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CYTOKINES AND SKELETAL MUSCLE WASTING

by

YAN ZHANG

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Abstract

Pro-inflammatory cytokines are important mediators in the regulation of diverse aspects of skeletal muscle function, however the muscle cytokine receptors mediating these functions are uncharacterized. Binding kinetics ($Kd=39\pm4.7\times10^{-9}$M, $Bmax=3.5\pm0.23\times10^{-12}$mol/mg membrane protein) of muscle tumor necrosis factor (TNF) receptors were obtained. Skeletal muscle was found to express mRNAs encoding numerous other cytokine receptors (Interleukin-1 types I and II receptors, Interleukin-6 receptor, Interferon γ receptor) by RT-PCR, however the receptors were beneath the limits of detection of radioligand binding assay (1 fmol/mg membrane protein). To determine if the cytokine receptor expression is subject to modulation, we examined the mRNA levels in muscle tissue from rats 24hr after intraperitonial administration of endotoxin using semi-quantitative RT-PCR. mRNA levels of TNF receptor type II and IL-6 receptor were up-regulated by endotoxin treatment The expression of mRNA for multiple cytokine receptors was also induced by TNFα as well as by a mixture of TNFα+IFNγ+endotoxin in cultured L6 cells *in vitro*. This induction suggests that the low levels of cytokine receptor expression appears to be complemented by a capacity for receptor induction, providing a clear mechanism for amplification of cytokine responses at the muscle level.

The stimulation of muscle with a mixture of cytokines and endotoxin resulted in induction of iNOS, as has been shown in cultured muscle cells. However, no stimulation of the process of muscle protein catabolism was observed in a various experimental conditions *in vitro* with cytokines alone, in combination or in the presence of endotoxin. These results suggest that cytokines activate nitric oxide (NO) synthesis but not protein

catabolism under the conditions studied. This would be consistent with the observation that neither inhibitors nor activators of NO production had any effect on protein breakdown in muscle.

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Chapter 1 Literature Review

The objective of this chapter is to outline our present knowledge of: 1) skeletal muscle wasting in diseases; 2) the proposed humoral mediators involved in protein breakdown in skeletal muscle, especially four pro-inflammatory cytokines, and their possible mechanisms; 3) the main proteolytic pathways in skeletal muscle and their role in muscle wasting; and 4) methods of studying protein metabolism in skeletal muscle. The review leads to the rationale and hypothesis of my thesis research.

1.1 Physiological Significance of Muscle Wasting in Diseases

Skeletal muscle is the largest tissue of the mammalian body. Its mass comprises 40~50% of the total body weight and it accounts for about 70-80% of the body's cell mass (Millward et al., 1984). The main function of skeletal muscle is to provide the mechanical effort that enables the organism to move within, and interact with, its physical environment under voluntary nervous control (Landon, 1992). In addition, it has a major role in the general metabolism of the organism. Skeletal muscle makes a large contribution to the body's content of water (80%) and intracellular ions, and functions as a main store of energy-rich compounds, a major glucose disposal site and is a reservoir of protein, and free amino acids in the body (Schmalbruch, 1985, Landon, 1992).

Protein balance in muscle is very important in overall energy homeostasis of the organism, since muscle contains most of the body's protein reserves. Net mobilization of muscle protein can provide amino acids for metabolism by other tissues, while net uptake

of amino acids by muscle and their incorporation into protein is a form of energy storage (Felig, 1975, Ruderman, 1975). A prominent physiological response in a variety of disease states, such as fever, sepsis, traumatic injury, burns, AIDS and cancer is a severe negative nitrogen balance, especially muscle wasting (Beisel, 1984, Argiles et al., 1992, Grunfeld and Feinfold, 1992). The accelerated proteolysis in muscle in disease states appears to be part of an integrated physiological response of the host to diseases. Enhanced protein breakdown in muscle contributes to the increased survival of the patient by providing gluconeogenic precursors, or substrates for direct oxidation, as well as amino acids for the synthesis of acute phase protein, immunoglobulins, and perhaps for wound repair (Tawa and Goldberg, 1994, Reeds et al., 1994).

When muscle wasting is prolonged or severe, emaciation results. This condition is known as cachexia. Since a loss of body mass exceeding 30% of the ideal body mass for a given individual may be fatal (Brennan, 1977), cachexia is an important clinical problem contributing directly to mortality and morbidity, and may reduce the ability of patients to tolerate aggressive therapies (Eden et al., 1983, Van Eys, 1982, Chlebowski, 1985). Depletion of skeletal muscle mass is also recognized as critical to overall survival of the patient and can prolong rehabilitation to normal physiological function following recovery (De Wys, 1985, reviewed by Argiles et al., 1992, Beulter, 1993, Tawa and Goldberg, 1994). However, even as we near the 21st century, the pathophysiologic and biochemical basis of muscle wasting remains exclusive.

1.2 Regulation of Protein Breakdown in Skeletal Muscle

In skeletal muscle, as in all tissues, protein synthesis and degradation are precisely regulated, and vary under different physiological and pathological conditions. This regulation is an area of rapidly expanding research efforts. Muscle wasting may be due to increased muscle catabolism and/or decreased protein synthesis. Although anorexia is often associated with cachexia (Baracos et al., 1995, Langstein et al., 1991, Strelkov et al., 1989), cachexia is distinct from simple starvation in several respects (Brennan, 1977, Espat et al., 1995). Through the use of pair fed controls (Strelkov, et al., 1989), it has been shown that anorexia does not explain the wasting of muscle protein and elevated protein breakdown observed in animal models. In addition, total parenteral nutrition trials in cachectic cancer patients appear to be of a limited clinical benefit as aggressive nutritional support did not change median survival rates or preserve lean body mass (Gough et al., 1996).

Muscle wasting is probably provoked by humoral mediators (Goldberg et al., 1988). The plasma of patients with trauma or sepsis had an increased ability to stimulate tissue protein breakdown compared to normal plasma (Clowes et al., 1983). Partially purified products from activated human monocytes could stimulate these proteolytic processes when added to isolated rat muscles (Baracos et al., 1983, Goldberg et al., 1988). It is very possible that there exist some catabolic factors in the products from activated monocytes or in the plasma of these patients, which act on muscle cells directly.

Cytokines are a family of small proteins mainly produced by activated macrophages, monocytes and lymphocytes, which includes interferons, interleukins, and tumor necrosis factor, among others. Together these factors form a complex network of

signals regulating the growth, differentiation, and function of almost every cell type. Cytokines are characterized by multiple functions and redundancy in biological activities and exert their effects at picomolar or femptomolar concentration, typically acting in an autocrine, or paracrine fashion. Tight control of the cytokine network is essential to maintain homeostasis and minimize nonspecific inflammatory injury in regions not implicated in cellular activation (Stewart and Marsden, 1995, Vilcek and Le, 1994, Foex, 1993). The four pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interferon gamma (IFNγ), have been proposed to regulate many biological functions in skeletal muscle (Table I-3), including muscle wasting in a various conditions (see review by Argiles et al., 1992, Moldawer et al., 1992, Smith and Tisdale, 1993, Tisdale, 1993, Toomey et al., 1995).

1.2.1 Cytokines

I. Introduction to Cytokines and their Receptors (Table I-1)

i. Interleukin-1 (IL-1)

IL-1 is one of the prototypic multifunctional cytokines. It acts on a variety of cell types and possesses a number of biological activities. Some of these include the ability to stimulate B cell function (Falkoff et al., 1984), fibroblast proliferation (Schmidt et al., 1982), to activate muscle proteolysis (Baracos et al., 1983, Gelin et al., 1991a, Belizario et al., 1991), and to induce fever (Kozak et al., 1998). IL-1 is mainly synthesized by

activated macrophages and monocytes, but also by many other nucleated cells (Stewart and Marsden, 1995).

There are three members of the IL-1 gene family: IL-1α, β and IL-1 receptor antagonist (IL-1Ra) (Dinarello, 1996). IL-1α and IL-1β represent two forms of the active protein and are derived from distinct genes (March et al., 1985). They are both synthesized as precursors with molecular weight of 31KD, and then processed to their mature forms of 17KD by specific cellular proteases. IL-1β represents the predominant free extracellular form, whereas most IL-1α is membrane bound (Kurt-Jones et al., 1985). This "membrane-bound " IL-1α is biologically active, and it appears to be anchored via a lectin interaction involving mannose residues (Dinarello, 1991, Brody and Durum, 1989). IL-1α and IL-1β share 26% amino acid identity and bind to the same IL-1 receptor (Oppenheim et al., 1986). IL-1 exerts its biological effects via its receptors on target cells, and these have been identified on a variety of cell types, including T-cell lines (Lowenthal and MacDonald, 1986), fibroblasts (Chin et al., 1987), and endothelial cells (Thieme et al., 1987). There are two distinct types of IL-1 receptor: IL-1RI and IL-1R II, which are 80KD and 60KD proteins, respectively. Structurally, the extracellular portion of IL-1RII is 28% similar in amino acid sequence to IL-1RI, however, the intracellular portion of IL-1RII comprises only 29 amino acids in contrast to the 215 amino acids of IL-1RI (Dinarello, 1996). The type I receptor transduces a signal, whereas the type II receptor binds IL-1 but does not transduce a signal, instead it inhibits IL-1 activity by acting as a decoy target for IL-1 (Colotta et al., 1994). The extracellular domains of the type I and type II IL-1R are found as "soluble" molecules in the

circulation and urine of healthy subjects and in inflammatory and other pathologic body fluids (Sims et al., 1994).

ii. Tumor Necrosis Factor alpha (TNFα)

TNF is a 17KD polypeptide and one member of a family of structurally related cytokines, including lymphotoxin alpha (TNFß or LTα) and lymphotoxin beta (LT ß) (Browning et al., 1993). Both TNFα and TNFα (lymphotoxin) are recognized by the same cell surface receptors and are associated with similar biological activities (Heller and Kronke, 1994). TNFα is a pleiotropic cytokine that can mediate a wide variety of biological effects. TNFα induces cytotoxicity in various tumor cells, stimulates the growth of fibroblasts and regulates the functions of endothelial cells, neutrophils T cells, and B-cells (Sugarman et al., 1985, Vicek et al., 1986, Nawroth and Stern, 1986, Shalaby et al., 1985), and TNFα is mainly produced by activated macrophages (Kelker et al., 1985). It is well known that TNFα plays an important role in the pathology of such diseases as septic shock, inflammation and cachexia (Argiles et al., 1992, Garcia-Martinez et al., 1997).

