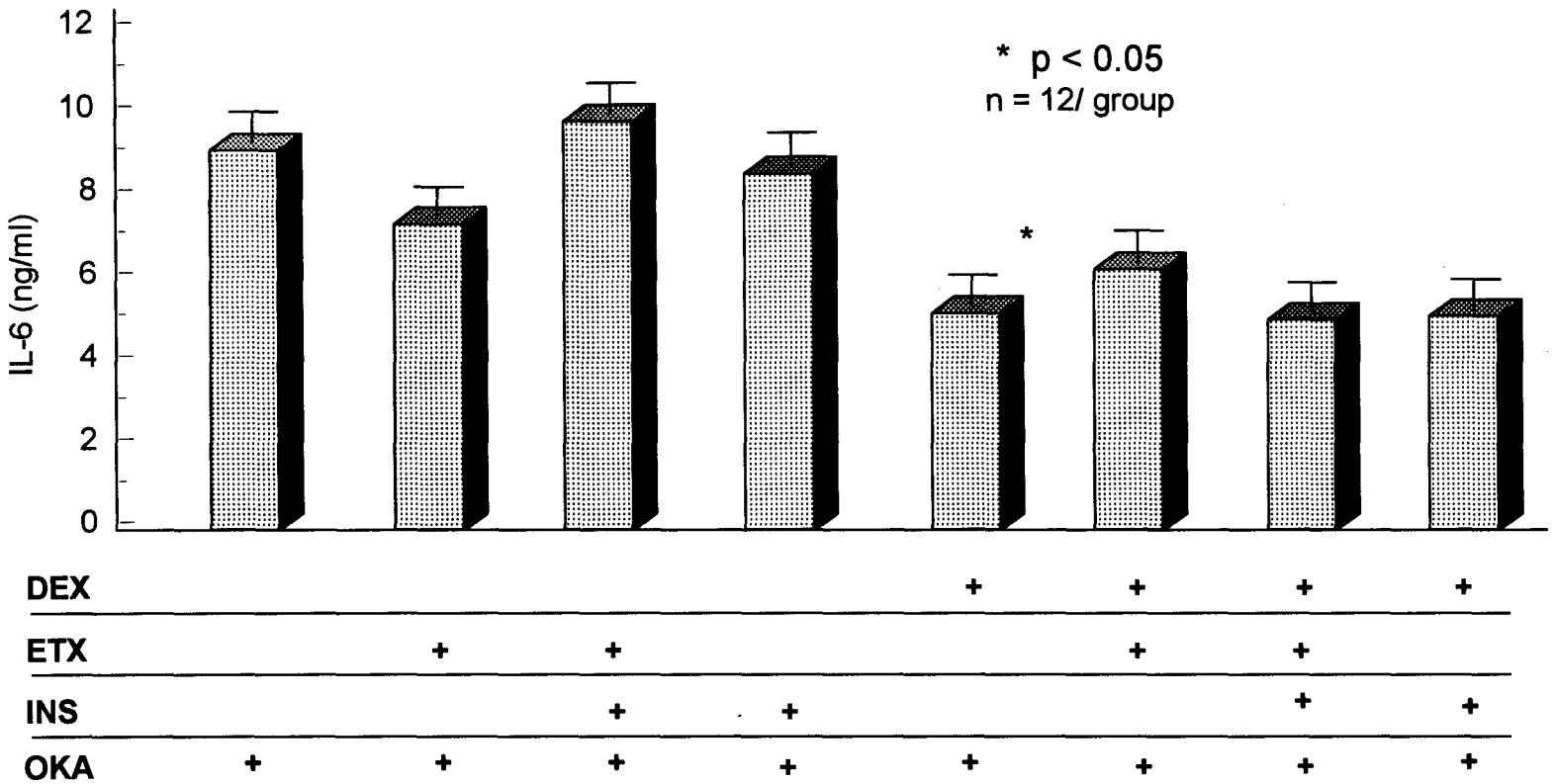


Fig. 42. The effect of okadaic acid, OKA, on IL-6 production by ANA1 macrophages . ANA1 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and OKA (10 ng/ml) were added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by ELISA.. + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares ETX/OKA with DEX/ETX/OKA.

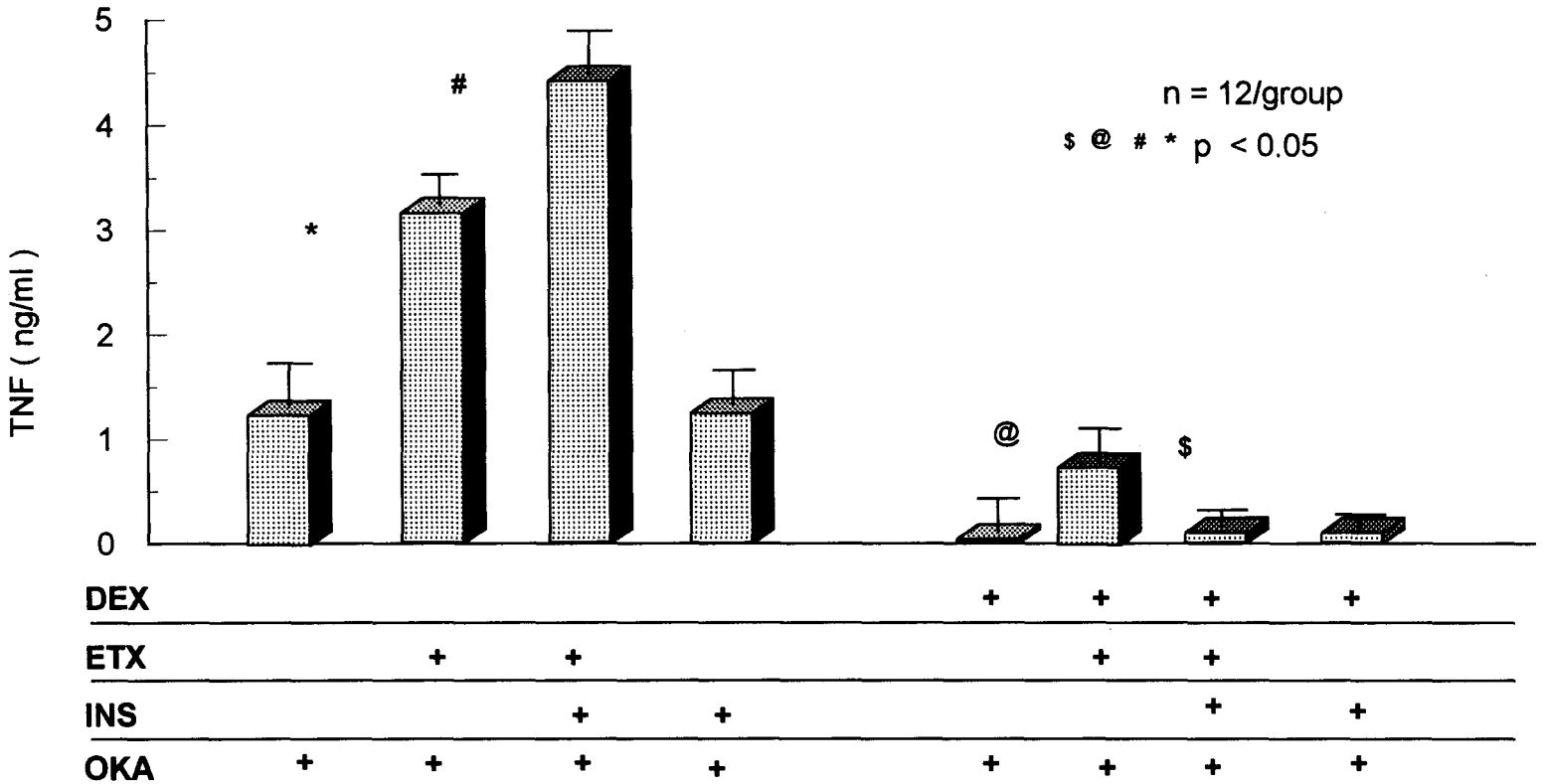


Fig. 43. The effect of okadaic acid, OKA, on TNF production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and OKA (10 ng/ml) were added to the cells. Media was harvested 16 hoursafter ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares OKA with ETX/OKA. # $p < 0.05$ compares ETX/OKA with ETX/INS/OKA. @ $p < 0.05$ compares ETX/OKA with DEX/ETX/OKA. $ $p ,< 0.05$ compares DEX/ETX/OKA with DEX/ETX/INS/OKA.

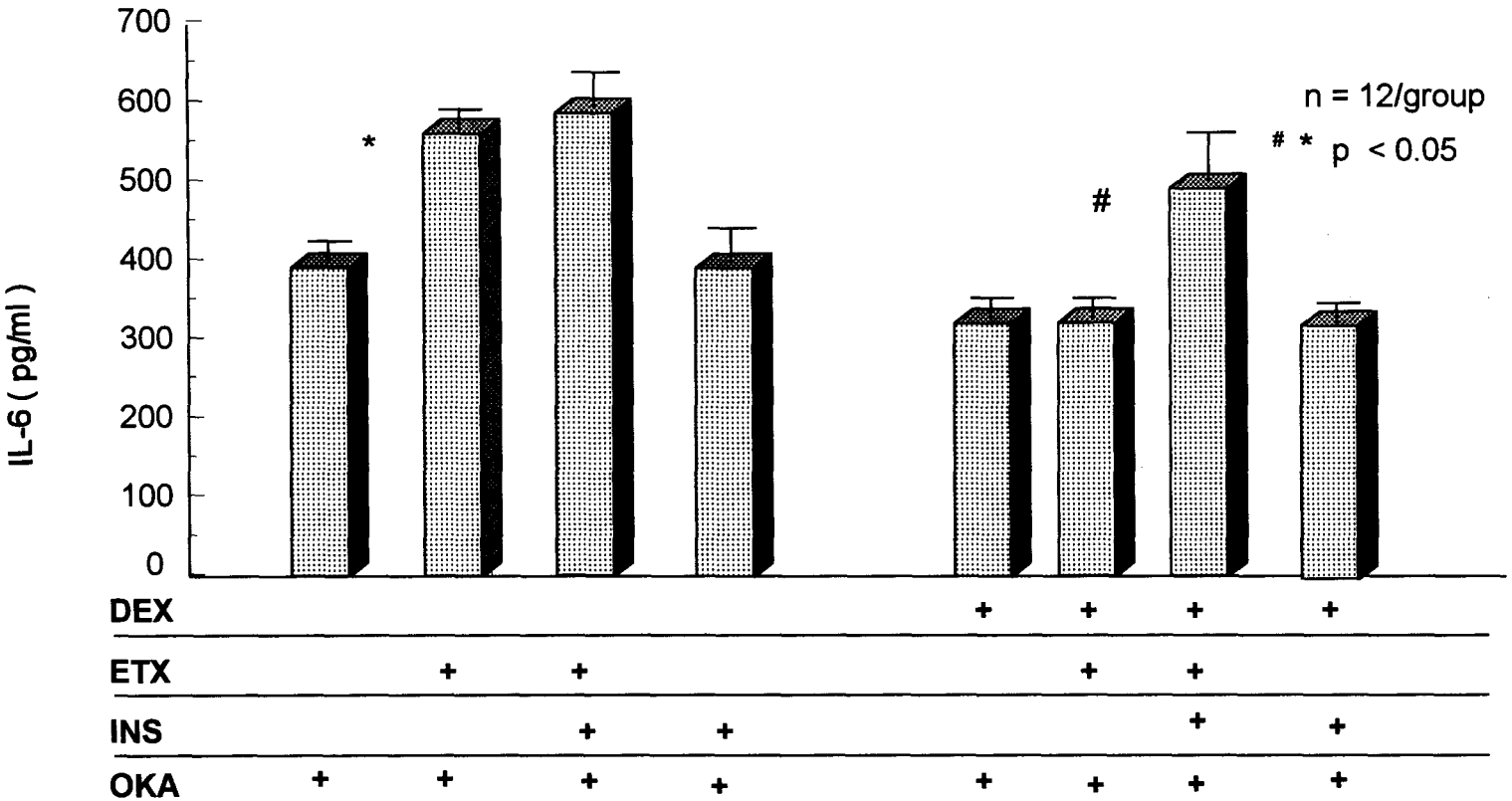


Fig. 44. The effect of okadaic acid, OKA, on IL-6 production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and OKA (10 ng/ml) were added to the cells. Media was harvested 16 hoursafter ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares OKA with ETX/OKA. # $p < 0.05$ compares DEX/ETX/OKA with DEX/ETX/INS/OKA.