TNFα initially exists as a 26KD type II cell-surface associated molecule anchored by an N-terminal hydrophobic domain (Jue et al., 1990, Kriegler et al., 1988). TNFα is subsequently processed by proteolytic cleavage to a 17KD peptide. Three 17KD molecules become non-covalently associated to form a homotrimeric complex that is biologically active (Smith and Baglioni, 1987) and the cytokine trimer binds to three

receptors, presuming triggering signal transduction by receptor clustering (Banner et al., 1993). Each of the three TNFα subunits makes contact with two adjacent receptor molecules, thus stabilizing the receptor trimer. TNF interacts with at least two distinct membrane-associated receptors, termed TNF-RI (55KD) and TNF-RII (75KD) which exhibit similar receptor binding affinities for TNF. Both receptors are present on virtually every cell type, excluding red blood cells (Tracey and Cerami, 1993) and they also exist in soluble forms that are present in the serum and urine of patients with sepsis, cancer and febrile illness (Tracey and Cerami, 1993).

iii. Interleukin-6 (IL-6)

IL-6 acts on a wide range of tissues, exerting growth inducing, growth-inhibiting and differentiation-inducing effects, depending on the nature of the target cells (Hirano et al., 1990). IL-6 was previously investigated under the designations: IFN-β2, B-cell stimulatory factor (HPGF or IL-HP1), hepatocyte stimulating factor (HSF) and monocyte granulocyte inducer type2 (MGI-2). Molecular cloning studies have shown that all of these molecules are identical (Heinrich et al., 1990). IL-6 is produced by a variety of cells after appropriate stimulation. Monocytes/ macrophages (Bauer et al., 1988), and endothelial cells (Jirik et al., 1989) are believed to be the major IL-6 producing cells in mammals. Human IL-6 mRNA is translated into a 212-amino acid precursor protein with a molecular mass of 26KD (Content et al., 1982). After removal of a 28-amino- acid signal peptide, the resulting 184-amino-acid protein is N- and O- glycosylated and subsequently secreted (Heineich et al., 1990).

The specific receptor for IL-6 is expressed on lymphoid and non-lymphoid cells (Coulie et al., 1989, Taga et al., 1987). The IL-6R cDNA encoded a protein consisting of 468 amino acids of 80 KD (Heinrich et al., 1990, Taga et al., 1989). In contrast to many other receptors, the cytoplasmic domain of the IL-6 receptor is not essential for signal transduction. However, a possible signal transducer (gp130) with a molecular mass of 130 KD could associate with the 80 KD IL-6R after the interaction of IL-6 with receptor and transduce the signal (Taga et al., 1989, Heinrich et al., 1990). There are at least two sites in the IL-6 molecule: one binds to gp80 (IL-6 receptor), while the other contacts the signal transducer gp130 (Brakenhoff et al., 1990). A soluble IL-6R (sIL-6R), lacking the transmembrane and intracytoplasmic domain, has been detected in the serum and in the urine of normal individuals. Naturally-occurring sIL-6R with a 50-55KD molecular mass is identical to that of the extracellular domain of membrane-bound IL-6R. The clinical relevance of the sIL-6R in immune and inflammatory disorders and plasma cell neoplasias remains to be clarified (Ohtani et al., 1995).

iv. Interferon gamma (IFNγ)

IFNs are a group of proteins with a wide array of biological effects on target cells, including antiviral activity, growth inhibitory effects, and several immunoregulatory activities. (Moore, 1981). The human IFN family of cytokines is comprised of two main types. Type I includes IFNα and IFNβ, which both appear to interact with a common binding site. Nearly all cells of the body are capable of producing

and secreting type I IFN. Type II IFN is also known as IFNγ, a 17 kD peptide, and is produced mainly by T lymphocytes and natural killer cells when the cells encounter foreign antigens. IFNγ probably binds to a distinct cellular receptor (Branca and Baglioni, 1981, DeMaeyer and DeMaeyre-Guignard, 1994).

IFNγ acts on cells via specific receptors. Its receptor has been demonstrated on various cells: fibroblasts (Novick et al., 1987), monocytes (Finbloom et al., 1985), and astrocytes (Rubio and Felipe, 1991). IFNγ receptor consists of at least two components, a 90KD glycoprotein that is able to bind IFNγ (Aguet et al., 1988) and a structurally related putative transmembrane protein (B) that is necessary for full receptor functionality (Soh et al., 1994). Signaling through the IFN receptor requires Jak1 and Jak2, two members of a family of tyrosine kinases. By analogy with other systems involving the Jak family kinases, the interferon gamma receptor and the Jak kinases probably exist in a preformed complex before ligand binding (Igarashi et al., 1994).

II. The Role of Cytokines in Muscle Wasting (Table I-2)

The production of IL1, TNFα (Costelli et al., 1993, Tessitore et al., 1993), and IL6 (Gelin et al., 1988) was elevated in patients and animals showing muscle catabolism. Early experiments of Baracos et al. (1983) suggested that partially purified human interleukin-1 (IL-1) increases proteolysis in isolated muscle preparations. Cytokines have been hypothesized to contribute to diverse cases of activated muscle protein catabolism ever since and this idea is supported by numerous studies showing increased protein

catabolism after *in vivo* cytokine administration. Acute treatment of rats with TNFα caused an enhanced tyrosine release and a decreased protein synthesis in soleus muscle (Garcia-Martinez et al., 1993b). Decreased protein synthesis was also observed in extensor digitorum longus (EDL) muscles (Garcia-Martinez et al., 1993b). *In vivo* administration of TNFα has also been shown to activate muscle protein degradation by other authors (Goodman, 1991). TNFα can increase the ubiquitinization of rat skeletal muscle proteins *in vivo* (Garcia-Martinez et al., 1993a). Total and myofibrillar protein breakdown rates were both increased by 49 and 134%, respectively in EDL from rats treated with IL-1 (Zamir et al., 1991). IL-6 administration *in vivo* acutely activated both total and myofibrillar protein in rat muscles (Goodman, 1994). Administration of repetitive sublethal doses of either recombinant human TNFα or recombinant murine IFNγ to rats caused a significant decline in food intake and body weight (Langstein et al., 1991). However, Kettelhut and Goldberg (1988) found no changes in the rat of protein synthesis in either incubated rat EDL or soleus muscles following recombinant TNFα treatment, neither did Moldawer (Moldawer et al., 1987). Furthermore, IL-1 induced no change in muscle protein degradation (Goodman, 1991) and was shown to increase proteolysis only synergistically with TNFα (Flores et al., 1989). Most studies *in vivo* suggest that cytokines activate muscle protein breakdown. Differences in muscle type, animal species, dose and model of cytokine administration may account for the instances where negative results were obtained.

The essential role of cytokines in the pathogenesis of hypercatabolic states was confirmed by the demonstration that specific antibodies/antagonists against cytokines can attenuate the catabolic effects of different disease states. Food intake and body

composition were improved in sarcoma-bearing mice given an anti-TNFα antibody compared to tumor-bearing controls (Sherry et al., 1989). Immunizing animals with antibodies against TNFα (Gelin et al., 1991a, Costelli et al., 1993) or IL1R type I (Gelin et al., 1991a) decreased protein degradation in the muscles of rats bearing the Yoshida ascites hepatoma (Costelli et al., 1993) and MCA fibrosarcoma (Gelin et al., 1991a). Antibodies to IL6 attenuated the anorexia, weight loss and muscle atrophy associated with a colon adenocarcinoma (Fujita et al., 1996, Strassmann et al., 1992). A systemic administration of both IL-1R antagonist and the mAb 35f5 directed against IL-1R type1 did not reverse weight loss in C-26-bearing mice, while intra-tumoral injections of IL-1RA significantly reduced the muscle wasting (Strassmann et al., 1993). Injection of anti-IFNγ in rats with a methylcholanthrene-induced sarcoma reduced anorexia and weight loss and increased survival time (Langstein et al., 1991). However, the reversal of the muscle wasting by IFNγ antibody was only partial and short-lived, this suggested that IFN is not the sole mediator. Interestingly, in both of the latter models, anti-TNFα did not modify muscle protein catabolism. IL-1 receptor antagonists was unable to reverse muscle wasting in rats bearing hepatoma Yoshida AH-130 (Costelli et al., 1993).

Mice deficient in p55 TNFRI were resistant to tumor induced protein breakdown in skeletal muscle (Llovera et al., 1998). The implantation of the Lewis lung carcinoma to both wild-type and gene-deficient mice for the TNFR I protein resulted in a considerable loss of carcass weight in both groups. However, muscle wastage in the gene-knockout mice was less than in the tumor-bearing wild-type. The increase in protein degradation in the tumor-bearing wild mice was accompanied by an enhanced expression of both ubiquitin and proteasome subunit genes, all of them related to the activation of the ATP-

dependent proteolytic system in skeletal muscle. Tumor-bearing gene-deficient mice did not show any increase in gene expression. In both groups, tumor burden resulted in significant increases in circulating TNFα. It was concluded that TNFα is responsible for the activation of protein breakdown in skeletal muscle of tumor-bearing mice (Llovera et al., 1998).

These results appear to implicate several specific cytokines in skeletal muscle wasting, but are difficult to interpret in that the cytokine involved seems to vary from tumor to tumor. As the studies cited above did not examine the presence of all four catabolic cytokines or use multiple anti-cytokine antibodies, it is unknown whether only the selective presence of a given cytokine is associated with muscle catabolism within a specific type of tumor. The results may indicate that four different cytokines can independently elicit a catabolic response. However known inter-relationships among cytokines suggest the alternative possibility that there may be a common mediator or interactions between cytokines. For example, the production of TNFα is integrally related to the other cytokines: TNFα can trigger the production of IL1, IL6 and IFNγ which propagate its effects; these cytokines modulate responses to TNF in a positive or negative fashion depending upon the system studied (Espat et al., 1995, Tracey and Cerami, 1993). Immunization with anti-TNFα attenuates the production of IL1 and IL6 (Fong et al., 1989). It has been suggested that many of the effects attributed to IL1 and TNF are actually mediated by IL6 (Espat et al, 1995, Starnes et al., 1990, Tracey and Cerami, 1993). IL6 can suppress IL1 and TNFα production, and serves as a feedback inhibitor for its own production (Espat et al., 1995, Neta et al., 1992). All of these findings make it difficult to distinguish each cytokine's function and physiological consequence *in vivo*.

III. The mechanism of cytokine induced muscle wasting

Despite the mounting *in vivo* evidence of cytokines' involvement in protein catabolism in diseases, a major deficit in our present knowledge is whether cytokines act directly on muscle wasting. Incubation experiments produced conflicting evidence and are difficult to interpret. Partially purified IL-1 (Baracos et al., 1983) and purified IL1α, β (Belizario et al., 1991) are reported to stimulate proteolysis in incubated muscle. In cultured muscle cells, IL6 induced proteolysis (Ebisui et al., 1995). Incubation of rat soleus with TNFα resulted in increased ubiquitin gene expression (Llovera et al., 1997). However, IL6 (Garcia-Martinez et al., 1994), IL1α or β, TNFα, IFNα, β, γ, or mixtures of these failed to activate muscle proteolysis *in vitro* in other similar studies (Goldberg et al., 1988, Goodman, 1991, Moldawer et al., 1987). The lack of response to cytokine's proteolysis-inducing activity *in vitro* made some researchers suggest that though cytokines have a role in muscle wasting, their effects is indirect and mediated via intermediators, such as hormones (insulin, glucorticoids), or prostaglandin E2.

Various hormones, including insulin, thyroid hormones, growth hormone, and glucocorticoids have been shown to affect the rates of protein synthesis and degradation in muscle (see Table I-4) (Tawa and Goldberg, 1994, Rooyackers and Nair, 1997). Release of cytokines can trigger release of a cascade of hormonal changes or modulate the sensitivity of tissue towards the anabolic hormones (Costelli et al., 1993, Tracey et al., 1990). Infusion of TNFα stimulates the production of a variety of catabolic hormones

including catecholamines, cortisol and glucagon in a dose responsive manner (Tracey et al., 1990). The catabolic hormones can partly simulate the metabolic pattern observed in injury or infection and may therefore participate directly at the muscle level. Several studies have explored this possibility. Using adrenalectomized animals, Mealy et al. (1990) demonstrated that an intact adrenal stress hormone response is necessary for negative N balance following TNF infusion. Muscle proteolysis induced by administration of TNF was partly mediated by glucocorticoids (Meally et al., 1990), but the effect of IL-1 was not mediated by these hormones (Zamir et al., 1991). The disturbance in hormone systems may therefore contribute to the negative nitrogen balance in skeletal muscle. Additionally, TNFα and IL-1 have been proposed to be associated with insulin/IGF-1 resistance (Hofmann et al., 1994, Saghizadeh et al., 1996, Lang et al., 1996). However, although some of the manifestations of cachexia may be induced by hormonal infusions of serotonin, bombesin, and combinations of the "stress" hormones (adenaline, cortisol and glucagon), no single combination induces all the manifestations of cachexia (Bessey et al., 1984, Watters et al., 1986). Attempts to correct these metabolic disturbances with infusions of anabolic hormones, such as insulin or growth hormone, in an effort to overcome relative insulin-resistance have met with limited success (Heber and Tchekmedyian, 1992, Pearlstone et al., 1994). Thus, while hormonal mechanisms may play some part in cachexia, their role has not been clearly defined.

TNFα and IL-1 are amongst the most potent agonists of peripheral prostaglandin production, particularly that of PGE_2 (Yucel-Lindberg et al., 1995, Hughes et al., 1999, Mollace et al., 1998). Increased PGE_2 appears important in a variety of instances of

cachexia and muscle wasting (Baracos et al., 1983, Tian and Baracos, 1989, Gelin et al., 1991b, Strelkov et al., 1989). When rat muscles were treated with PGE_2, protein synthesis was not affected, but protein degradation increased by about 22% (Rodemann et al., 1982). Treatment of chicks infected of E. coli. with naproxen, an inhibitor of prostaglandin production, decreased body and muscle weight losses, and significantly inhibited muscle protein wasting compared to similar chicken not treated with naproxen (Tian and Baracos, 1989). Of particular interest in the context of cytokines are results obtained using the Yoshida ascites hepatoma, a tumor associated with a marked activation of muscle protein degradation. Administration of an anti-TNFα IgG to tumor-bearing rats inhibited most of the accelerated protein degradation in skeletal muscle, clearly indicating a role for TNFα in this model (Costelli et al., 1993). Systemic administration of inhibitors of prostaglandin synthesis including naproxen (Strelkov et al., 1989) and acetylsalicylic acid (Tessitore et al., 1994) also inhibit the elevated catabolism. These results imply that TNF-dependent muscle protein degradation is dependent upon the synthesis of PGE_2, although this remains to be directly tested. Partially purified IL-1 stimulated the net release of tyrosine in skeletal muscle via PGE_2 (Baracos et al., 1983). However, Moldawer et al. (1987) reported that purified IL-1α and TNFα stimulated the production of PGE_2 without any impact on skeletal protein balance. Additionally, IL-1β failed to induce PGE_2 release in skeletal muscle in other study (Goldberg et al., 1988). The role of PGE_2 as a direct mediator of cytokines' proteolysis-inducing activity remains unclear. Furthermore, the role of PGE_2 in regulation of muscle proteolysis is the subject of controversy. Hasselgren et. al. (1990) did not find that PGE_2 activated protein breakdown in incubated muscles or that inhibitors of prostaglandin synthesis reduced muscle protein degradation in septic rats. Such difference may be due

to the varying animal models, drugs, doses and modes of administration used in studies of this kind.

A direct role of cytokines on muscle protein breakdown can not be ruled out. The pro-inflammatory cytokines regulate other biological functions in skeletal muscle directly: stimulating synthesis of nitric oxide by IFNγ and TNFα or IL-1 β (Okuda et al., 1997, Williams et al., 1994, Bedard et al., 1997); regulating glucose uptake by TNFα (Rangnanthan and Davidson, 1996, Yamasaki et al., 1996); inducing insulin resistance by TNFα and IFNγ (Ranganathan and Davidson, 1996, Bédard et al., 1997); facilitating myoblast differentiation by IL-6 (Okazaki et al., 1996); inducing expression of leukocyte antigen and cell adhesion proteins in myoblasts (Michaellis et al., 1993, Kalovidouris et al., 1993), altering nerve-induced redistribution of acetylcholine receptors by IFNγ (Kelic et al., 1997); and inducing the expression of ferritin H mRNA by TNFα and IL-1 (Wei et al., 1990). Local production of certain cytokines: TNFα IL-1β, and IL-6 by skeletal muscle or myotubes has been reported and been related to several pathophysiologic conditions, such as diabetes, polymositis, myopathy, muscle injury and regeneration (Kami and Senba, 1998, Kurek et al., 1996, Saghizadeh et al., 1996, Belec et al., 1997, Bartoccioni et al., 1994). Cytokines typically act in a paracrine/autocrine fashion, therefore local cytokine concentrations may be more important than circulating levels in inducing a specific physiological response. Thus, cytokines play a very crucial role in the functional integrity of skeletal muscle.

The mechanism of action of cytokines appears to be mainly at the level of gene expression (Foex and Shelly, 1996). This may explain that the ubiquitin mRNA (a key

component of proteolysis pathway in skeletal muscle) level increases in soleus muscle treated by TNFα (Llovera et al., 1997). Alternatively, cytokines may function via stimulation of synthesis of other intermediators, such as nitric oxide (NO), which act on skeletal muscle directly (see Figure I-2). This mechanism has been demonstrated in regulation of glucose uptake by cytokines in skeletal muscle cells (Bedard et al., 1997). TNFα+IFNγ stimulated the expression of inducible nitric oxide synthase (iNOS) and NO release in L6 cells, which was concomitant with increased basal glucose uptake and hampered insulin-dependent glucose uptake. Treatment of muscle with nitric oxide (NO) donors can reproduce the effects and inhibitors of NO synthesis can restore insulin sensitivity (Kapur et al., 1997, Bedard et al., 1997). NO works as a direct mediator of cytokines in induction of insulin resistance.

Nitric oxide is a short-lived free radical, synthesized from L-arginine by a family of enzymes known as NO synthases (NOSs) (Moncada et al., 1991) and has been established as a novel mediator of various biological processes. The regulation of signaling occurs largely at the level of NO biosynthesis through regulation the enzymatic activity of NOSs. Individual muscle fibers express one or both of the constitutive NO synthase (NOS) isoforms (neuronal and endothelial NOSs) under normal conditions, which both produce NO at low rates under resting conditions (Frandsen et al., 1996). The third NOS isoform, inducible NOS (iNOS), is not typically expressed in resting muscle cells and is regulated by a complex network of cytokines, endogenous hormones, microbial products, and lipopolysaccharides (see Figure I-2) (Kapur et al., 1997, Williams et al., 1994, Thompson et al., 1996, Okuda et al., 1997, Bedard et al., 1997). iNOS produces NO in greater amounts at higher rates over a prolonged period compared

with the constitutive isoforms (Lincoln et al., 1997). Cytokines can stimulate the synthesis of NO and expression of iNOS in many cell types (Okuda et al. 1997, Williams et al., 1994, Mohaupt et al., 1995, Robbins et al., 1997, Freitag et al., 1996, Pollard et al., 1993) including skeletal muscle cell lines (Okuda et al, 1997, Williams et al., 1994, Bedard et al., 1997). NO has been proposed to be an important mediator of other cytokine functions, such as TNFα induced inhibition of phosphatidylcholine (PC) synthesis by human pneumyocytes (Vara et al., 1996), and IL-1's cytotoxity on ovarian cells (Ahsan et al., 1997). Recent studies indicate that muscle metabolism is sensitive to NO at several sites, including glucose uptake, glucose oxidation, glycolysis, glycogen synthesis, mitochondrial oxygen consumption and creatine kinase activity and amino acid oxidation (Etgen et al., 1997, Ranganathan et al., 1996, Roberts et al., 1997, Young et al., 1997). The production of iNOS and NO were increased in a TNFα associated animal cancer cachexia model (Thompson et al, 1996, Liu et al., 1993, Buck et al., 1996). Muscle wasting was partly prevented by feeding animals L-NMMA, a NOS inhibitor, in this model (Buck et al., 1996). It is very likely that cytokines influence protein metabolism in skeletal muscle via nitric oxide.

Compared to other cell types, it takes longer time for cytokines to activate muscle tissue for stimulation of iNOS expression and NO release, and a mixture of cytokines is necessary for maximal stimulation (Williams et al., 1994). Inducible nitric oxide synthase (iNOS) mRNA expression in myotubes was seen after at least 6 h incubation and was still present after 24 h (Okuda et al., 1997). The protein product was detectable after 12 h stimulation by cytokines (Okuda et al., 1997). However, such induction can happen within 2 h in marcrophages (Williams et al., 1994). If cytokines activate protein

breakdown in skeletal muscle via gene regulation, as reported by Llovera et al. (1997), or synthesis of new mediator, such as NO, these would explain the length of time it takes cytokines to achieve a biological response. Most of the previous muscle incubation experiments were completed in 2-3 hrs, which is a widely accepted time period for muscle incubation and allows the energy status of the muscle to remains stable. Previous studies may have failed to demonstrate a direct action of TNFα and other cytokines on incubated muscle because the incubation time was insufficient to see a significant increase in proteolysis (Ebisui et al., 1995, Llovera et al., 1997). Therefore, a direct mode of action of cytokines in muscle wasting can not yet be ruled out.

Recently, a new cancer cachectic factor from the MAC16 tumor cell (a transplantable colon adenocarcinoma tumor cell line) homogenate has been characterized (Beck and Tisdale, 1987, Todorov et al., 1996, Cstiuk et al., 1997). This new factor decreased protein synthesis by 50% and increased protein degradation by 60% in incubated gastrocnemius muscle (Todorov et al., 1996). The catabolic factor had also been detected in the urine of cancer patients with weight loss (Todorov et al., 1996). But the interaction between the cytokines and this factor is not known.

1.2.2 Pathway for protein degradation in skeletal muscle

Muscle wasting in cachexia is due to increased muscle protein catabolism and/or decreased protein synthesis. The mechanisms of protein synthesis in mammalian cells and bacteria have been extensively investigated and are now well understood. By

contrast, significant progress in understanding the mechanisms and regulation of intracellular protein degradation has been slow, and despite dramatic recent advances, many biochemical features of this process and its importance in human disease remain unclear. A number of observations indicate that the nitrogen loss during sepsis results primarily from accelerated degradation of cell proteins, primarily in skeletal muscle, with normal or even enhanced rates of protein synthesis (Clowes et al., 1983, Baracos et al., 1983, Goldberg et al., 1984, Jepson et al., 1986). Therefore, the focus in this section is on the pathway of protein breakdown.