responsible for mRNA degradation. Figs. 45, 46 and 47 demonstrate the effect of cycloheximide addition to the treatment groups. Fig. 45 illustrates the effect of CYC on IL-6 secretion by ANA1 cells. CYC, surprisingly caused superinduction of IL-6 in the ETX/CYC treatment group. Insulin addition to this group, ETX/CYC/INS also notably enhanced IL-6 secretion. Dexamethasone significantly suppressed IL-6 production in the DEX/ETX/CYC group in comparison to ETX/CYC treatment. DEX/ETX/INS/CYC treatment did not alter IL-6 production above that observed with the DEX/ETX/CYC group.

Figs. 46 and 47 demonstrate the effect of CYC on TNF and IL-6 secretion in peritoneal macrophages. CYC caused "superinduction" of TNF synthesis above that observed for ETX alone (4.43 ± 0.387 ng/ml vs. 1.61 ± 0.22 ng/ml). Insulin addition to this group abolished TNF synthesis. Surprisingly, DEX/ETX/INS/CYC treatment elicited a profound increase in TNF concentration (5.47 ± 0.72 ng/ml) not observed with DEX/ETX/CYC treatment.

Fig. 47 illustrates the effect of CYC on IL-6 production by peritoneal macrophages. Although there are significant differences when comparing the treatment groups to each other, in relationship to the results from the original treatment groups illustrated in fig. 33, CYC severely inhibited IL-6 secretion.

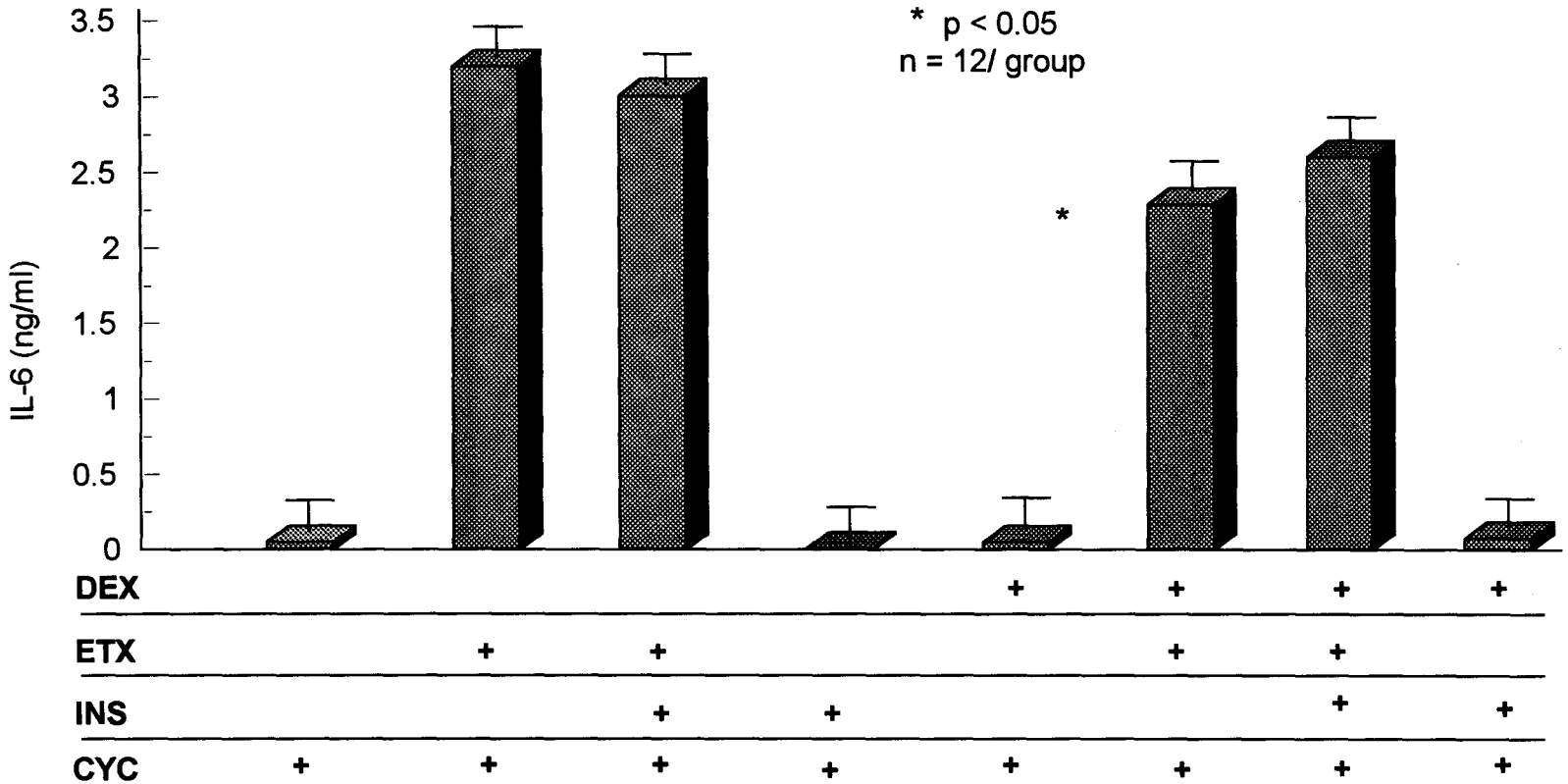


Fig. 45. The effect of cycloheximide, CYC, on IL-6 production by ANA1 macrophages . ANA1 macrophages were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and CYC (10 ug/ml) were added to the cells. Media was harvested 16 hours after ETX and analysed for IL-6 by ELISA.. + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares ETX/CYC with DEX/ETX/CYC.

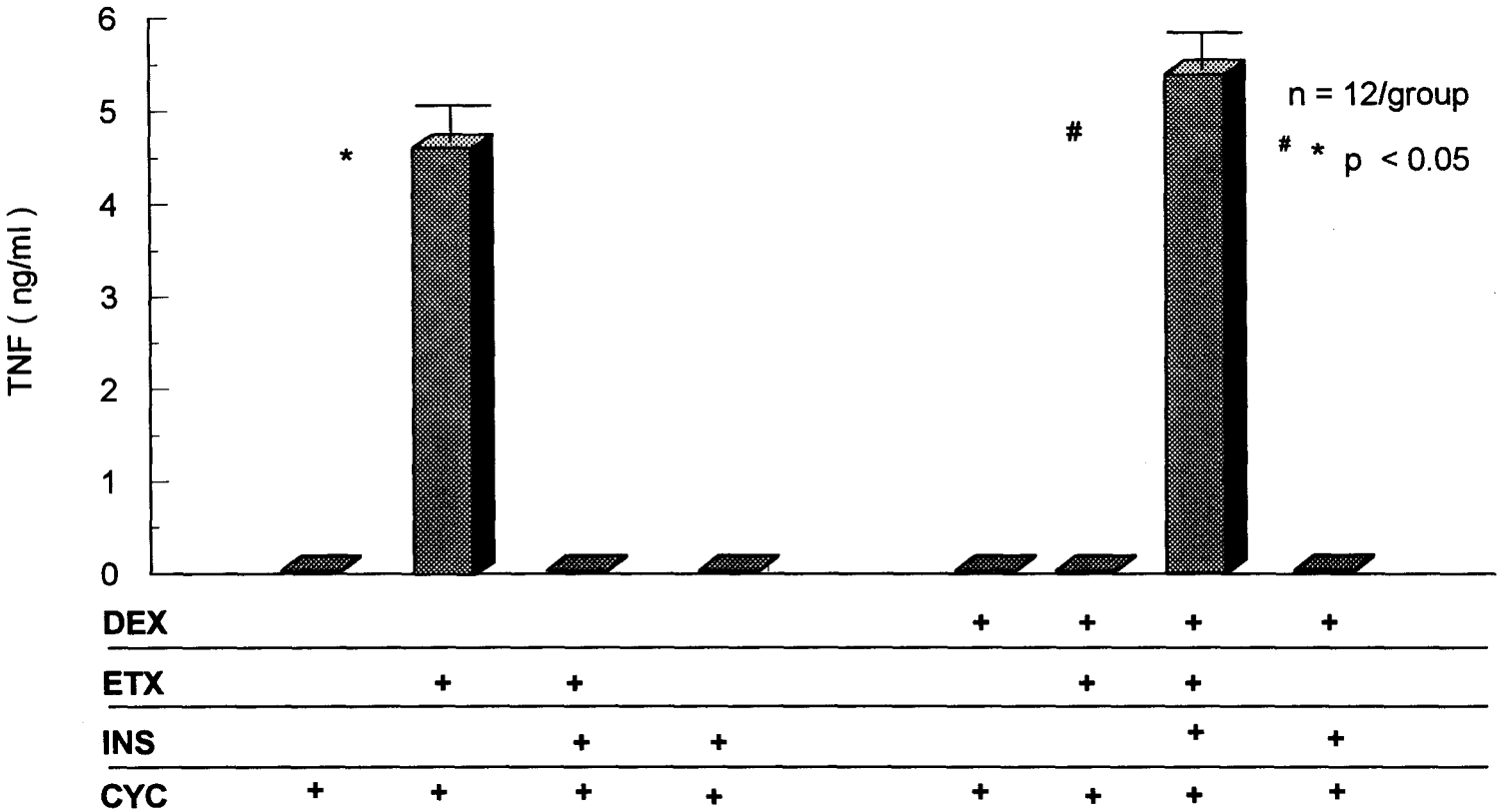


Fig. 46. The effect of cycloheximide , CYC, on TNF production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and CYC (1 ug/ml) were added to the cells. Media was harvested 16 hoursafter ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares ETX/CYC with ETX/INS/CYC. # $p < 0.05$ compares DEX/ ETX/CYC with DEX/ETX/INS/CYC.