1.2.2.1 General Review

Muscle, like other tissues, contains multiple intracellular proteolytic systems, including the lysosomal proteases, Ca^{2+}-dependent proteases and the ubiquitin-proteasome proteolytic pathway (Attaix et al., 1994, Attaix et al., 1998). The best-known proteolytic system is the lysosomal pathway. Lysosomes are particularly abundant in the liver, and play a major role in the degradation of hepatic long-lived proteins (Mortimore et al., 1992). By contrast, skeletal muscle contains few lysosomes. Skeletal muscle also contains two Ca^{2+}-activated proteases, calpains I and II (Murachi, 1989). Their *in vivo* function remain unclear in normal cells, but there are a few biological models in which significant changes in calpain activities occur (Ilian and Forsberg, 1992). An activation of Ca^{2+}-dependent proteolysis in tumor-bearing and fasted rats seems very unlikely (Wing and Goldberg, 1993). In addition, lysosomes and Ca^{2+}-activated proteases are not involved in the degradation of myofibrillar proteins (Waterlow et al., 1987, Lowell et al., 1986, Furuno et al., 1990), which represent approximately 60-70% of muscle protein, and

most of the increase in total protein breakdown appears to result from enhanced hydrolysis of myofibrillar proteins (Zamir et al., 1991, Goodman, 1991, Hasselgren et al., 1989). Skeletal muscle also contains a soluble ATP-ubiquitin dependent proteolytic system (Fagan et al., 1987), which is widely believed to catalyze the selective breakdown of short-lived and abnormal proteins (Rechsteiner, 1991). However, recent data indicate that in striking contrast to either the lysosomal or the Ca^{2+}-dependent processes, the activation of the ubiquitin-proteasome pathway is mainly responsible for the muscle wasting that prevails in various animal models of cachexia as well as in some human disease (Attaix et al., 1994, Baracos et al., 1995, Mitch and Goldberg, 1996, Attaix et al., 1998).

1.2.2.2 ATP-dependent Ubiquitin-Proteasome Proteolytic System (Figure I-1):

Degradation of a protein via the ubiquitin pathway usually involves two distinct steps: signaling of the protein by covalent attachment of muscle ubiquitin molecules and degradation of the targeted protein with the release of free and re-utilizable ubiquitin by the 26S proteasome (Hershko and Ciechanover, 1992).

I. Ubiquitin-conjugating system

Conjugation of ubiquitin to protein destined for degradation proceeds, in general, in a three-step mechanism. Initially, the C-terminal Gly of ubiquitin is activated by ATP

to a high-energy thiol ester intermediate in a reaction catalyzed by the ubiquitin-activating enzyme (E1). Following activation, ubiquitin carrier protein or ubiquitin-conjugating enzyme (E2) transfers ubiquitin from E1 to the substrate that is bound to a ubiquitin-protein ligase (E3), which plays a role in the selection of substrates targeted by the ubiquitin-proteasome system. In many cases, however, E2 transfer activated ubiquitin directly to the protein substrate (Rechsteiner, 1991, Ciechanover, 1994, Hass and Siepmann, 1997).

The structure of the ubiquitin-conjugating system appears to be hierarchical. A single E1 carries out, most probably, activation of ubiquitin required for all modifications. Several major species of E2 enzymes (14-, 17-, and 20-KDa) (Jentsch, 1992), and of E3 enzymes have been identified (Ciechanover, 1994). It is unclear which E2, and E3 enzymes are involved in the degradation of the bulk of muscle proteins in muscle wasting. So far, only the expression of the 14KDa E2 was found regulated in muscle wasting (Attaix et al., 1998).

II. Proteasome

The 26S proteasome was first purified from rabbit reticulocyte lysate by Hough et al (1987). It is formed by the association of the 20S proteasome with two 19S regulatory complexes (Baumeister et al., 1998). The 700Kda 20S protease complex is the core catalytic unit of the proteolytic machinery (Driscoll and Goldberg, 1990), which comprises about 1% of the proteins in mammalian cells (Eytan et al., 1993). 20S proteasomes are composed of at least 14 different types of subunit (22-34Kda), which are

encoded by members of the same gene family. The mammalian proteasome has several distinct peptidase activities (i.e. trypsin-like chymotrypsin-like, peptidyllylutamyl-peptide bond hydrolase activities etc.) (Rivett et al., 1995). The 19S "regulatory" complex may confer specificity and control (Rechsteiner et al., 1993) and is composed of at least 15 different subunits (Dubiel et al., 1995). Several components in the regulatory complex are ATPases and are believed to provide energy for the assembly of the 26S proteasome, the unfolding of protein substrates and possibly their injection into the catalytic chamber of the proteasome and the breakdown of ubiquitylated proteins into peptides (Baumeister et al., 1998).

III. Regulation of ATP-dependent ubiquitin-proteasome proteolytic pathway during muscle atrophy.

Incubating the atrophying EDL muscles from tumor-bearing rats in the presence of inhibitors of the lysosomal and the Ca^{2+}-dependent proteolytic pathways did not suppress the enhanced proteolysis. In contrast, concomitant ATP depletion suppressed the increased protein breakdown (Baracos et al., 1995. Temparis et al., 1994). Muscles of Yoshida ascites hepatoma (YAH)-bearing rats showed increased levels of ubiquitin mRNA and mRNA for multiple subunits of the proteasome in Northern hybridization analysis and increased levels of Ub-conjugated proteins and a 27-KDa proteasome subunit in western blot analysis (Baracos et al., 1995). Evidence for increased expression of multiple components of this pathway (ubquitin, 14Kd E2 and proteasome subunits) was also observed in various conditions, including acidotic rats (Mitch et al., 1994),

septic rats (Garcia-Martinez et al., 1995), trauma (Baracos and Attaix, unpublished observations), rats with Yoshida sarcoma (Temparis et al. 1994) and colon-26 adenocarcinoma (Fujita et al., 1996), and even in head trauma patients with muscle wasting (Mansoor et al., 1996). The gene expression of those components has become an indication of the activity of this proteolytic system (Attaix et al., 1998).

Cytokines appear to have an important role in regulation of this proteolytic pathway. An acute intravenous administration of TNFα resulted in an increase in the levels of ubiquitin mRNAs in rat skeletal muscle (Garcia-Martinez et al., 1995). Implantation of the ascite Yoshida AH-130 hepatoma resulted in increases in muscle ubiquitin gene expression associated with activated protein breakdown in skeletal muscle (Llovera et al., 1996). Administration of anti-murine TNFα IgG to tumor-bearing rats abolished the increase in muscle protein degradation and ubiquitin gene expression observed in the control tumor-bearing rats (Llovera et al., 1996). The implantation of the Lewis lung carcinoma to both wild-type and gene-deficient mice for the TNFR I protein resulted in a considerable loss of carcass weight and a significant increase in circulating TNFα in both groups. The increase in protein degradation in the tumor-bearing wild-type mice was accompanied by an enhanced expression of both ubiquitin and proteasome subunit genes. However, tumor-bearing TNFRI-gene-deficient mice did not show any increase in gene expression (Llovera et al., 1998). Anti-IL6 receptor antibody also prevented muscle atrophy in colon-26-adenocarcinoma-bearing mice and the expression of poly-Ub were significantly suppressed compared with those of the tumor-bearing mice (Fujita et al., 1996). Additionally, TNF directly induced the expression of ubiquitin mRNA in rat soleus muscles (Llovera et al., 1997) and IL-6 treatment increased the

expression of proteasome subunits and the activity of 26S proteasome in C2C12 myotubes (Ebisui et al., 1995). It is concluded that the ubiquitin system for protein degradation could have a very important role in the mechanism triggered by cytokines which are responsible for enhanced muscle proteolysis in various pathological states.

1.3 Methods for the studying protein metabolism in muscle

Measurement of muscle protein dynamics (protein synthesis and breakdown) is an important aspect when studying muscle protein metabolism. Muscle protein dynamics regulate both the quantity and quality of muscle protein, and quantification of their activities is of great importance to elucidate mechanisms involved in changes of muscle mass and function.

1.3.1 *In vivo* methods

A variety of *in vivo* methods have been used, and some are subject to a number of potential artifacts. Rates of synthesis of specific proteins or average proteins were estimated from the incorporation of radioactivity in muscle protein, and rates of degradation were estimated from the loss of radioactivity in pre-labeled muscle protein. Urinary urea or total nitrogen excretion are often regarded as an index of muscle protein breakdown, but these measurements actually represent processes of amino acid catabolism and will be influenced by amino acids released from non-muscle tissues and from the diet (Kettelhut et al., 1988, Goldberg and Odessey, 1972). One useful method for estimating rates of degradation of certain muscle proteins *in vivo* is the measurement

of urinary N-methylhistidine (MeHis) excretion (Young and Munro, 1978). This amino acid is a constituent of actin and myosin. It is formed by a post-translational modification of certain histidine residues in these proteins. After release from protein breakdown it is neither reutilized for protein synthesis nor catabolized, therefore its release in urine reflects breakdown of these contractile proteins (Young et al., 1972). However, actin and myosin also exist in other tissues, such as smooth muscle in the gut, and since gut has a high turnover it may also contribute to the excretion of MeHis (Rennie and Millward, 1983). Finally, data on MeHis excretion are interpretable only if renal function is not impaired and if data are related to muscle protein mass (Tawa and Goldberg, 1994).

1.3.2 *In vitro* methods

Measurement of protein synthesis and protein degradation *in vitro* affords the investigator the opportunity to study these processes under a strictly defined set of conditions. The effects of specific inhibitors, hormones or other agents can be tested readily. The major limitation of *in vitro* measurements is that they are relatively unphysiological. The perfused hindlimb preparation and the *in vitro* isolated muscle preparation are two of the most frequently employed methods (Bonen et al., 1994, Tischler et al., 1992). Hindlimb perfusion offers the closest approximation to the *in vivo* situation, which allow delivery of oxygen and substrates via an intact intraorgan circulatory system. However, it is technically more difficult and more expensive to set up and maintain. Different perfusion preparations are not directly comparable, inasmuch as blood flow to hindlimb muscles can differ considerably between experiments. With

incubated muscle systems, diffusion of oxygen, fuels, and metabolites can present a problem. It is best to select a thin muscle, such as the rat epitrochlearis or flexor digitorum brevis, for metabolic studies because such muscles can rely on oxygen diffusion without becoming very hypoxic (Bonen et al., 1994, Gorski et al., 1986, Gulve et al., 1993, Tawa and Goldberg, 1994). Generally, isolated muscle preparations are in a highly catabolic state in which the overall rate of protein degradation exceeds that of protein synthesis. This does not represent the physiological condition. However, isolated muscle preparation still show qualitative responses reflecting *in vivo* perturbations, such as changed protein synthesis and protein degradation in the presence of insulin and other metabolic mediators (Fulks et al., 1975, Baracos et al., 1989). A great deal of valuable data regarding the mechanisms and regulation of these metabolic processes has been obtained from this method. Overall, *in vitro* studies with isolated muscles are technically simple, have low start-up and maintenance costs, and also permit exceptional experimental control.

Rates of protein synthesis are generally determined by measuring rates of incorporation of [14C]-tyrosine or phenylalanine into muscle protein, after correcting for the specific activity of intracellular amino acid pools. Rates of protein degradation are measured by following the net release of tyrosine or phenylalanine from cell proteins (Fulks et al., 1975). Tyrosine is especially useful, because it is easily measured fluorometrically and is neither synthesized nor catabolized by muscle. Its production by isolated muscles reflects net protein breakdown. Total protein degradation can be determined by measuring net tyrosine release in the presence of an inhibitor of protein synthesis, such as cycloheximide or it can be calculated from simultaneous measurements

of net protein balance and rates of protein synthesis. The measurement of tyrosine release reflects the breakdown of all classes of proteins but does not distinguish between the breakdown of myofibrillar proteins and nonmyofibrillar components. To evaluate the breakdown of myofibrillar proteins, which correspond to 60-70% of all proteins in skeletal muscle, the rate of release of N-methylhistidine can be measured *in vitro*. However, the low concentration of MeHis results in a low accuracy using the current techniques. The various approaches described above measure only the breakdown of the bulk of cell proteins that have half-lives of many hours to days. Such techniques treat the muscle protein as a single homogenous entity. While this assumption has allowed valuable physiological insights into the overall regulation of protein turnover, it ignores the wide range of half-lives of individual muscle proteins.

Measurement of changes in the ATP-dependent ubiquitin proteasome pathway provides an alternative method for evaluating proteolysis in catabolic conditions in skeletal muscle (Llovera et al., 1997, Baracos et al., 1995), especially measurement of gene expression of the components of this specific pathway in muscle wasting conditions (reviewed by Attaix et al., 1998).

1.4 Hypothesis and objectives of the research

In summary, cytokines play a very critical role in maintaining homeostasis in skeletal muscle. As proteins, cytokines can not penetrate the plasma membrane and must exert their biological function via binding to specific membrane cytokine receptors. The

previous studies support the direct actions of cytokines on skeletal muscle. Unfortunately, skeletal muscle cytokine receptors have been barely studied to date. TNFα binds specifically to myotubes (Beutler et al., 1985), and mRNAs for TNFRI and TNFRII exist in skeletal muscle (Hofmann et al., 1994). However, no study has been undertaken at the protein level in mature skeletal muscle tissue to prove the presence of specific cytokine receptors. Therefore, characterization of cytokine receptors is the first fundamental step towards understanding their role in regulation of metabolism in skeletal muscle. My first experiment was designed to identify the presence of four cytokine receptors: TNFα receptors, IL-1, IL-6, and IFNγ receptors.

Whether these receptors have a functional role in regulating muscle's metabolism still remains questionable. One way of demonstrating this is by stimulating cells or tissues to see if the cytokine receptors are subject to regulation at the level of gene expression. Modulation of receptor is one mechanism of controlling a molecule's function. Injection of bacteria and endotoxin to animals, can induce the release of many cytokines (IL-1, IL-6, TNFα) (Bagby et al., 1991, Espat et al., 1994, Webel et al., 1997, Estrada et al., 1998). Muscle's functionality and metabolism undergo substantial changes in endotoxemia, such as expression of inducible nitric oxide synthase (iNOS) expression (Okuda et al., 1997, Thompson et al., 1996), insulin resistance (Kupar et al., 1997), and muscle wasting (Goodman, 1991, Tian and Baracos, 1989). It is likely that these cytokines (IL-1, IL-6, TNFα and IFNγ) are responsible for those changes in muscle, at least *in vivo* (Michie et al., 1988). Animals undergoing endotoxemia can thus be used as an animal model to assess the physiological role of cytokine receptor in skeletal muscle. The modulation may be carried on by cytokines themselves since it is a mechanism that

they manipulate their own function: downregulation or upregulation, dependent on the system studied (Aggarwal et al., 1994, Kalthoff et al., 1993, Rubio, 1994, Tsujinoto et al., 1987, Bader and Nettesheim, 1996, Hoffmann et al., 1994, Israel et al., 1986). In muscle, a tissue with apparently low levles of cytokine receptors (from my preliminary result), up-regulation of receptor gene expressio would be anticipated. Therefore, we propose the first hypothesis:

Skeletal muscle expresses the receptors for TNFα, IL-1, IL-6, and IFNγ *in situ*, and that cytokine receptor gene expression is increased in skeletal muscle in endotoxemia and by cytokine stimulation.

It still remains unclear if cytokines can stimulate protein breakdown directly. Previous studies provided conflicting results and indicated the problem associated with *in vitro* studies was that the incubation time might have been insufficient for a full response to cytokine treatment (Ebisui et al., 1995, Llovera et al., 1997) as cytokines may activate protein breakdown in skeletal muscle via upregulation of genes of proteolytic pathway, especially ubiquitin-proteasome pathway (Llovera et al., 1997) or work through activating synthesis of a second mediator, namely nitric oxide. Additionally, compared to other cell types, skeletal muscle is a tissue responding relatively slowly to cytokines (Williams et al., 1994). It may thus take long incubation time to observe the proteolysis-inducing activity of cytokines in skeletal muscle. To observe direct cytokine action on protein catabolism, the challenge is to find a suitable *in vitro* incubation condition with long duration to evaluate cytokine's action on muscle protein metabolism. According to our experience with chicken muscle (Baracos et al., 1989) and previous experiments done

by Wallberg-Henriksson (1987) and Gulve et al (1991), the protein metabolism remain stable up to 9 hrs or longer (< 30 hr) with optimized incubation conditions: appropriate muscle type; continuous oxygenation of the medium; presence of anabolic hormones (e.g. insulin); and nutrients (glucose, amino acids). Under these conditions, it is possible to increase the duration of incubation with cytokines.

As the regulation by cytokines may occur at gene transcription level, especially the genes involved in ubiquitin-proteasome pathway, detection of the mRNA levels of those genes could be used as an alternative method, in addition to measurement of tyrosine release, to monitor the change of protein catabolism in response to cytokine (Llovera et al., 1997, Baracos et al., 1995)

Much remains to be learned, however, about the relationship between NO and protein metabolism, and its underlying mechanisms. Whether it is the direct mediator of these four catabolic cytokines or it works via interfering with other molecule's function in various muscle wasting condition, is not known yet. NO is capable of modulating insulin and IGF-regulated glucose metabolism in skeletal muscle and was proposed to be involved in insulin resistance conditions (Kapur et al., 1997, Young et al., 1997). Another aspect of insulin/IGF-1 function is regulation of protein metabolism (increase protein synthesis/ decrease protein breakdown), and resistance in these also occures in cachectic conditions (Hasselgren et al., 1992, Hasselgren et al., 1987, Hobler et al., 1998). The interference of NO with insulin-regulated protein metabolism on skeletal muscle has not been examined.

Cachexia is often associated with anorexia and decreased food intake (Strelkov et al., 1989, Baracos et al., 1995, Langstein et al., 1991). However, the observed activation of protein degradation is not attributable to the decrease in food intake (Strelkov et al., 1989), it is possible that the function of cytokines might be influenced by the nutritional status of host animals as caloric deprivation has been found to facilitate stimulated TNF release by monocytes *in vitro* (Vaisman et al., 1989). However, fasting alone failed to affect the systemic concentration of cytokine in animals (Smith et al., 1993). It is not clear if fasting could affect the sensitivity of muscle towards cytokine action. No experiment has been performed to study the effect of caloric deprivation on cytokines function on skeletal muscle, which will be elucidated in my study.

Based on the above statements, the second and third hypotheses were proposed:

The second hypothesis: Cytokines may induce proteolysis in skeletal muscle directly via upregulation of genes of ubiquitin-proteasome pathway, and this might be influenced by the fasted state of the animals.

The third hypothesis: Alternatively, cytokines may exert their proteolysis-inducing activity via stimulation of iNOS gene expression and nitric oxide release. The regulation by NO may partly inhibit insulin's action on protein syntheis and degradation.

The specific objectives of my research were:

1. To identify and characterize the complement of cytokine receptors in skeletal muscle by: a) ligand binding assay on purified sarcolemma and b) RT-PCR on total RNA from muscle tissue and purified cell lines;

2. To recognize the potential physiological role of cytokines in skeletal muscle by assessing the expression pattern of four catabolic cytokine (TNFα, IFNγ, IL-6, Il-1β) receptors in muscle in response to stimulation with endotoxin and cytokines. We attemped to determine if cytokine receptor gene expression on skeletal muscle is modulated *in vivo* and whether the modulation involves cytokines themselves.

3. To re-evaluate the direct action of cytokine-induce muscle wasting by using a modified long term *in vitro* muscle incubation model and monitoring the mRNA levels of components in ubiquitin-proteasome proteolytic pathway, in fed and fasted animals.

4. To investigate whether cytokines can stimulate the iNOS expression in differentiated skeletal muscle tissue and whether nitric oxide, a product of cytokine-stimulation, can regulate muscle protein metabolism directly.

Significance of my research

It is increasingly clear that cytokines orchestrate the body's response to neoplastic diseases, injury and probably most other inflammatory illnesses. A large research effort has been spent in the last two decades in an attempt to understand how these protein

mediators function. A more comprehensive understanding of the biological effects of cytokines may lead to advancement in the therapy and treatment of cachexia.

Table I-1: Cytokine family

Cytokines	Molecular weight	Main producer	Receptors
TNFα	17 kD (precursor: 26 kD)	Macrophages	TNFRI (55 kD), TNFRII (75 kD), soluble TNFRI and TNFRII.
IL-1α IL-1β	17 kD (precursor : 30 kD)	Macrophages and monocytes	IL-1RI (80 kD), IL-1RII (decoy receptor, 60 kD), soluble IL-1RI and IL-1RII.
IL-6	24 kD (precursor: 26 kD)	Monocytes, macrophages and endothelial cells	IL-6R (80 kD), gp130 (130kD), soluble IL-6R (50-55 kD)
IFNγ	17 kD	Activated T-lymphocytes	IFNγ R (90 kD)

Table I-2. The role of cytokines in mediating muscle protein breakdown

cytokines	Experimental results
TNFα	Plasma level of TNFα increased in tumor bearing animals showing muscle catabolism (Costelli et al., 1993, Tessitore et al., 1993) Injection of TNFα or TNFα-transformed cells increase muscle proteolysis (Goodman, 1991, Garcia-Martinez et al., 1993) Anti-TNFα treatment partly inhibited protein loss in muscle (Sherry et al., 1989, Gelin et al., 1991a, Costelli et al., 1993). Mice deficient in p55 TNFRI were resistant to tumor induced protein breakdown in skeletal muscle (Llovera et al., 1998). TNFα increased ubiquitin mRNA content of in incubated soleus muscle *in vitro* (Llovera et al., 1997). Pure recombinant TNFα failed to induce protein breakdown *in vitro* (Goodman, 1991, Goldberg et al., 1988)
IL-1	Plasma level increased in tumor bearing animals showing muscle catabolism (Costelli et al., 1993) Increase total and myofibrillar protein breakdown *in vivo* (Zamir et al., 1991) Increase proteolysis in synergy with TNFα *in vivo* (Flores et al., 1989) Partially purified human IL-1 and purified IL-1α and IL-1β increase proteolysis directly in incubated muscle (Baracos et al., 1983, Belizario et al., 1991). IL-1 could not induce protein breakdown *in vitro* (Goldberg et al., 1988) IL-1R antagonist failed to reverse cachexia in Yoshida AH-130 hepatoma-bearing animals (Costelli et al., 1995)

(Continued)

cytokines	Experimental results
IL-6	High concentration of IL-6 in the circulation of mice bearing a sarcoma (Gelin et al., 1988) IL-6 administration activated total and myofibrillar protein *in vivo* (Garcia-Martinez et al., 1994) Anti-IL6 antibody attenuated weight loss and muscle atrophy in tumor-bearing rats (Fujita et al., 1996) IL-6 increased ubiquitin-proteosome pathway activity directly in C2C12 muscle cells (Ebisui et al., 1995) IL-6 failed to induce protein breakdown *in vitro* (Garcia-Martinez et al., 1994)
IFNγ	Administration of murine IFNγ to rats caused a significant weight loss (Langstein et al., 1991). Injection of CHO/IFNγ tumor cells induced cachexia (Matthys et al., 1991) Injection of anti-IFNγ antibody reduced weight loss and increased survival time (Langstein et al., 1991). IFNγ failed to induce protein breakdown *in vitro* (Goldberg et al., 1988).