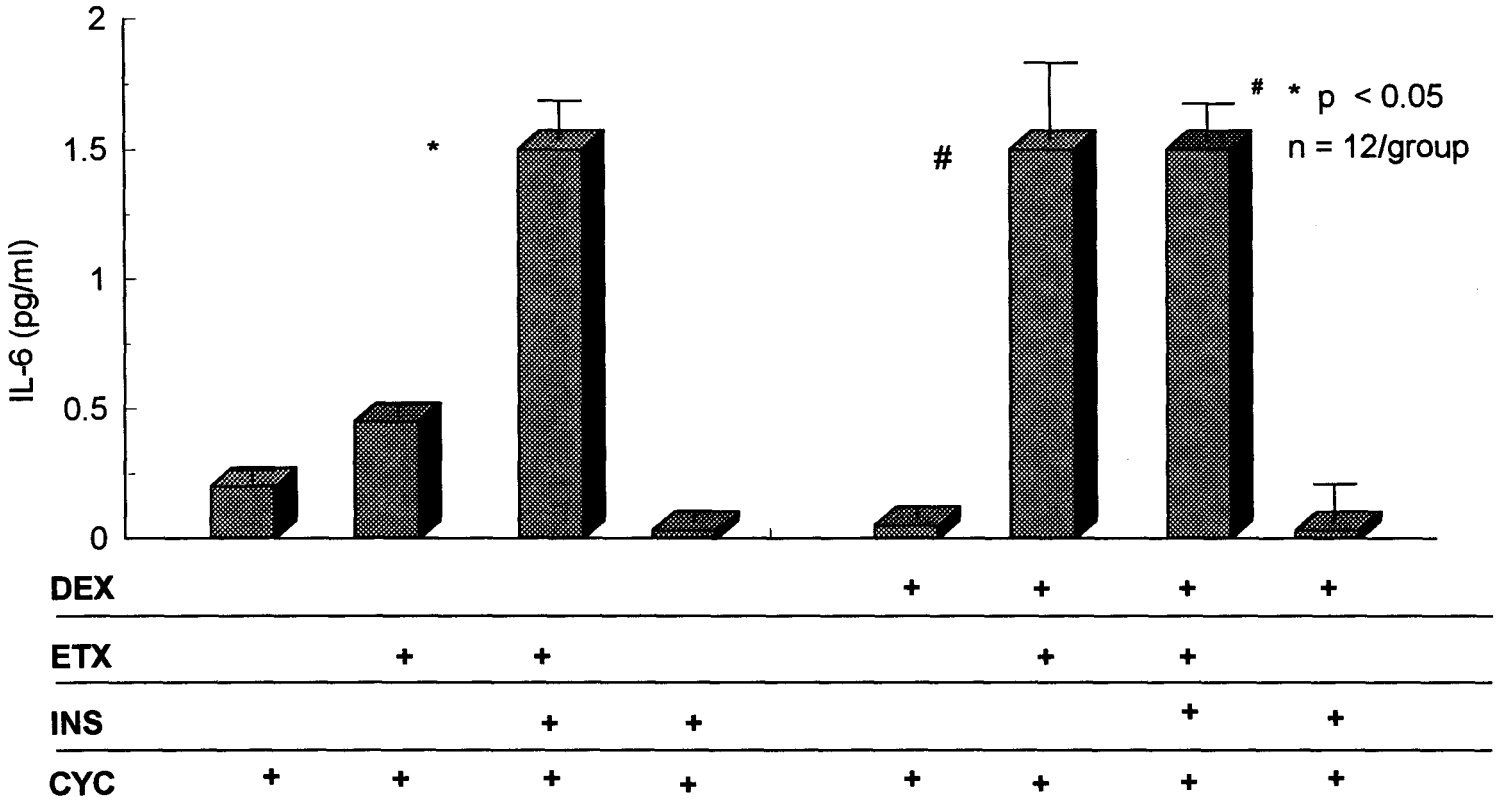


Fig. 47. The effect of cycloheximide , CYC, on IL-6 production by peritoneal macrophages . The cells were pretreated with dexamethasone, DEX, (100 nM) or saline 3 hours prior to endotoxin, ETX, (1 ng/ml) administration. One hour after ETX, insulin, INS, (100 uU) and CYC (1 ug/ml) were added to the cells. Media was harvested 16 hoursafter ETX . + designates the agents given. The absence of + designates that saline was given . * $p < 0.05$ compares ETX/CYC with ETX/INS/CYC. # $p < 0.05$ compares DEX/CYC with DEX/ETX/CYC.

CHAPTER V

DISCUSSION

A. *In Vivo* Projects

1. The Effect of Exogenous Insulin Administration and Endogenous Insulin Secretion on Lethality and Glucose Homeostasis

a) Mortality Effects

This study was initially designed to examine metabolic and mortality differences observed in response to endotoxin and to determine if insulin would alter dexamethasone protection of endotoxic lethality. This preliminary *in vivo* experiment was essential so that the effects of the various treatments could be observed in a conscious unrestrained model. The rats were fasted overnight to stabilize plasma insulin at low levels. Dexamethasone was administered at 0.33 mg/kg. This dose was proven to protect rats against endotoxic lethality in a study by Yelich et al., 1987, (358). This dose also represents an analogous dose of methylprednisolone (MP) that has been used in clinical studies (47, 164) considering that MP is approximately one tenth the therapeutic strength of dexamethasone. In comparison to the clinically recommended therapeutic dose of dexamethasone (5 mg/kg) and the amount administered to animals in other

studies (18), the dose of dexamethasone used in this experiment is significantly reduced. The administration of dexamethasone three hours before endotoxin administration enabled the intraperitoneal injection to be effectively absorbed. Hinshaw et al.,1978, (164) have shown that glucocorticoid treatment can be protective if administered prior or even concurrently with endotoxin.

The dose of endotoxin chosen for this experiment was 20 mg/kg. This amount of endotoxin was determined from pilot studies to be midrange lethal dose of 66% for this particular lot of endotoxin. In comparison to Buchanan's (51,52) experiments (1mg total) this is a much larger dose. In comparison to other species the rat is extremely resistant to endotoxic challenge. Although, there is no recorded effective lethal dose for man, the amount administered to human volunteers in clinical studies is usually in the nanogram range, resulting in physiological alterations such as increased fever, blood pressure and TNF production (116, 270). The preparation of endotoxin used in this study is the Boivin extraction with approximately 18% protein existing with the lipopolysaccharide coat. Raetz et al., 1991 (267) have suggested that the protein is important for the activation of macrophages with subsequent cytokine production and antigenic response by macrophages.

The insulin dose used was chosen after examination of Buchanan's work. As illustrated in table 6, 0.5 U was as effective as 1U and 0.25U yielded results no different from endotoxin alone. Insulin was administered one hour post endotoxin. During this time period after endotoxin administration plasma insulin

and plasma glucose concentrations usually increase. This response has been described as the "insulin resistant" hyperglycemic phase of endotoxicosis (112). Insulin was administered during this period to determine if the animal was truly resistant to the action of insulin in insulin dependent tissues. As illustrated in fig. 15 the administration of insulin one hour after endotoxin, ETX/INS, increased mortality from 66% to 100%. This combination was extremely and consistently lethal with animals succumbing within 2 hour after insulin administration. This was not an unexpected finding as Buchanan and Filkins,1976, (51) previously demonstrated that endotoxin and insulin coadministration resulted in increased mortality in comparison to endotoxin alone. Mortality in his study was increased from 25% to 100% by insulin administration.

The protective effects of dexamethasone pretreatment against endotoxic lethality have been well described (36,37,164,167). Dexamethasone therapy is believed to stabilize plasma glucose, blood pressure and modulate the inflammatory response during endotoxicosis (358). In this study dexamethasone effectively reduced mortality from 61% to 6.25% . However, insulin administration to dexamethasone-treated endotoxic rats was able to reverse protection increasing mortality from 6.25 to 75 %. The bowel of the DEX/ETX/INS group at autopsy was noted to have significant areas of petechiae and focal necrosis and appeared deep purple in color in contrast to the pink color of the bowel of saline treated rats. These observations were similar to those of the ETX and the ETX/INS groups and are consistent with death due to endotoxicosis. The

appearance of the bowel of animals dying from insulin-induced hypoglycemia is distinct as the peritoneum and intestines exhibit areas of blanching and white strangulation.

Tolbutamide, a sulfonylurea derivative used therapeutically for the stimulation of insulin secretion in Type II late onset diabetes was utilized in this study to determine if the increase in mortality was due specifically to exogenous insulin administration and also to determine if endogenously produced insulin would elicit the same response and reverse dexamethasone protection against endotoxicosis. Although tolbutamide alone did not result in any deaths, ETX/TOL mortality was 100%. Buchanan and Filkins, 1976, (51) demonstrated previously that the addition of tolbutamide to endotoxin animals treated with an LD_{20} resulted in increased mortality to 100%. As illustrated in our experiment, dexamethasone protection was also reversed by tolbutamide administration. However, mortality was only increased to the level of the ETX group. The amount of tolbutamide administered stimulated only a modest amount of insulin secretion from the pancreas and thus plasma concentrations of insulin were reduced. Since insulin, either from an exogenous or endogenous source, was able to alter mortality, the endotoxic rats were not truly unresponsive to insulin effects.

These findings suggest that insulin is a key hormonal regulator during endotoxic shock. The ability of insulin to enhance endotoxin's ability to increase mortality and negate dexamethasone's protective effects cause concern if extrapolated to the clinical setting. Therapeutically administered substances which

increase endogenous insulin such as glucose, or total parenteral hyperalimentation could alter dexamethasone protection by initiating insulin secretion a competitive cascade with endogenous or exogenously administered glucocorticoids. Glucocorticoid therapy in two highly cited clinical trials (47,166) has been shown to be ineffective, although in almost all nonhuman studies glucocorticoids have proven to be protective. The failure of the predicted outcome in the clinical trials has been attributed to severity of injury of the patients as well as to the time of administration of glucocorticoids (167). Bone et al., 1987 (47) and Hinshaw et al. 1985 (166) did not examine the effects of therapies such as hyperalimentation and dextrose administration and plasma insulin was not measured in these studies.

b) Plasma Metabolite Effects

The early phase of a septic challenge is characterized by two distinct phases of glucose metabolism (107,112). Initially a transient " insulin resistant" hyperglycemic phase occurs within two hours post endotoxin. This phase is termed "insulin resistant" because an abnormal increase in plasma insulin without a subsequent decrease in the already abnormally elevated plasma glucose level is observed (107). Insulin dependent tissues such as fat and muscle are unable to take up glucose in the presence of insulin due to the presence of alterations in glucose transporter activity (193). The exact mechanism of the impaired transporter activity has not been uncovered, however, endotoxin-stimulated

production of tumor necrosis factor has been implicated as a possible mediator (214,337).

The hyperglycemic phase terminates abruptly and is followed by a rapid onset lethal hypoglycemic phase (107,143). Gluconeogenesis by the liver is significantly impaired during sepsis and the glucose demand by non-insulin dependent tissues such as the brain and the cells of the immune system is elevated (193,223). Non-insulin dependent utilization of glucose is approximately 70-85% of total body disposal (193,222). Other tissues responsible for this uptake during sepsis include liver, spleen, lung, ileum and skin. Insulin dependent tissues such as muscle have also increased glucose needs (222). Therefore, the peripheral glucose requirements exceed the glucose output by the liver and hypoglycemia occurs.

Plasma glucose levels of the ETX/INS and of the ETX/TOL rats were significantly reducedin this study in comparison with the ETX treatment group. Since blood samples were obtained approximately 3 hours after endotoxin administration the plasma glucose concentration of the ETX treatment group would be expected to be slightly decreased as illustrated in fig. 16. The addition of the insulin or tolbutamide to the ETX groups during the hyperglycemic phase exacerbated the ETX induced hypoglycemia.

Hepatic gluconeogenesis is significantly impaired during sepsis (36,37). The key enzyme responsible for gluconeogenesis, PEPCK, is upregulated under normal conditions by cortisol and glucagon and is dominantly suppressed by

insulin and phorbol esters (242,243,254). In sepsis, even though plasma cortisol and glucagon concentrations are elevated, PEPCK activity is impaired (11,12,306). McCallum and Hill, 1992, (161) have shown that PEPCK activity is inhibited both at the activity and the transcriptional level by the cytokines TNF and IL-6. Our data suggest that the addition of insulin or tolbutamide to endotoxic rats may have act to further impair PEPCK activity and decrease glucose output by the liver.

Dexamethasone pretreatment resulted in a persistent hyperglycemia and has been shown to maintain plasma glucose at euglycemic to hyperglycemic levels for an extended period of time post endotoxin challenge (358). Both insulin and tolbutamide treatment of DEX/ETX rats negated the hyperglycemia and induced a profound hypoglycemia. This observation supports the role of insulin as a dominant regulator of plasma glucose homeostasis.

During sepsis an increase in plasma lactate is considered to be a marker of metabolic distress (202,230). Endotoxin is thought to alter pyruvate dehydrogenase and mitochondrial activity resulting in uncontrolled lactate production (330). Van Lambalgen et al., 1988, (330) demonstrated in a canine model that endotoxin increases plasma lactate significantly within 30 minutes. Salleh et al. 1990, (279) also demonstrated that in cecal ligation and puncture models of sepsis plasma lactate levels increased 100% over those of sham controls.Fong et al., 1990, (116) found that although no significant increase in plasma lactate occurred iln human volunteers administered endotoxin an

increased efflux of lactate from skeletal muscle was observed. A recent report (202) suggests that macrophages are capable of utilizing lactate as a fuel during stress and that lactate transporters exist on the surface of these cells.

Plasma lactate levels in saline treated rats was increased by endotoxin administration from 1.0 mmol/L to 3 mmol/L. The addition of insulin or tolbutamide resulted in an intense hyperlacticacidemia. Dexamethasone pretreatment reduced plasma lactate concentrations by possibly increasing the delivery of glucose or oxygen to the cells. Insulin administration increased plasma lactate concentrations of the DEX/ETX/INS and the DEX/ETX/TOL groups by reducing the available glucose through the inhibition of gluconeogenesis.

During the first hour after an endotoxin challenge plasma glucose increases rapidly with a simultaneous increase in plasma insulin concentration. There is also substantial evidence that the pancreas hypersecretes insulin in response to a glucose challenge during sepsis. Buchanan and Filkins, 1976, (52) demonstrated in rats that within 90 minutes after an injection of endotoxin, insulin increases to a maximum of 120 μU/ml, and then rapidly decreases .As depicted in our experiment, plasma insulin in the ETX group was elevated above that of the control rats. Other authors have noted similar elevations in plasma insulin concentrations after an LPS challenge or in cecal ligation and puncture models (11,12,279). Results of studies involving septic patients have shown an inconsistency with respect to the presence or absence of elevated plasma insulin concentrations (64,116,122). This discrepancy could be due to the administration

of dextrose or glucose to maintain plasma euglycemia which, in turn, alters the plasma insulin profile.

Even with the administration of pharmacological levels of insulin to the rats in this study the peak insulin observed was approximately 120 μU/ml. This value is within the range of plasma insulin observed during endotoxin-stimulated insulin secretion. Tolbutamide did not increase plasma insulin levels as dramatically as anticipated, however, a significant increase in mortality was observed. These observations suggest that an increase in insulin turnover occurred in the endotoxic animals and that a very small concentration of insulin can exert a lethal effect. Tolbutamide may also produce effects unrelated to insulin secretion however, tolbutamide administration alone did not cause death.

2. The Effect of Restoring Euglycemia on Mortality

Although the observations from the bowel pathology did not indicate death as a result of hypoglycemia, the plasma glucose concentrations were so diminished that hypoglycemia was postulated as the cause of death of the treatment groups. If plasma glucose concentration could be re-established in these groups by the administration of dextrose, would the prognosis be altered ?

The stabilization of euglycemic plasma glucose concentrations in these animals without significantly altering the model created a dilemma. The appropriate model would have been to surgically implant catheters and glucose clamp the animals at a stable plasma glucose concentration. However, the effects

from anaesthesia, surgery and recovery would alter the original model. Therefore, the administration of dextrose intraperitoneally was chosen. As illustrated in fig. 16, plasma glucose was significantly increased by dextrose therapy in the ETX/INS and DEX/ETX/INS groups at the time of death without significant reductions in mortality. Surprisingly, the administration of dextrose to the ETX group resulted in 100% mortality. Buchanan and Filkins, 1976, (51) have shown that administration of glucose significantly increased mortality from 10 to 60 % if given one hour post endotoxin. Satomi et al. 1985, (284) also confirmed this observation in mice. The stimulation of insulin secretion by dextrose injection in these studies was postulated to cause the increased mortality. Therefore, from Satomi's studies and from our results, insulin administration to endotoxic rats did cause hypoglycemia and contributed to death, however, insulin may have stimulated other physiological or immunological pathways resulting in the demise of the animals.

3. The Effect of PMA and H-7 on Mortality

PKC activation has been implicated in the development of endotoxemia. PMA, an activator of PKC is known to stimulate the physiologic sequelae observed during sepsis, including macrophage differentiation and cytokine production (345,347). Inaba and Filkins, 1991, (177) demonstrated that endotoxin lethality could be exacerbated by the injection of PMA into rats. They also showed that inhibitors of PKC, such as H-7 or polymyxin B, attenuated insulin induced

hypoglycemia and the rise in insulin during IVGTT (178).

PMA was used to in place of insulin and insulin was combined with H-7 to observe if the effect of insulin was by a PKC activation. As demonstrated in the Inaba study, endotoxin was extremely lethal when combined with PMA. Dexamethasone pretreatment was also unable to protect against PMA induced lethality, as was demonstrated with insulin. H-7 in combination with insulin did not alter the significant mortality due to insulin. Although, this finding suggests that PKC activation may not be involved, the effects of *in vivo* administration of H-7 are complex. H-7 administration *in vivo* at the dosage used in this study also induces alterations in plasma glucose and blood pressure. Also H-7 is not an entirely specific inhibitor of PKC and it also affects PKA activity.

B. Cytokine Production *In Vivo*

As the administration of dextrose did not alter mortality in the experiments discussed above, the effects of hormonal modulation of other responses was examined. Endotoxin stimulates the production of an assortment of biologically active mediators, such as prostaglandins, leukotrienes, hormones and immunological cytokines. The production of TNF by macrophages is a critical initial response to endotoxemia. Coincidently, the initial increase in plasma TNF occurs in parallel with plasma hyperglycemia and hyperinsulinemia (182,185). TNF then disappears from the circulation as other cytokines such as IL-1 and IL-

6, begin to appear (116,185). The decline in plasma TNF levels also coincides with the onset of endotoxin-induced hypoglycemia .

This study examined the insulin and dexamethasone modulation of TNF and IL-6 production *in vivo* using the treatment protocol described in the mortality experiments. In order to facilitate serial blood sampling the rats were anesthetized with pentobarbital. The administration of pentobarbital alters the sympathetic response and catecholamine production of anesthetized animals by inhibiting parasympathetic activity in the brain. Increased plasma catecholamines alter heart rate and metabolic responses by stimulation of beta adrenergic receptors. Plasma glucose and lactate concentrations in this study were slightly different to those observed in conscious animals possibly due to the effects of anesthesia.

Plasma TNF was increased significantly 60 minutes after endotoxin administration. Peak plasma TNF levels occurred at 90 minutes, with a decline to near basal levels by 180 minutes post endotoxin. This time course and pattern of TNF production appears to be a consistent with many studies and has been described in several species (78, 182, 185, 271, 324,325). Insulin administration to the endotoxic rats did not alter the amount of TNF produced , however, the plasma TNF level was sustained in contrast to the declining plasma TNF of the endotoxic group. Since rats in the insulin post-treated endotoxic group died prior to the conclusion of the study, the trend to increasing or persisting TNF concentrations could not be measured. The effect of insulin on TNF production could be by either direct or indirect modulation of TNF. The insulin-endotoxic

group also exhibited profound hypoglycemia and lacticacidemia. Elevated plasma TNF concentrations have been previously correlated with significantly reduced plasma glucose levels. Satomi et al., 1985 (284) have demonstrated that the administration of glucose 30 minutes prior to or Xsimultaneouslywith endotoxin reduced plasma TNF, however, glucose administered after endotoxin had no effect on TNF.

The plasma IL-6 amounts in the endotoxin-insulin group was also significantly elevated at the 120 minute time point. Elevations of plasma IL-6 are known to occur approximately 2 to 4 hours after LPS stimulation (200,317). In comparison to the endotoxin group, the plasma IL-6 levels were markedly increased. The appearance of elevated plasma IL-6 concentrations has been associated with a fatal outcome in rats and in humans (60,307). Recently, Casey et al., 1993, (60) verified that in a population of septic patients who died, the only factor that correlated with a poor prognosis was elevated IL-6 levels. The effect of insulin could be theorized as due to either direct or indirect modulation of TNF and IL-6 production. From *in vitro* studies evidence exists that insulin and endotoxin alter TNF, IL-1 and IL-6 production by cells (34,84,172). The indirect effect could be via the production of other cytokines such as IL-1 and IFNγ. Interleukin 1 has been shown to increase insulin secretion by the pancreas (70), however, the *in vivo* effects of insulin on IL-1 production have not been investigated. IFNγ synergizes with endotoxin to produce elevated amounts of TNF and IL-6 (85,212). Although IFNγ was not measured in this study, insulin

may have altered this lymphocyte produced cytokine. Insulin is important for the upregulation of insulin receptors on T-lymphocytes and subsequent immunobiological activity. Helderman et al., 1992, (156) has demonstrated that insulin-stimulated macrophages produce a monocyte-derived insulin receptor factor (MIRRF) which increases insulin receptors on T-lymphocytes.