Table I-3. Biological activities of cytokines on skeletal muscle:

Cytokines	Biological function on skeletal muscle	Reference:
TNFα	Protein metabolism	Frost et al., 1997
	Myogenesis	Miller et al., 1988
	Glucose uptake	Yamasaki et al., 1996, Rangnanthan and Davidson, 1996
	Insulin resistance	Rangnanthan and Davidson, 1996
	Iron homeostasis	Torti et al., 1988, Wei et al., 1990
	NO synthesis, in synergy with IFNγ	Okuda et al., 1997, Bedard et al., 1997
IL-1	Iron homeostasis	Wei et al., 1990
	Muscle damage after exercise	Cannon et al., 1989
	NO synthesis, in synergy with IFNγ	William et al., 1994
IL-6	Muscle damage after eccentric exercise, prolonged running;	Ostrowski et al., 1998, Brunsgard et al., 1997
	Differentiation of myoblasts	Okazaki et al., 1996
	Muscle regeneration	Kami and Senda, 1998, Kurek et al., 1996
IFNγ	Muscle differentiation	Kalovidouris et al., 1993
	Immune interaction with lymphocytes	Michaellis et al., 1993, Kalovidouris et al., 1993
	NO synthesis, in synergy with TNFα, or IL-1	William et al., 1994, Okuda et al., 1997, Bedard et al., 1997
	Distribution of acetylcholine receptors	Kelic et al., 1997

Table I-4. Summary: hormonal regulation of skeletal muscle protein metabolism (adapted from Rooyackers and Nair, 1997 and Tawa and Goldberg, 1994).

Hormone	Protein synthesis	Protein degradation	Protein balance
insulin	↑	↓	↑↑
Growth hormone	↑	↔	↑
IGF-1	↑	↓	↑
Thyroid hormone			
Euthyroid levels	↑↑	↑	↑
Hyperthyroid levels	↑	↑↑	↓
Testosterone	↑	?	↑
glucocorticoids	↓	↑↔	↓
glucagon	↔	↔	↔
epinephrine	↔	↓	↑

Figure I-1. ATP-ubiquitin pathway (Adapted from Tawa and Goldberg et al., 1994)

Three enzymes, E1, E2, E3 are involved in the formation of Ub-protein conjugates. *Ub activation*: A single ATP is necessary for the initial activation of the carboxyl terminus of Ub by Ub-activating enzyme E1. A high energy thiol-ester bond is formed between the C-terminal glycine of Ub and E1. Ub is then transferred to the active site of thiol group by one the several carrier proteins (E2s). *Ub ligation*: The proteolytic substrate is recognized by an Ub-protein ligase E3 which mediates transfer of Ub from E2 to the protein. Multiple Ub moieties may be covalently bound to each other and to one or more lysines on the protein substrate through isopeptide bonds between the carboxyl terminus of Ub and the ε-amino group of lysine. The addition of Ub to preexisting Ub side chains leads to the formation of high molecular weight Ub-protein conjugates. *ATP-dependent proteolysis*: The protein substrate is degraded by the 26S proteasome complex, which is formed from the 20S proteasome and additional regulatory polypeptides in the presence of ATP. This complex produces short peptides and regenerates free Ub by an associated isopeptidase activity. The released Ub can be reutilized in degradation of other proteins.

Figure I-1

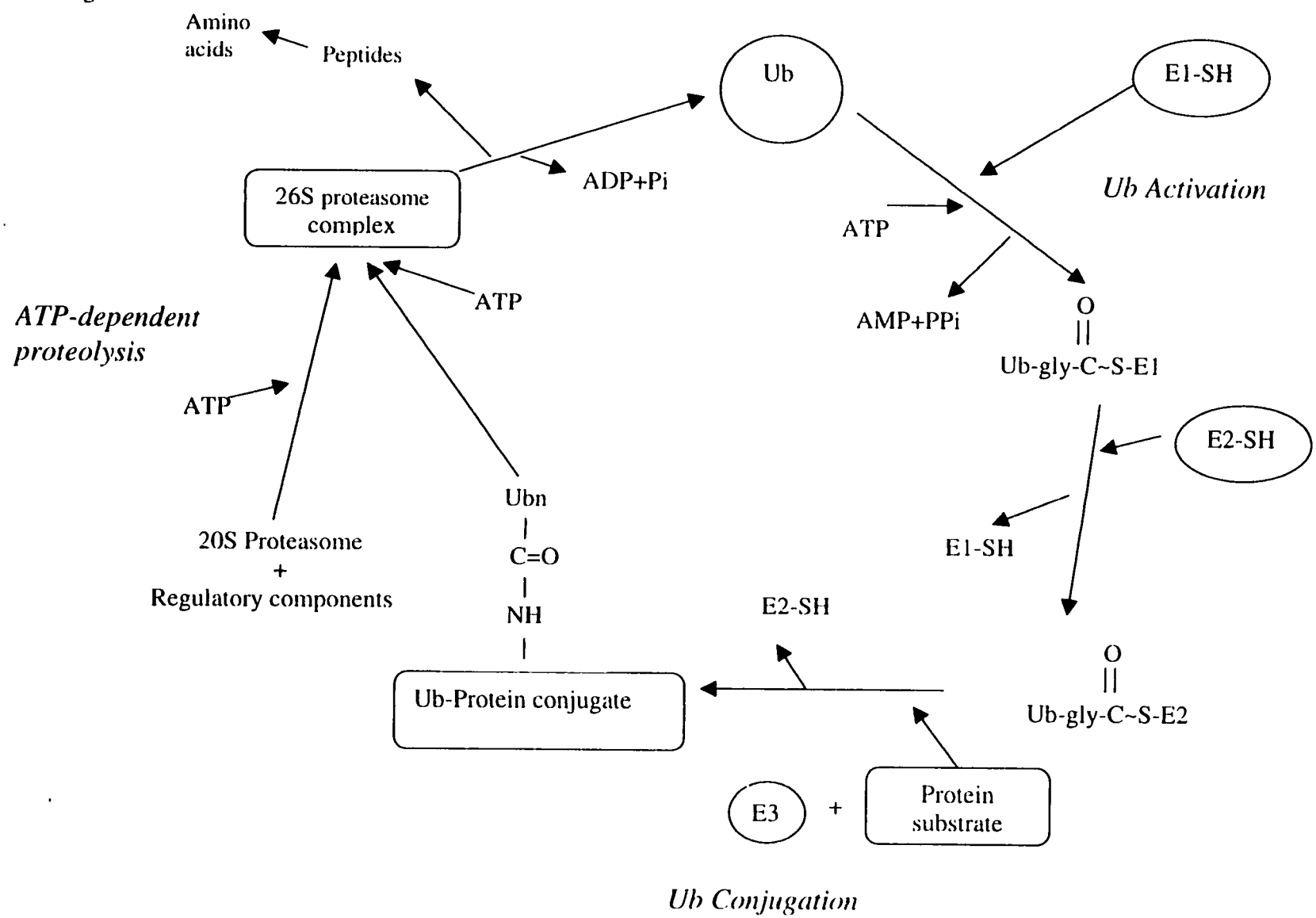
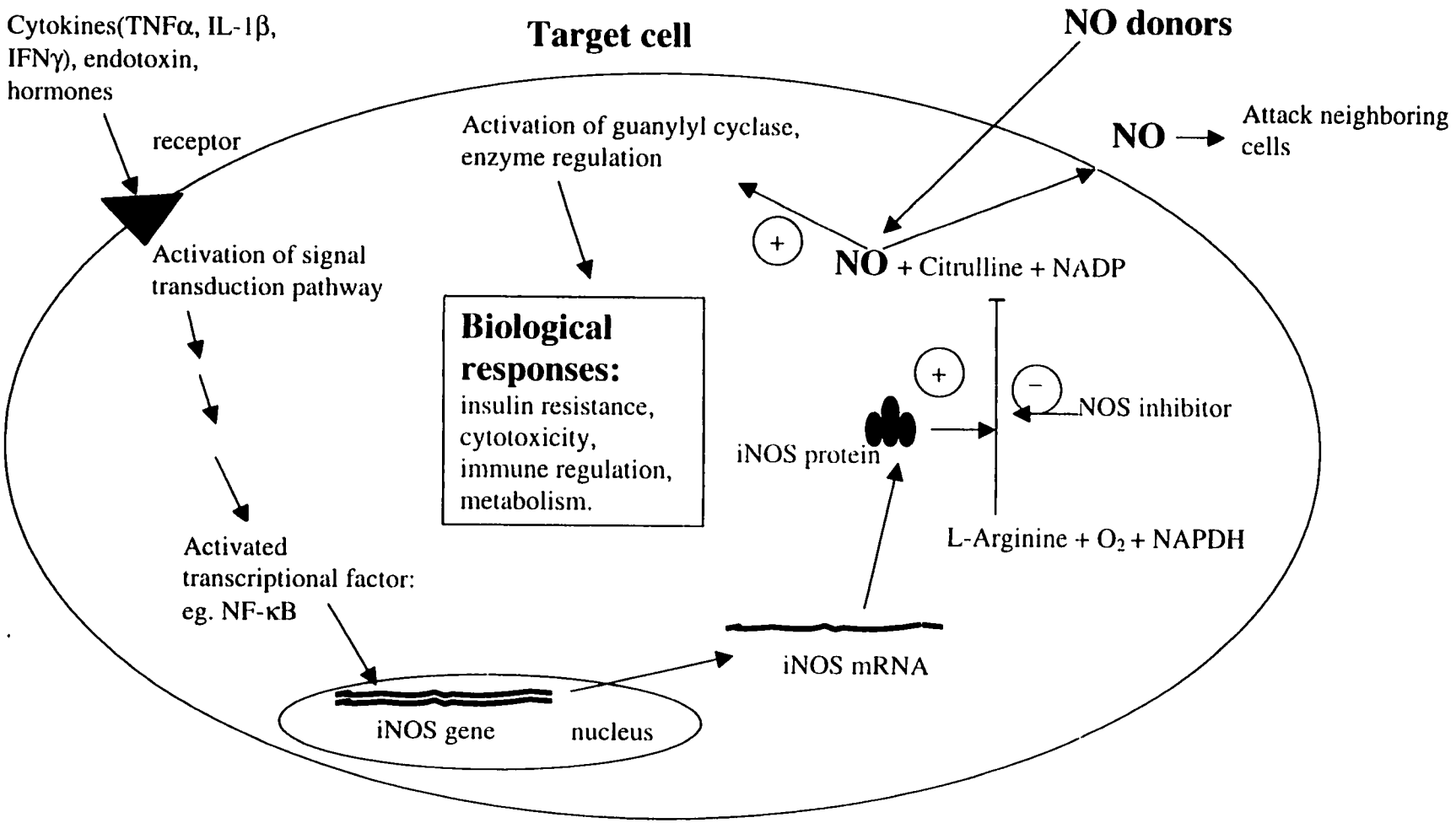

Amino acids
Peptides
Ub
E1-SH
26S proteasome complex
ADP+Pi
Ub Activation
ATP
ATP-dependent proteolysis
ATP
AMP+PPi
O
Ub-gly-C~S-E1
ATP
E2-SH
Ubn
E1-SH
20S Proteasome
+
Regulatory components
C=O
NH
E2-SH
O
Ub-Protein conjugate
Ub-gly-C~S-E2
E3
+
Protein substrate
Ub Conjugation

Figure I-2. Nitric oxide (NO) production and action (Stamler et al., 1992, Xie and Nathan, 1994). Inducible nitric oxide synthase (iNOS), whose mRNA expression can be increased by various stimuli, such as cytokines and endotoxin, converts L-arginine to NO. NO has the capacity to modulate the activity of proteins through reversible reaction with available functional groups, notably cofactor Fe and thiols. The modulated activities of certain enzymes result in the biological effects.