Dexamethasone pretreatment significantly suppressed plasma TNF and IL-6. Waage, 1987, (339) has shown that maximal suppression of plasma TNF occurs if dexamethasone is administered 3 to 8 hours prior to endotoxin. Dexamethasone can also be administered simultaneously with endotoxin *in vivo* and effectively reduces TNF production, however, a delay of only 20 minutes after endotoxin will not prevent TNF production (167). Dexamethasone suppression of TNF production occurs at the posttranslational level (44,340) whereas dexamethasone suppression of IL-6 occurs at the transcriptional level (76). The effect of dexamethasone on TNF or IL-6 secretion has not been examined. Insulin administration to dexamethasone-endotoxic rats resulted in a restoration of plasma TNF concentrations, with no marked effect on plasma IL-6. The rise of plasma TNF occurred rapidly after insulin injection. This observation indicates that insulin can affect either posttranscriptional processing of TNF mRNA or the actual secretion of the TNF protein.

TNF and IL-6 administration have been shown to cause hypoglycemia and death in experimental animals (93,337). A strong inverse correlation is observed in endotoxin treated mice when plasma TNF and glucose concentrations are

examined (284). The administration of a hypoglycemic dose of insulin to a group of saline treated rats was shown in this study to not alter plasma TNF or plasma IL-6 concentrations.

C. *Ex Vivo* Studies

Several authors have demonstrated that the liver is a vital organ for the maintenance of physiological stability during sepsis (39,118,134,333,341). The liver is responsible for the production of glucose as well as the removal, neutralization and processing of endotoxin (190,302). The subsequent presentation of endotoxin on the surface of antigen presenting cells results in the activation of the immune functions and cytokine production by the resident Kupffer cells (15,57,101,217). The hepatocytes and endothelial cells are also capable of producing cytokines in response to LPS (102,169). The Kupffer cells however, are believed to be the primary producers of TNF, IL-6 and IL-1 in the liver (180).

To investigate the role of the liver in cytokine production the isolated perfused liver (IPL) was used as a model for TNF and IL-6 production. The physiological and metabolic functions of the liver are regulated by elevated portal concentrations of insulin and glucocorticoids, therefore, the effects of these hormones were also examined. The IPL has been a model for examination of the various aspects of hepatic function for over 100 years (191,207,342,351). This model offers several advantages over *in vivo* and currently widely used *in vitro*

techniques. In comparison to *in vivo* studies the direct effects of a substance on liver function can be tested without interference from neural and hormonal factors secreted in response by other organs (139). Also, multiple samples of perfusate can be taken over time without impairing organ function (351). The advantage of this technique over *in vitro* studies is that the architecture and integrity of the environment of a specific cell population, for example the Kupffer cell is maintained (139,351). The discrepancies and variations in cytokine production observed in many studies may be a reflection of the matrix of cells regulating the production of cytokines by specific cells.

In this experiment a recirculating system was chosen so that the accumulation of TNF and IL-6 could be measured. Initially we postulated that the high flow rates required to deliver adequate amounts of oxygen to the cells would wash out any TNF or IL-6 produced. By using this system the rates of production could also be measured. In many IPL systems RBCs are added to maintain oxygenation (207). We did not use RBCs because of possible interference in TNF and IL-6 production . Since TNF and IL-6 are labile proteins which have very short half-lives in plasma and from our experience with other protein such as insulin which adheres to the glassware and tubing, 5% albumin was chosen from pilot studies to be an appropriate amount to maintain stable detectable levels of TNF and IL-6. Figs. 23 and 24 indicate that both TNF and IL-6 were stable during the perfusion period. In order to achieve detectable levels within a perfusion volume of 100 ml, 70 and 20 ng of TNF and 50 ng of IL-6 were required. Only one

perfusion at each concentration of cytokine was performed due to the expense and availability of the cytokines. The TNF and IL-6 produced by the liver may have contributed to the lethality observed in the *in vivo* experiments. Therefore the livers were isolated from rats which had been administered dexamethasone, endotoxin and insulin according to the protocol as described previously in this dissertation.

Fig. 25 illustrates the TNF production over 60 minutes with several treatments. This is the first study to demonstrate directly the ability of the liver to produce TNF. Therefore, observations from the literature to support these findings are taken from *in vitro* and *in vivo* studies. TNF production by the saline treated rats was not observed until after 50 minutes of the perfusion period. This result was unexpected. From earlier unpublished work in our laboratory the production of TNF at this time point has been consistent and repeatable. Initially this response was postulated to be due to contamination by endotoxin. However, Limulus testing determined that the perfusion apparatus was free of endotoxin. Surgical conditions as well were used to maintain sterility. The alterations in liver integrity due to the perfusion are usually not observed until 3 to 4 hours after perfusion. This study was only 60 minutes in duration and no alterations in liver flow or integrity were observed. Ghezzi ,1992, (134) has stated that TNF is required for normal liver regeneration and significant quantities of TNF are produced early in response to hepatectomy. A more probable cause for this increase in TNF production is due to the removal from its normal milieu of

hormones and growth factors which suppress TNF production. Without suppression by such hormones as corticsterone, TNF could be produced in an unregulated manner. As demonstrated in this study, exogenous dexamethasone treatment completely suppresses this response.

ETX treatment in the rat caused a significant early increase in TNF production. Production was still increasing by the end of the experimental period. The approximate rate of TNF production was 0.711 pg/min/g of liver in almost a linear manner. Dexamethasone pretreatment of endotoxic rats was also shown to suppress liver TNF production. Many papers show, indirectly, that the liver is capable of producing TNF after endotoxin stimulation (101,102,134,180); however, there is little direct evidence that the liver is an actual contributor to the circulating TNF pool (206). Byerly et al.,1989,(54) have shown that TNF message in the liver is expressed one hour after i.v. administration of *E coli.* Ulich et al.,1990, (324) and 1991, (325) have indicated that TNF mRNA increase occurs as early as 15 minutes after endotoxin exposure and that this increase in mRNA can be suppressed by dexamethasone treatment. However, in studies using CAT reporter constructs in transgenic mice, Girior et al. 1991, (136) and 1992, (137) have shown that the liver does not produce TNF. This discrepancy could be due to the incomplete expression of the construct in the transgenic animal. Hunt et al.,1992, (169) have shown by *in situ* hybridization that hepatocytes and endothelial cells of mice produce TNF. Results of *in vitro* studies with isolated Kupffer cells have shown that endotoxin stimulates TNF production and that dexamethasone is a

powerful inhibitor of TNF and IL-6. Karck et al., 1992 (180) demonstrated that 100 nM dexamethasone and PGE_2 were both effective in reducing TNF production by 85% and that dexamethasone could be administered up to 30 min. after LPS and still alter TNF production.

Fig. 26 clearly demonstrates the effect of insulin on TNF in the isolated perfused liver. The initial TNFlevels of the ETX/INS were group significantly elevated in comparison to TNF levels in ETX alone; however, the rates of production were the same. Insulin posttreatment did not alter dexamethasone suppression of TNF production. This observation indicates that dexamethasone *in vivo* is extremely effective in reducing TNF production. The liver could be a target organ for glucocorticoid suppression of TNF production. From the earlier *in vivo* experiments it appears that the contribution of TNF to the circulating pool from insulin stimulation could be from another cell population that cannot be suppressed as effectively as those in the liver. As the initial TNF response in the ETX/INS group was elevated although the rates of production were parallel, perhaps another tissue source of TNF production was no longer contributing when the liver was isolated .

Several authors have demonstrated that the liver is an important organ for the production of IL-6 (15,76,95,101,102,131,180,252). Endotoxin stimulated Kupffer cells, hepatocytes and endothelial cells are capable of producing IL-6. The production of IL-6 results in the of acute stimulation of acute phase protein synthesis by the liver, (26,56,103,245,329) as well as the induction of fever and

the synthesis of ACTH from the pituitary (235).

Interleukin 6 production by the IPL is illustrated in fig. 27. Initial IL-6 production was suddenly curtailed at approximately 20 minutes into the perfusion. At this point in time the liver was continuing to produce TNF. TNF has been shown to be a potent stimulator of IL-6 production. The production of factors by the liver may account for the sudden decrease in IL-6 production liver. Stadler et al., 1993,(303) have recently demonstrated that nitric oxide production inhibited the production of IL-6 by isolated Kupffer cells. Dexamethasone pretreatment significantly inhibited IL-6 production by the endotoxic liver. Insulin did not alter the dexamethasone suppression. These findings indicate that the liver produces IL-6 and that glucocorticoids are dominant down regulators of synthesis.

D. *In Vitro* Studies

1. Cell Lines

The use of isolated specific cell populations to investigate the mechanism of a particular physiological response has become an extensively used technique. Investigation of the effects and possible mechanisms of insulin and glucocorticoid regulation of TNF and IL-6 production at the *in vitro* level were examined in four different macrophage populations. Advocates of *in vitro* experimentation describe the primary advantage of cell culture techniques as reducing the number of confounding factors and removing biological variability associated with *in vivo*

studies. From these *in vitro* studies new questions at a different level of biological complexity were introduced. Initial experiments were undertaken in the ANA1 cells, a transformed macrophage cell population primarily due to the ease of growth and maintenance of these cells. ANA1 cells also express on their cell surface several of the immunological receptors and antigens characteristic of peritoneal macrophages (75). Since the ANA1 cells have not been well described, another more widely used macrophage cell line (RAW 264.7) was also tested.

The dose of endotoxin (1 ng/ml) was chosen from the results of preliminary dose-response studies to determine an effective amount that would stimulate TNF and IL-6 production. The administration of 100 nM dexamethasone was chosen from several reports as effective in suppressing TNF and IL-6 in several cell lines. U937 cells treated with 100 nM dexamethasone show a 90 % suppression of TNF and IL-6 production. The insulin amount was chosen from a study by Cavalot et al., 1992 (61). Insulin at 100 μU/ml is a physiological dose of insulin that was reported to alter lymphocyte chemotaxis. This dose is also similar to the plasma level of insulin achieved during endotoxicosis in the *in vivo* studies.

Endotoxin stimulated significant production of TNF and IL-6 by the ANA1 macrophages. In contrast to our *in vivo* and *ex vivo* results ETX/INS suppressed both TNF and IL-6 production. Doherty et al., 1992, (84) demonstrated that ETX/INS increased both TNF and IL-6 production by peritoneal macrophages. They employed serum free medium and a larger amount of both endotoxin and insulin. Possible differences between this study and the results of Doherty could

be due to unknown growth factors in the serum or concentration differences of hormones administered. This indicates the effects observed *in vivo* may not be direct effects of insulin and that other modulating substances are involved. Dexamethasone pretreatment of endotoxic ANA1 cells did not significantly suppress TNF production but completely abolished IL-6 production. Han et al., 1991, (147, 148)) demonstrated that dexamethasone regulates TNF production by altering the rate of mRNA degradation. This observation suggests that the ANA1 cells process TNF mRNA differently. Insulin post treatment of DEX/ETX cells was effective in restoring IL-6 production. In many cell types such as fibroblasts and endothelial cells TNF production must precede IL-6 production. In ANA1 cells IL-6 production appears to be independent of initial TNF synthesis. This uncoupled IL-6 production has also been observed in J774.1 macrophages and PD3881 macrophages (211).