Figure I-2.


Cytokines(TNFα, IL-1β, IFNγ), endotoxin, hormones
receptor
Target cell
NO donors
Activation of guanylyl cyclase, enzyme regulation
NO → Attack neighboring cells
Activation of signal transduction pathway
NO + Citrulline + NADP
Biological responses:
insulin resistance, cytotoxicity, immune regulation, metabolism.
NOS inhibitor
iNOS protein
L-Arginine + O2 + NAPDH
Activated transcriptional factor: eg. NF-κB
iNOS mRNA
iNOS gene
nucleus

Chapter 2 Materials and Methods

Reagents recombinant cytokines: murine IL1β, human IL6, human/rat TNFα, and human/rat IFNγ were obtained from Peprotech (Peprotech Inc. Rocky Hill, NJ). I^{125}-labeled cytokines: murine IL1β, human IL6, human TNFα and IFNγ were purchased from Du-Pont-New England Nuclear (Boston, MA). Endotoxin (LPS: Escherichia coli O55:B5), aprotinin, leupeptin, pepstatin A, phenylmethylsulphonyl fluoride, wheat germ agglutinin, sodium nitroprusside (SNP), PlasmidPure MINIPREP KIT, porcine insulin and BSA (fraction V) were purchased from SIGMA (St. Louis, MO). DAN [2,3-Diaminonaphthalene was from Sigma-Aldrich. Iscove's modified Dulbecco's medium (IMDM), Minimum essential (Eagle) medium (MEM), fetal bovine serum (FBS), penicillin, streptomycin, Taq polymerase, TRIzol reagent, restriction endonucleases and Random-primer-labeling kit were purchased from GIBCO (Grand Island, NY). Expend^{TM}-reverse transcriptase was from Boehringer Mannheim (Laval, Quebec). P^{32}-dATP was a product of Amersham (Oakcille, Ontario). Nitrocellulose membrane was bought from MSI (Westborough, MA). Geneclean II kit was a product of Bio 101 (Vista, CA). P^{32}-dATP and P^{32}-CTP were products of Amersham (Oakcille, Ontario). Nitrocellulose membrane was bought from MSI (Westborough, MA) and Zeta-probe GT Genomic Tested Blotting Membrane from Bio-Rad (Hercules CA). Riboprobe system T7 was from Promega (Madison WI). N^G-Nitro-L-arginine-Methyl-Ester-HCl (L-NAME) was purchased from CALBIOCHEM (La Jolla, CA).

Experimental animals Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Female and male Sprague-Dawley rats from a colony

maintained at the University of Alberta were used. Rats were housed in individual wire mesh cages in a temperature (24°C) and humidity (80%) controlled room. Animals were on a 12h light-12h dark cycle and fed laboratory chow (Continental Grain, Chicago, IL) containing 24% crude protein energy of diet. Muscle was dissected from rats after CO_2 asphyxiation and immediately used for incubation or frozen in liquid nitrogen and stored at -80°C.

Cell culture The murine fibroblast tumor cell line L929 and epithelial tumor cell line NOB-1 were provided by Dr. L. Guilbert (University of Alberta, Edmonton, Alberta, Canada) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). The murine OKT4 cell line (CRL-8002) and human Hela 229 cell line (CCL-2.1) were purchased from American Type Culture Collection (Rockville, Maryland). OKT4 cells and Hela cells were cultured in Iscove's modified Dulbecco's medium plus 20% FBS and Minimum essential medium (Eagle) medium with 10% FBS, respectively. L6 cells were grown and maintained in monolayer culture in α-MEM containing 2 % (v/v) fetal bovine serum in an atmosphere of 5 % CO2 at 37°C. L6 myoblasts were plated in 10 cm dishes at 20,000 cells/ml and were used after complete differentiation to myotubes (7 days post-plating). The L6 cell line was derived from neonatal rat thigh skeletal muscle cells and retains many morphological, biochemical and metabolic characteristics of skeletal muscle (Yaffe, 1968). All culture media contained 1 % (v/v) antibiotic/antimycotic solution (10,000 units/ml penicillin, 10,000 µg/ml streptomycin and 25 µg/ml amphotericin B).

Membrane preparation (Appendix 1) Plasma membranes from skeletal muscle were isolated using a procedure reported by Ohlendieck et al. (1991) as in our previous work with muscle insulin and IGF-1 receptors (Liu et al., 1994). The procedure is based on sub-cellular fractionation by differential centrifugation, density gradient centrifugation, and wheat germ agglutination. The plasma membrane fraction has been extensively characterized by using immunologic markers for sarcolemma, transverse tubules and sarcoplasmic reticulum (Ohlendieck et al., 1991). In brief, the procedure allows isolation of highly purified sarcolemma essentially devoid of sarcoplasmic reticulum and T-tubular markers (Ohlendieck et al., 1991). The membrane after the density-gradient-centrifugation step was suspended in PBS buffer, and stored at -70°C for further analysis.

Plasma membranes from cell lines were isolated using the procedure described by Bird et al. (1988). In brief, about 10^8 cells were centrifuged at 500g for 5 min, the pellet was resuspended in 5 ml of hypotonic buffer, and then disrupted in Dounce glass-glass homogenizer. The homogenate was made up to 35 ml with 0.25M sucrose containing 5 mM Tris-HCl and 1 mM $MgCl_2$, and centrifuged at 150,000g for 30 min. The pellet was resuspended in PBS and stored at -70°C. Membrane protein was determined after solublization with 1 N NaOH by the method of Bradford using BSA (Sigma, fraction V) as a standard (Bradford, 1976). Crude plasma membranes from cells already known to express specific cytokines receptors were used as positive controls.

Radioligand Binding assay (Appendix 2) Direct binding assays were conducted as described by Bird et al. (1988) with small modifications. Fifty µl of porcine or murine membrane suspension (2µg/µl, after density-gradient centrifugation) was combined with

the I^{125}-labeled cytokines: murine IL1β, human IL6, human TNFα and IFNγ in micro-centrifuge tubes at increasing concentrations in triplicate to a final volume of 100 µl. Nonspecific binding is defined as radioactivity bound to membrane fractions in the presence of 200-fold excess of unlabeled cytokines. The mixture was incubated in a shaking water bath for 4 hr at 25°C. The binding reaction was stopped by centrifugation at 12,000g for 5 min after agglutination of the vesicles with 100 µl of 1 mg/ml wheat germ agglutinin. The supernatant was discarded and the pellet was washed twice with chilled 0.25M sucrose. The tips of the tubes containing the pellets were cut off and counted in a Packard Cobra Auto-Gamma counter.

Competitive binding assay is used to calculate the binding parameters. The procedure was the same as the direct binding assay except that the membrane was incubated in the binding buffer with a constant amount of I^{125}-labeled cytokine and increasing concentrations of unlabeled cytokine. The Kd and Bmax value were obtained from binding data with the assistance of GraphPad program.

RT-PCR Total RNA was extracted by guanidinium isothiocyanate/phenol/chloroform method with TRIzol Reagent based on the method developed by Chomczynski and Sacchi (1987). RNA for PCR was harvested from differentiated rat muscle tissue, as well as from the cultured rat muscle cell line L6. Primers were based on published sequences in the literature or designed by ourselves according their mRNA sequences submitted to the National Gene Bank with the assistance of computer programs, Genejokey II from BIOSOFT (Ferguson, MO) and Amplify1.2. The primer sequences are listed in Table II-1 and are specific for the rat genes.

For the qualitative assay, total RNA (1μg) was reverse-transcribed into cDNA in the presence of 50U Expand™ reverse transcriptase (RT), 1 mM dNTP mixtures, RNase inhibitor (1U/μl), 0.5 μM sequence specific anti-sense primer, 10 mM DTT, and 1× Expand™ RT buffer (first strand) in a total volume of 20 μl by the procedure recommended by the manufacturer (Boehringer Mannherim). The PCR reaction system contained 5 μl 10× PCR buffer, an aliquot of RT (5μl-10 μl) product, 1.5μl 50 mM $MgCl_2$, 1 μl 10 mM dNTP mixture, 1.25 units Taq DNA ploymerase, 2 μl each 10 μM sense and antisense primers in a total volume of 50 μl. Amplification was carried out on IL-1RI, II, IL-6R, IFNγ R, TNFRI and GAPDH as follows: 1×(3 min at 94 °C, 1 min at 60 °C, 3 min at 72 °C), 45×(30s at 94 °C, 30s at 55 °C, 45s at 72 °C), 1× (7 min at 72 °C). For IL-1RII and TNFRII, the "Hot-start" protocol provided by GIBCOBRL was employed: 1×(3 min at 94°C) prior to addition of Taq polymerase, followed by addition of enzyme at 80 °C, then 35×(45s at 94 °C, 30s at 60 °C, 1.5 min at 72 °C), 1× (10 min at 72 °C). A portion (20 μl) of RT-PCR product was electrophoresed in 1% agarose gel in TBE buffer. The gel was stained with ethidium bromide and photographed with Gel-Doc 1000 (BIO-RAD, Hercules, CA).

Restriction enzyme digestion of PCR products The PCR products were purified with Geneclean Kit (Bio 101 Inc.) from agrose gels. Restriction enzymes (5-10 units) and buffers adequate to optimize the reactive condition for each restriction enzyme were added to the purified PCR product. The volume was adjusted to 20 μl with DNase-free water. These reaction mixtures were incubated at 37°C for 2hr and electrophoresed in 1% agrose gel in TBE buffer. The gel was stained and photographed as described above.

Semi-quantitative RT-PCR assay To examine the possibility of semiquantitative analysis using RT-PCR, the samples with different amounts of total RNA (0.1 -0.5 μg) were assayed for cytokine receptor mRNA by RT-PCR. The RT-PCR condition is described above except the cycle number was reduced (IL-1RI: 24, IL-1RII: 26, IL-6R: 24, TNFRI and IFNR: 22, TNFRII: 28) so that PCR reaction was conducted in the exponential phase. All the PCR reactions were repeated in triplicate. Glyceraldehydephosphate dehydrogenase (GAPDH) gene was used as an internal control and its level was evaluated in the same RNA samples as described (Bedard et al., 1997). The signal was detected by Southern blotting. After 1% agarose gel electrophoresis, the PCR product was transferred to nitrocellulose membranes and hybridized overnight with a random-primer ^{32}P-labeled specific cDNA probe, which was generated by PCR reaction and purified with Geneclean kit from agarose gel. The hybridized filters were then washed, exposed to Fuji Phosphoimage plates, and analyzed using a Fuji Bioimaging analyzer BAS1800.

Muscle incubations Immediately after dissection, epitrochlearis muscles were incubated at 34°C in 3.0 ml of Krebs-Ringer bicarbonate (KRB) buffer (119 mM NaCl, 4.8mM KCl, 1.25 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.24 mM NaH_2PO_4, 1.0 mM $CaCl_2$), containing 2 mM HEPES/NaOH (pH7.4), supplemented with 8 mM glucose, 0.1% BSA, with or without the presence of cytokines, insulin, amino acids or other reagents as indicated in the text. The medium was continuously bubbled with 5% CO_2/95% O_2. Muscles were routinely pre-incubated at 34°C for 0.5 to 1 hr, then transferred to fresh media and

incubated further. The length of the incubation was altered as indicated in the legends, with transfer of tissues to fresh media. Muscles were either frozen in isopentane cooled in liquid nitrogen for ATP and phosphocreatine analysis or in liquid nitrogen immediately and stored at -80°C for other analysis. For measurement of protein synthesis, muscles were placed in ice-cold 2% perchloric acid (PCA) at the end of the incubation. Incubation media were acidified by the addition of PCA to a final concentration of 2% (w/v) and stored at -20 °C until analyzed. Whenever endotoxin was present in the medium, the 2% (v/v) FBS retenate filtered by Millipore Ultrafree-MC centrifugal M.W. 10,000 filter units (Bedford, MA) was added to the medium in each group because the stimulation by endotoxin may be serum dependent (Ulevitch and Tobias, 1995).