Endotoxin stimulated both TNF and IL-6 production by RAW 264.7 macrophages. In comparison to the ANA1 cells the RAW cells produced significantly more TNF and a lesser amount of IL-6. ETX/INS treatment did not alter TNF or IL-6 production although this treatment inhibited both TNF and IL-6 production in ANA1 macrophages. Dexamethasone was effective in suppressing the production of both TNF and IL-6 and insulin posttreatment did not alter the amount of either cytokine. These results indicate that two macrophage cell-lines under similar treatment conditions exhibit differential responses in cytokine production. Divergence in the observed response could be due to the specific

alterations in DNA due to the virus used to immortalize the cells. These changes in turn might alter the regulation of transcription and posttranscriptional processing of mRNA. Dissimilar populations of receptors for glucocorticoids or insulin could also explain the variations in cytokine production between the two cell lines. Salkowsky and Vogel, 1992, (280) demonstrated that glucocorticoid receptor populations in macrophages are upregulated by LPS, thus enhancing the suppressive ability of dexamethasone. The choice of a particular cell line for examination of TNF and IL-6 production will, therefore, determine the specific response; therefore, caution should be used in extrapolating the results to the *in vivo* scenario.

2. Primary Macrophage Cells

The effect of insulin and glucocorticoid modulation of cytokine production was also tested *in vitro* utilizing two primary cell populations, peritoneal macrophages and Kupffer cells. Since these cells were isolated directly from the rat peritoneal cavity and liver, TNF and IL-6 production by these cells should better reflect the *in vivo* and *ex vivo* production of the cytokines.

Peritoneal macrophages responded to endotoxin stimulation by producing TNF and IL-6. In contrast to the established cell lines, TNF and IL-6 production by peritoneal macrophages was significantly less. ETX/INS treatment did not significantly alter TNF or IL-6 production although a trend of increased IL-6 concentration was observed. The peritoneal cells could possibly be stimulated

maximally by this concentration of endotoxin and further stimulation by the addition of insulin would not result in further production of TNF. Both TNF and IL-6 production was abolished by dexamethasone pretreatment. Glucocorticoid receptor populations are increased in peritoneal macrophages by LPS stimulation (280). Therefore dexamethasone treatment effectively suppresses TNF and IL-6 production.

Insulin post-treatment of the DEX/ETX group resulted in a mild increase in TNF and a more substantial augmentation of IL-6 production. Insulin receptors are present on the surface of peritoneal macrophages (29,30,364). However, since macrophages are considered non-insulin dependent cells , insulin is not required for glucose metabolism in these cells (223,224). The function of the insulin receptors on the surface of macrophages is unknown. The insulin receptor density can be reduced by certain stimuli such as infection by *Propionium acne* bacterium (29). The effect of endotoxin stimulation on insulin receptor concentration in macrophages has not been studied. The reversal of dexamethasone-suppressed TNF and IL-6 production by insulin has not been documented. Interferon γ has been shown to reverse dexamethasone suppression of TNF production in human monocytes (205).

Kupffer cells were extremely difficult to isolate and maintain in a cell culture environment . In comparison to the other cell types, Kupffer cells require a medium with high glucose and the addition of buffering agents for growth (35,186,349). The recommended addition of dexamethasone and insulin required

for basal maintenance of these cells was not added to the media. The absence of these hormones may have affected the initial recovery of the cells following isolation. Kupffer cells in culture constitutively produced IL-6. This has also been observed by Feder et al., 1993 (101). Both hepatic endothelial cells and Kupffer cells were shown to spontaneously produce both IL-1 and IL-6 in a time dependent manner (101) with maximal levels attained after 10 hours in culture.

Endotoxin addition stimulated an increase in TNF and IL-6 production. Insulin addition surprisingly, reduced TNF and IL-6 concentrations in the medium of endotoxin stimulated Kupffer cells. Rae et al., 1992, (265) described an increase in PGE_2 binding to its receptor by insulin in $P388D_1$ macrophages. PGE_2 is a potent inhibitor of TNF production in several types of macrophages (56,57,345). The inhibition of TNF production by PGE_2 in Kupffer cells has been described by Karck et al. 1988, (180).

Dexamethasone posttreatment in our study significantly reduced TNF and IL-6 concentrations in the medium. Insulin induced only a weak recovery of TNF and IL-6 production in the LPS-DEX treated cells.

The contrasting responses of peritoneal macrophages and Kupffer cells to hormonal modulation of cytokine production could be attributed to many factors. The density of insulin and glucocorticoid receptors might be different as well as alterations in receptor density as a result of endotoxin stimulation. Modulation by other factors such as PGE_2 could be of differential importance to the cells. Since insulin is recommended for growth of Kupffer cells and liver sinusoidal

concentrations of insulin are normally higher than in plasma, Kupffer cells may have exhibit a modified response in terms of TNF and IL-6 production. The Kupffer cell is normally intimately associated with endothelial cells as well as other cells of the hepatic sinusoid (341). One can speculate that Kupffer cell responses are largely dictated by the other cells present. Peritoneal macrophages are probably more independent on stimuli from other cells.

3. Mechanisms of Insulin Action on TNF and IL-6 In Vitro

In order to elucidate the possible mechanisms of insulin and dexamethasone regulation of TNF and IL-6 production by macrophages, specific pharmacological agents which alter second messenger pathways were added to the cell culture medium and the effects on IL-6 production by ANA1 macrophages and TNF and IL-6 production by peritoneal macrophages were examined.

Protein kinase C activation has been implicated as a possible second messenger system required for endotoxin stimulation of macrophage cytokine production as well as macrophage differentiation (320,345,347). PMA, an activator of PKC has has been reported in several other studies to stimulate IL-6 production by macrophages (76, 220). ETX/PMA treatment did not further increase IL-6 production. This finding indicates that either maximal stimulation of IL-6 production had been obtained or that ETX treatment down regulated PKC, thus preventing maximal stimulation by PMA. As was demonstrated with insulin, PMA, was able to reestablish IL-6 production which was suppressed by

dexamethasone. Mengozzi et al., 1991, (220) demonstrated that PMA administration was more effective than IFNγ in restoring IL-6 production in LPS tolerant mice . Insulin restoration of IL-6 production by ANA1 cells may be via PKC activation.

Peritoneal macrophages were also stimulated to produce TNF and IL-6 by PMA. ETX/PMA stimulation increased TNF but not IL-6 production. As was demonstrated with ANA1 cells, PMA was able to reverse the effect of dexamethasone suppression of TNF production. The action of PMA in this experiment is similar to that observed when macrophages are treated with IFNγ. Dunham et al. 1990, (86) have shown that IFNγ treatment of macrophages reverses the suppressive effects of both TGFβ and glucocorticoids on TNF production. Leudecke and Cerami, 1990, (205) also concluded that INFγ can restore TNF secretion inhibited by dexamethasone. Interferon γ may exert its modulatory effects via PKC , however, this has not been established. Insulin could also be exerting its effects on cytokine production by a mechanism similar to that of IFN γ.

To determine if insulin was acting via a PKC mechanism , H-7, an inhibitor of PKC was added with insulin to the ANA1 cells and the peritoneal macrophages. H-7 did not alter cytokine production by either cell type. These findings suggest that PKC activation is not involved in insulin activation of cytokine production. However, H-7 has been shown to exert a differential effect on TNF production based on the dose administered to the cells (66). The dose used in our study may

have not been effective in modulating the effect of insulin.

Indomethacin inhibits the production of prostaglandins by inhibiting cycloxygenase activity. PGE_2, produced by macrophages via an autocrine mechanism, suppresses the production of TNF. By the inhibition of PGE_2 synthesis, indomethacin facilitates an unregulated increase in TNF production by macrophages. Indomethacin treatment was able to stimulate IL-6 synthesis by ANA1 macrophages. Callery et al. 1990, (56) have demonstrated that indomethacin treatment of Kupffer cells results in an increase in IL-6 production. In our study ETX/INDO treatment did not further increase IL-6 production, indicating that production at this dose was maximal. Indomethacin did alter ETX/INS induced inhibition of IL-6 production. Therefore, insulin in this treatment group possibly affected PGE_2 binding. Dexamethasone was able to suppression the stimulation of IL-6 production by indomethacin. Dexamethasone also inhibits the production of phospholipase A_2 which in turn decreases PGE_2 synthesis. These results indicate that dexamethasone inhibits transcription of IL-6.

Previous reports have shown that the inhibition of PGE_2 by indomethacin increases TNF and IL-6 production in peritoneal macrophages (209,292). Indomethacin treatment of peritoneal macrophages in our experiment did not result in an alteration of TNF or IL-6 production. The ineffectiveness of indomethacin may have been due to the dose or preparation of indomethacin at the time of the study. The addition of PGE_2 and the subsequent addition of indomethacin in our system would act as a positive control to test the

indomethacin preparation.

Insulin and IGF-I are structurally homologous peptides with distinct receptors that are structurally homologous (262,363). Insulin is also able to bind to IGF-I receptors at elevated physiological concentrations (179,262,364). IGF-I acting as a growth factor for many cell types has been well documented (121, 263). Recently, IGF-I treatment of macrophages was demonstrated to result in an increase in superoxide production (125).

In this study IGF-I was added to ANA1 cells and TNF and IL-6 production were examined. In contrast to ETX/INS treatment , ETX/IGF-I stimulated substantial increases in TNF and IL-6 production. Peritoneal macrophages also treated with ETX/IGF-I produced substantial amounts of both TNF and IL-6. The intracellular signalling events for both IGF-I and endotoxin remain unresolved. IGF-I could possibly effect PKC.

IGF-I was unable to reestablish only IL-6 production in dexamethasone pretreated endotoxic ANA1 cells in contrast to insulin. However, IGF-I was able to restore both TNF and IL-6 in DEX/ETX peritoneal macrophages. Insulin was partially able to inhibit the IGF-I augmentation of TNF and IL-6 production by the peritoneal cells. This effect could have been due to insulin occupation of IGF-I receptors that would result in a blunted response.

Okadaic acid is a specific inhibitor of serine phosphatases (231,310). Phosphorylation of intracellular proteins is postulated to be an important step in the stimulation of TNF and IL-6 production (310). Phosphatases act to

dephosphorylate these proteins and, thus, prevent the continuous production of TNF and IL-6 (310). Okadaic acid addition to macrophages in culture results in augmented TNF production. This effect appears to be mediated at the posttranscriptional level (310). The effects of okadaic acid on IL-6 production remain undocumented.

In this study okadaic acid was shown to stimulate IL-6 production by ANA1 cells. ETX/OKA did not result in an amplified response in comparison to the ETX group. Possibly, the maximal amount of IL-6 that could be produced by these cells had been achieved. Although, ETX/OKA/INS did not cause a statistically significant increase in IL-6, again indicating maximal production was attained, okadaic acid was able to reestablish IL-6 production suppressed by ETX/INS treatment. Okadaic acid addition to the media was also shown to restore IL-6 production in DEX/ETX treated ANA1 cells.

Okadaic acid addition to endotoxin stimulated peritoneal macrophages resulted in increases in both TNF and IL-6. TNF, but not IL-6, production was found to be increased in the ETX/INS/OKA group. Recently, Begum et al., 1993 (32) demonstrated that insulin regulates the activity of phosphatases A_1 and A_2 at the posttranslational level. Also, insulin altered the phosphorylation of S6 in HepG2 cells (260). Therefore, insulin may exert its effect on TNF production at the translational level. Okadaic acid was also able to overcome dexamethasone induced suppression of both TNF and IL-6 production. Insulin addition to the DEX/ETX/OKA group resulted in a suppression of TNF and an increase in IL-6.

Cycloheximide has been used in *in vivo* and in *in vitro* experiments to superinduce the production of cytokines, primarily TNF (65,247). Cycloheximide prevents the synthesis of RNAse I which is responsible for the rapid degradation of accumulated TNF mRNA (44). The effect of superinduction appears to be specific for TNF , whereas, IL-6 mRNA is not directly affected (65,247).

IL-6 production by ETX/CYC ANA1 cells was suppressed, indicating that cycloheximide affected IL-6 during transcription. However, in this study, cycloheximide addition to the medium superinduced the production of TNF by endotoxin stimulated peritoneal macrophages. Further superinduction was observed with the addition of cycloheximide to the DEX/ETX/INS group. Consequently, the mechanism by which insulin overcomes dexamethasone suppression of cytokine production could be by posttranslational modification. Insulin could affect the synthesis of RNAse I, and thereby stabilizing mRNA. Insulin addition to hepatocytes alters the rate of mRNA degradation (19).

In summary, figs. 48, 49 and 50 depict the hypothesized influence of dexamethasone and insulin with respect to TNF and IL-6 production in the three models used in these studies. *In vivo* ,insulin, in the presence of endotoxin, could modulate the production of TNF and IL-6 directly. Also, indirect modulation of cytokine production could occur by insulin-stimulated production of MIRRF by macrophages. MIRRF could stimulate the production of IFNγ by T cells. IFNγ would then synergize with endotoxin and as well as reverse the dexamethasone supression of TNF and IL-6 synthesis. In the isolated perfused liver

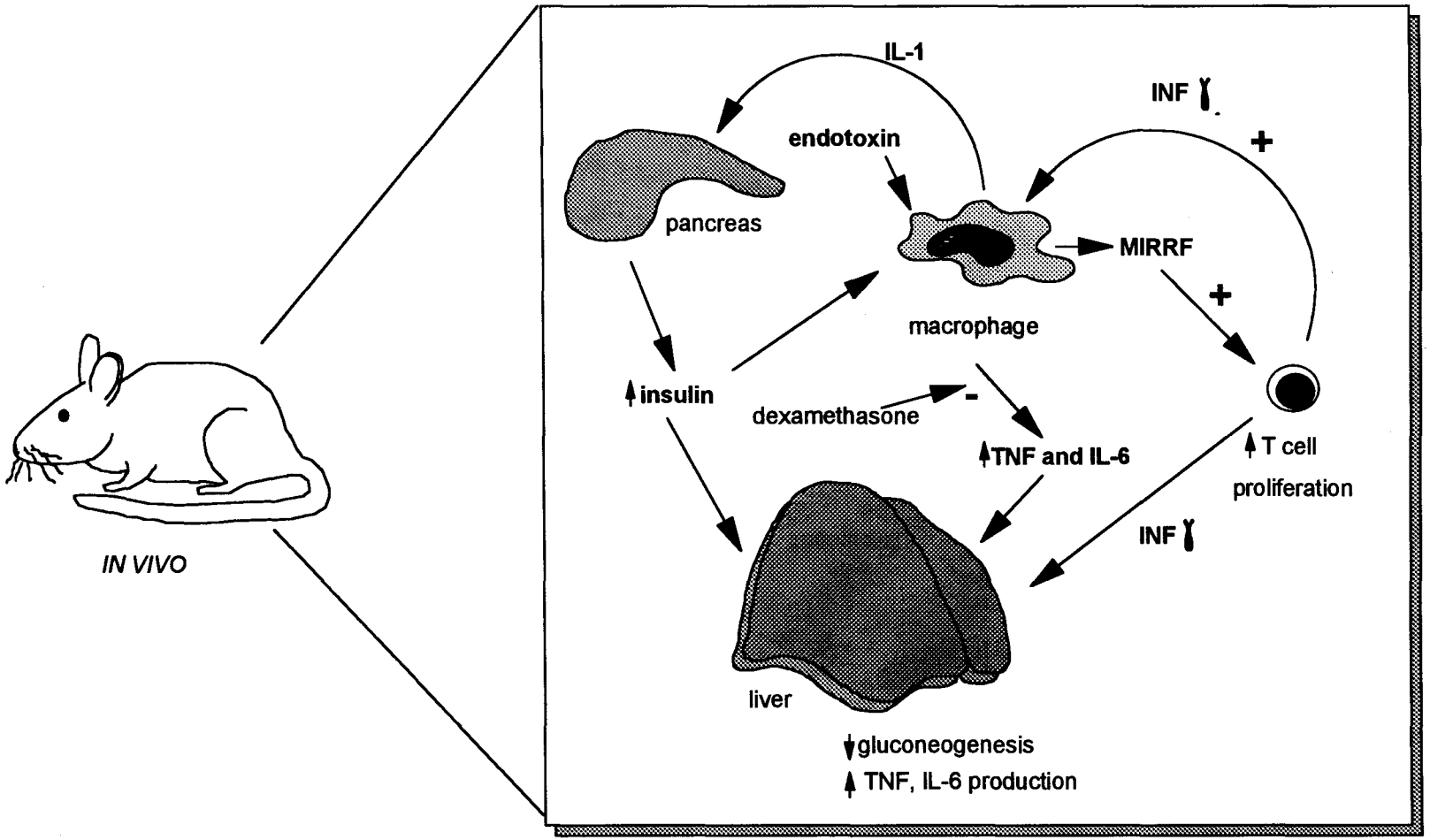


Fig 48. Postulated mechanism for the results observed for the *in vivo* studies.

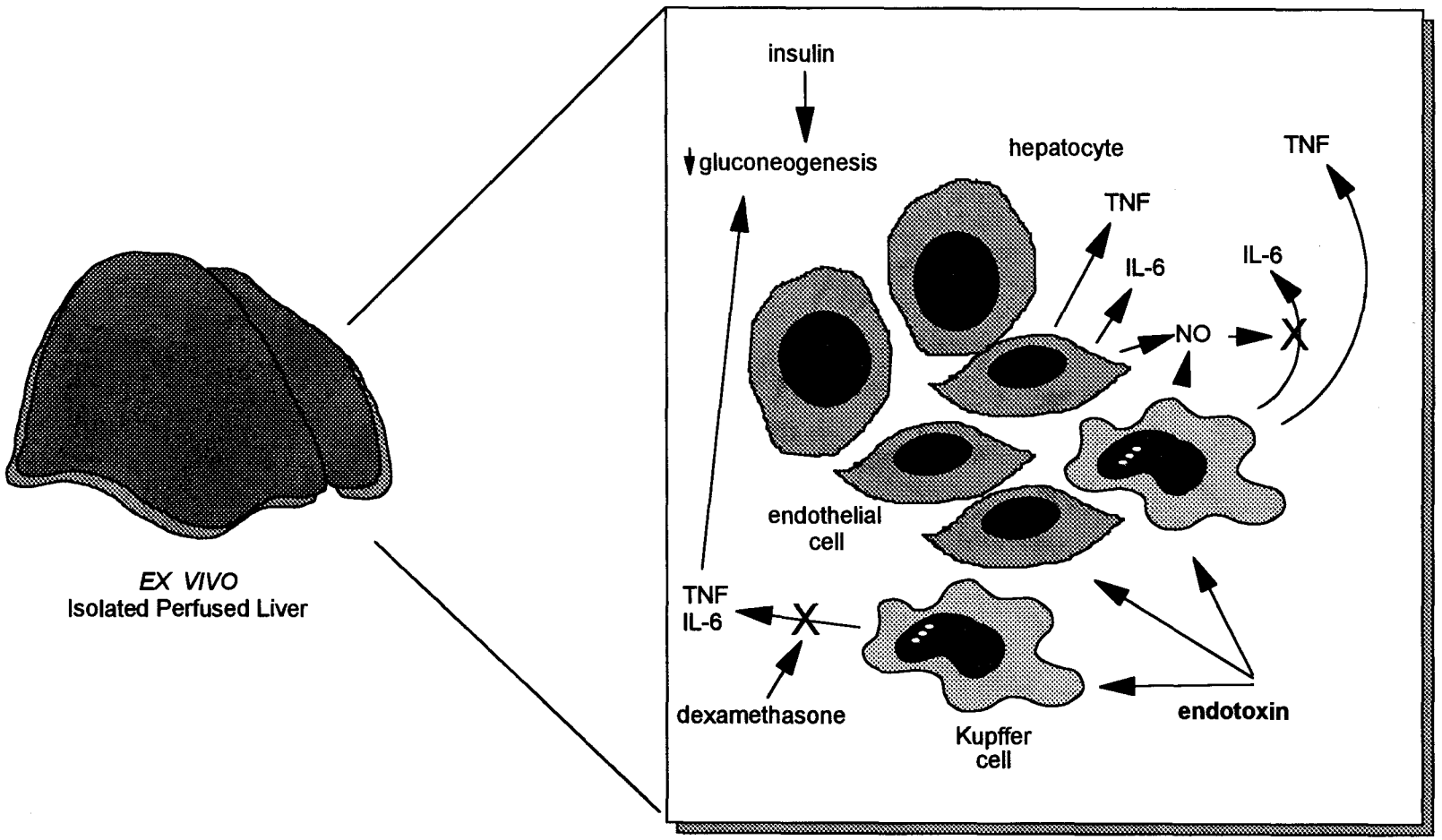


Fig. 49. Postulated mechanism for the results observed for the *ex vivo* isolated perfused liver studies.

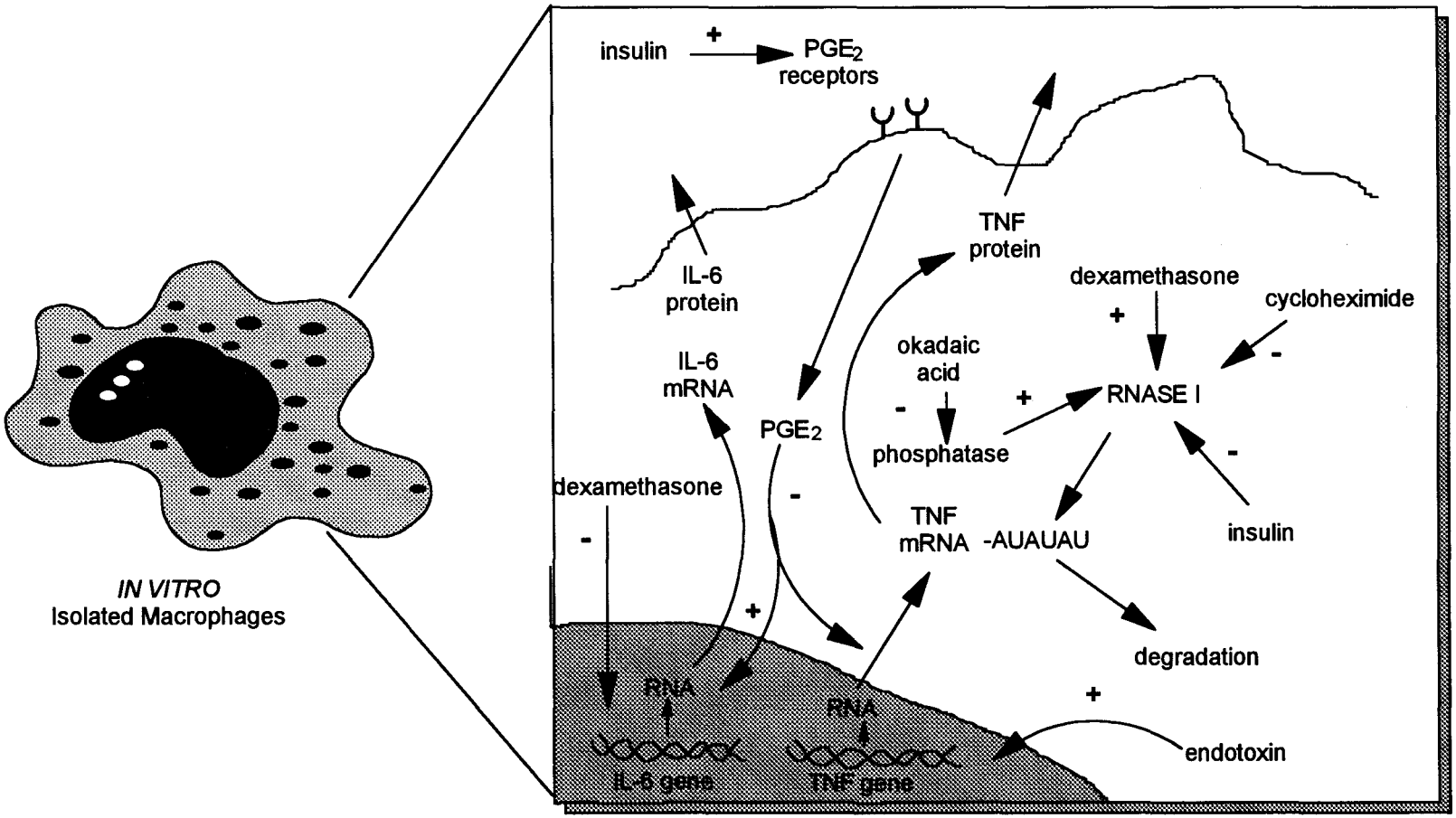


Fig. 50. Postulated mechanisms for the results observed for the *in vitro* experiments with the isolated macrophages.

dexamethasone appears to be a dominant regulator of TNF and IL-6 production as insulin did not stimulate TNF or IL-6 production. A factor, possibly NO, inhibited IL-6 production during the perfusion. *In vitro* dexamethasone was shown to suppress both TNF and IL-6 production. The supression could have occurred at the transcriptional or translational levels. Insulin may have restored cytokine production by altering the activity of RNAse I.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The major goal of this dissertation research was to examine the interactions between two humoral mediators, glucocorticoids and insulin, and to evaluate the consequence of both on cytokine production during endotoxin-induced sepsis. Initially, the combined effects of insulin and dexamethasone were tested on mortality and metabolic parameters of sepsis in an *in vivo* conscious rat endotoxic model. The regulation of TNF and IL-6 production *in vivo* by insulin and dexamethasone was also explored. The use of the isolated perfused liver provided an opportunity whereby TNF and IL-6 from a specific organ could be measured. Lastly, the regulation of TNF and IL-6 production was examined in isolated macrophages. This *in vitro* system enabled the possible mechanisms of the hormonal interaction on TNF and IL-6 production to be explored.

The following conclusions were derived from the data in these experiments:

From the *in vivo* studies:

1) Insulin from both exogenous and endogenous sources exacerbated endotoxin-induced mortality. Its presence induced a rapid demise associated with significant hypoglycemia and hyperlacticacidemia.

2) Dexamethasone pretreatment significantly reduced endotoxin-induced mortality and stabilized plasma lactate concentrations and euglycemia.

3) Insulin from both endogenous and exogenous sources reversed the protective action of dexamethasone pretreatment and a profound hypoglycemia and hyperlacticaidemia was observed.

4) Since the administration of insulin during the "insulin resistant" phase of the endotoxic time course resulted in an increase in mortality, these animals were responsive to the action of insulin.

5) Although marked hypoglycemia was observed, the rats died with hypoglycemia as opposed to of hypoglycemia as the administration of dextrose did not significantly alter mortality.

6) The phorbol ester, PMA , was also able to exacerbate endotoxic mortality similar to insulin. Dexamethasone protection was also negated by PMA administration.

7) Since H-7, an inhibitor of PKC was unable to alter the mortality of the insulin-endotoxin and dexamethasone-endotoxin-insulin treated rats, PKC activation was not involved in insulin-induced mortality.

8) Endotoxin administration *in vivo* stimulated the production of both TNF and IL-6. The administration of insulin did not alter the early pattern of cytokine production; however, it did cause a sustained increase in both TNF and IL-6 at 120 minutes post endotoxin.

9) Dexamethasone pretreatment blocked the elevation in circulating levels

of TNF and IL-6 *in vivo*. Insulin was able to reverse the suppression of TNF but not IL-6 in this model.

10) The insulin treated endotoxic animals exhibited hypoglycemia over the time course of the experiment. Insulin-induced hypoglycemia alone did not stimulate TNF or IL-6 production *in vivo,* suggesting that hypoglycemia was responsible for TNF or IL-6 production by the endotoxic rats.

From the isolated perfused liver:

11) TNF production was not observed in saline control animals until 45 minutes of the perfusion. Dexamethasone pretreatment blocked this increase in TNF production. This finding implicates glucocorticoids as stern regulators of cytokine production under basal conditions.

12) Endotoxin stimulated both TNF and IL-6 production by the isolated perfused liver.

13) The addition of insulin resulted in an initial significant increase in TNF production above that of endotoxin alone. Since that rate of production during the perfusion was parallel to that of endotoxic group, another source of TNF production outside the liver is affected by insulin coadministration.

14) Dexamethasone was able to suppress both TNF and IL-6 production by the endotoxic liver. In this model insulin was unable to restore TNF or IL-6 production. This finding suggests that glucocorticoids are possibly dominant regulators of cytokine production by the liver.

15) Since insulin altered TNF production *in vivo* but was ineffective in the isolated perfused liver, the liver may not be the site of cytokine production regulated by insulin.

From the *in vitro* studies:

16) Endotoxin stimulated TNF and IL-6 production by all four macrophage cell types tested. The transformed cell-lines, ANA1 and RAW 264.7 cells were generous producers of TNF and IL-6 in comparison to the primary cells, the peritoneal macrophages and the Kupffer cells. Differential effects were exhibited even been the two transformed lines. In three of the cell types, except the ANA1 cells IL-6 production appears to be linked to the initial synthesis of TNF.

17) Insulin added to endotoxin-stimulated macrophages also had differential effects dependent on the cell type used. Inhibition of TNF and IL-6 production was observed with the ANA1 cells and the Kupffer cells. There was no stimulation or depression of TNF or IL-6 production by the RAW 264.7 or the peritoneal macrophages.

18) Dexamethasone pretreatment significantly reduced TNF and IL-6 production in all cells except the ANA1 cells, where only IL-6 was significantly suppressed.

19) Insulin administration to the dexamethasone-protected-endotoxic macrophages reversed TNF and IL-6 production by the primary cell lines and IL-6 production by the ANA1 cells.

20) Protein Kinase C activation was not involved in the insulin-induced effects on TNF and IL-6 production, as H-7 was unable to effect the cells production of either cytokine. PMA as observed *in vivo* mimicked the insulin response in these cells.

21) Prostaglandin synthesis was also not responsible for the reversal of dexamethasone protection, however, indomethacin treatment was able to reverse the endotoxin-insulin induced inhibition of cytokines suggesting a role of insulin in modulating PGE_2 induced TNF suppression.

22) The effects observed by the substitution of IGF-1 for insulin suggest that although these peptides are structurally similar they exert differential effects on TNF and IL-6 production by macrophages.

23) The second messenger pathway affected by insulin appears to involve the regulation of phosphatase activity.

An overall conclusion that can be drawn from the results documented in this dissertation is that even though a similar treatment protocol was applied to three levels of experimentation the results obtained are very different and in some cases conflicting. The observations derived from *in vitro* and *ex vivo* examination alone cannot always be successfully extrapolated to the *in vivo* environment.

Future experiments may involve examination of the role of interferon γ or the activation of other second messenger pathways, such as tyrosine kinase phosphorylation, and the transcriptional and posttranscriptional regulatory activities initiated by insulin. Future results may provide valuable insight into

possible biological and pharmacological avenues which may be implemented as modulators of TNF and IL-6 production in the septic patient. However, Gram-negative infection perturbs the behavior of every organ-system of the hospitalized septic syndrome patient. The research focus which examines the interactions between systems (eg. endocrine system and the immune system) as opposed to the intricacies of an individual system will probably provide the therapeutic and clinical modalities that will save lives.

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VITA

Elisabeth Lilli Hahn, the daughter of Elith and Theresia Hahn was born in Toronto, Ontario, Canada on August 26, 1959. She attended elementary and secondary school in Orillia, Ontario, Canada. She attended the University of Guelph in 1978 and graduated in 1982 with an honors Bachelor of Science in Biology and Nutritional Science. From 1983 to 1987 Liz was employed as the Indirect Calorimetry Coordinator in the Division of Clinical Nutrition at The Hospital for Sick Children, Toronto.

In 1987, she began her graduate career at the University of Guelph in Nutritional Science under the supervision of Dr. Henry S. Bayley and was awarded a Master's of Science (M.Sc.) in 1989.

Liz began the Ph.D program in the Department of Physiology under the supervision of Dr. J.P. Filkins in 1989. She was award an alternate Schmitt Award in 1992, and nominated for the Loyola Presidential Medallion in 1993. Liz was also the recipient of a Shock Society Travel Award and a Society for Leukocyte Biology Award as a finalist in the Presidential Award Competition. She was the Physiology Departmental Representative from 1989-1992, Secretary of the Graduate Student Council in 1990 and served as the President of the Graduate Student Council at the Medical Center Campus 1992-1993.

PUBLICATIONS

A. Articles

Vaisman, N., Levy, L., Pencharz, P.B., Tan, Y., Soldin, S., Canny, G., Hahn, E. 1987. The Effect of Salbutamol on Resting Energy Expenditure In Patients with Cystic Fibrosis. *J. Ped.* **111**: 137-139

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B. Abstracts

Vaisman, N., Rossi, M., Goldberg, E., Hahn, E., Clarke, R., Pencharz, P.B. 1987. Abnormal Substrate Utilization on Refeeding Patients with Anorexia Nervosa .*Pediatric Research* April 1987

Vaisman, N., Pencharz, P.B., Levy, L., Tan, Y., Soldin, S., Canny, G., Hahn, E. 1987. The Effect of Salbutamol on Resting Energy Expenditure in Patients with Cystic Fibrosis. *Pediatric Research* April 1987

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APPROVAL SHEET

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

9-16-94
Date

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