Measurement of muscle protein turnover in vitro Rates of protein synthesis and net protein balance were measured by phenylalanine incorporation and tyrosine release, respectively, as described in detail previously (Baracos et al., 1989). Rates of total protein degradation were determined by difference between protein synthesis and net catabolism. When protein synthesis was measured, [^{3}H]-phe (0.1 μCi/ml) and phe (1 mM) was added to the medium. In the studies reported here, one muscle from each animal was used for the determination of protein synthesis and the same incubation media was used to determine the net tyrosine release.

Assessment of the expression level of ubiquitin and E2-14KD gene by northern blotting Total RNA (7.5 μg) isolated from epitrochlearis muscles was loaded onto 1% agrose-formaldehyde gel containing ethidium bromide and run for ~5hrs at 100v. The RNA was

then checked visually for intactness of the 28S and 18S ribosomal RNA bands and equivalent amounts of RNA loading. RNA was transferred to nitrocellulose membranes (MSI, for cDNA probe) or Zeta-probe membrane (Bio-Rad, for Riboprobe) by capillary action overnight. RNA was fixed by baking in a vacuum oven for 2 hrs at 80 °C.

Probes for ubiquitin (Ribo-probe) and E2-14KD (cDNA probe) were kindly provided by Professor J. Ozga (University of Alberta) and Professor S.S. Wing from University of McGill. The E2 sequence was inserted at the SmaI site in the multicloning region in a pTZ18R (Pharmacia) plasmid backbone and the ubiquitin gene is inserted at the Kpn site in a pGEM-4 plasmid (Promega) respectively. The plasmids were transfected into XLI-Blue MR supercompetent cells purchased from Stratgene. The transformed bacteria were grown in LB broth at 37°C overnight. The plasmids were isolated with PlasmidPure MINIPREP KIT (SIGMA). The cDNA fragment of E2-14KD was isolated by digestion by restriction endonucleases (5 µl plasmid+1 µl BamH1 + 1µl KpnI + 3µl 10×Buffer4 (GIBCOL) +20 µl d.d. H_2O) for 2h at 37°C. The plasmid for ubiquitin was linearized with EcoRI (5 µl plasmid+1 µl EcoRI + 3 µl 10×Buffer3 (GIBCOL) +21 µl d.d. H_2O) for 2h at 37°C. The DNA fragments were purified from agrose gel by the Geneclean Kit (Bio 101 Inc.). The cDNA probe was labeled using the Random-Primer-DNA-labeling kit (GIBCO) and Ribro-probe was labeled using *in vitro* Riboprobe System T7 labeling kit (Promega). The detailed labeling, prehybridization/hybridization and washing procedure for each probe are attached in Appendixes 4-5. After hybridization, the membrane was exposed to Kodak X-film or phosphorimage plate. The level of specific transcripts was evaluated quantitatively using scanning densitometry (Bio-Rad) or Fuji Phosphorimager.

NO_2 Detection by a fluorometric assay Fifty microliter of DAN [2,3-Diaminonaphthalene] (0.018 mg/mL in 0.22 M HCl) was added to 150 µL of test sample. Vortex and incubate the reaction mixture for 10 minutes at 20 °C. The reaction was stopped by adding 50 µL of 0.61 N NaOH. Samples were read with a fluorometer with excitation at 365 nm and emission at 450 nm. Four to five different concentrations (100-5000 nM) of sodium nitrite were used to generate a standard curve.

Other assays Muscle ATP and phosphocreatine contents were determined according to Lowry and Passonneau (1972). Tissue glycogen content was measured according to the method described by Lo et al. (1970). The concentration of phe in the incubation medium and in the intracellular fraction were determined by High Performance Liquid Chromatography (Sedgwick et al., 1991). The analysis of tyrosine was determined by fluorometric assay (Waalkes and Udenfriend, 1957).

Data analysis The results of each treatment are presented as means±SEM. Differences were analyzed statistically by the paired/unpaired Student's t-test, ANOVA or the regression analysis, wherever applicable.

Table II-1. Summary of primer sequence information:

Gene	Primer sequence	Accession #
TNFRI	sense:5'-CCTGATTTCCATCTACCTCTGACTTTGAGC-3' anti-sense:5'-CACTGGAAATGCGTCTCACTCAGGTAGCG-3'	M63122
TNFRII	sense: 5'-GATGAGAAATCCCAGGATGCAGTAGG-3' anti-sense: 5'-GCTACAGACGTTCACGATGCAGG-3'	U55849
IL-1RI	sense: 5'-AGATGGAAGGACCTATGATG-3' anti-sense: 5'-TGCAGCATCTGACGACAGGA-3'	M95578
IL-1RII	sense: 5'-GGCAAGGAATACAACATCAC-3' anti-sense: 5'-TGGTTGTCAGTCGGTAGCTT-3'	Z22812
IL-6R	sense: 5'-TGCCAACCTTGTGGTATCAGCC-3' anti-sense: 5'-TGAAGACACAGAGAAGCAATCC-3'	M58587
IFNγ R	sense: 5'-CCTAAGGTCAATGTGAGTCAGG-3' anti-sense: 5'-GAAGAGAAGAGGAGCAACGAGC-3'	U68272
TNF α	sense: 5'-TGCCTCAGCCTCTTCTCATTCC-3' anti-sense: 5'-TGAGGAGCACATAGTCGG-3'	X66539
IL-1β	sense: 5'-CCTCAATGGACAGAACATAAGC-3' anti-sense: 5'-TCTGAGAGACCTGACTTGGC-3'	M98820
IL-6	sense: 5'-TTCCAGCCAGTTGCCTTCTTGG-3' anti-sense: 5'-CCGAGTAGACCTCATAGTGACC-3'	M26744
GAPDH	sense: 5'-TATGTCGTGGAGTCTACTGGCG-3' anti-sense: 5'-CTCTTGCTCTCAGTATCCTTGC-3'	X02231

Accession #: is the number used to access the corresponding gene sequence stored in GenBank of National Center for Biotechnology Information.

Table II-2. Amino acid profile in incubation media

Amino acid	*conc. (μM)*	Amino acids	*Conc. (μM)*
Asn	*70*	Ile	*100*
Thr	*300*	Leu	*170*
Ser	*280*	Lys	*600*
Asp	*15*	His	*80*
Glu	*200*	Trp	*120*
Pro	*180*	Arg	*80*
Gln	*600*	Val	*200*
Gly	*400*	Tau	*200*
Ala	*350*	Orn	*90*
Cys	*40*	Cit	*80*
Met	*70*	*α–aminobutyrate*	*15*

The concentration indicated here is the final concentration of amino acids in incubation media, which resembles the plasma concentration of amino acids in rats (Watkins and Rannels, 1980), except Phe and Tyr.

Chapter 3 Experiment I: cytokines and endotoxin regulation of cytokine receptors in skeletal muscle

3.1 Introduction to study design

The experiment was designed to test the hypothesis that skeletal muscle may express receptors for TNFα, IL-1, IL-6 and IFNγ *in situ*, and that cytokine receptor gene expression is modulated in skeletal muscle in endotoxemia and by cytokine stimulation.

Traditional radio-labeled ligand binding assay was first employed on purified sarcolemma to detect high affinity binding sites. However, binding assays require large amounts of muscle membrane, the binding assays were first conducted on porcine sarcolemma. Commercially available ^{125}I-labelled cytokines are either human or murine, however there is sufficient homology between gene sequences or amino acid sequences of human, mouse and porcine cytokines/cytokine receptors for binding to take place (Lee et al., 1998, Richards et al., 1991, Dijkmans et al., 1990, Vandenbroeck et al., 1993). However, determination of the protein isoforms of TNF receptor in muscle is complicated by the fact that TNFR II is species-specific and only human I^{125}-TNFα is commercially available. Human TNFα can not bind to murine TNFRII (Lewis et al., 1991). Rats and mice are closely related and it is likely that human TNFα only binds rat TNFRI. This excludes the possibility of ligand blotting after SDS-PAGE as a means of testing for TNF receptor isoforms in mouse or rat muscle. Furthermore, there was no commercially available specific anti-rat-TNFR that can be used for Western-blotting.

Northern-blotting to test the expression of TNFR isoforms is another direct approach available and two TNFα receptor mRNAs have been found in skeletal muscle in a previous report (Hofmann et al., 1994). We used RT-PCR to confirm the isotypes of TNFRs in skeletal muscle.

RT-PCR is adopted as a sensitive method to detect low expression level of mRNAs encoding cytokine receptors. Muscle tissue consists of multiple cell types other than skeletal muscle cells. In order to test whether cytokine receptors are expressed a rat muscle cell line (L6) provided by Dr. A. Marette of Laval University was used to clarify whether muscle cells *per se* express cytokine receptors.

To determine whether cytokines and endotoxin can regulate the receptors on skeletal muscle, L6 mytotubes were incubated with cytokine or cytokines+endotoxin and animals were injected with a sub-lethal dose of endotoxin to induce endotoxemia. TNFα is viewed as an inducer for other cytokines. TNFα usually appeared first in the plasma after endotoxin or bacterial injection, followed by IL-1 and IL-6 (Espat et al., 1994, Shalaby et al., 1989, Starnes et al., 1990, Tracey and Cerami, 1993, Webel et al., 1997). *In vivo* treatment with anti-TNF antagonists also blocked the release of other cytokines (Tracey and Cerami, 1993). Therefore if there are some changes in cytokine receptor expression levels in muscle in endotoxin-injected animals, TNFα might be considered as one of the potential initiating factors *in vivo*. So the regulation of cytokine receptors by TNFα was studied *in vitro* first. It has been established that synergism among cytokines exists for specific biological responses: stimulation of nitric oxide (NO) release by

skeletal muscle requires an interaction between IFNγ and TNFα or IL-1β (Bedard et al., 1997, Williams et al., 1994), and further combination of cytokines with endotoxin enhanced the reaction (Bedard et al., 1997). To investigate the effect of the synergy on modulation of cytokine receptors, L6 cells were treated with TNFα+IFNγ+endotoxin.

Incubation for 24h was sufficient to observe the response of iNOS to cytokines in L6 cells in a previous study (Bedard et al., 1997, Okuda et al., 1997) and was used in our study. Semi-quantitative RT-PCR was applied to those RNA samples. The expression of GAPDH gene was constant in cytokine treatment and was used as an internal control to correct for minor variation in RNA loading (Bedard et al., 1997).

3.2 Experiments

Experimental animals For endotoxin treatment, rats were anesthetized by Somnotol (5.5mg/100g weight) and injected intraperitonially with endotoxin (400 μg /Kg weight) or equal volume of sterile saline. This dose is well below lethal levels for the rat, but could induce certain well-characterized symptoms including anorexia, lethargy, weight loss and muscle wasting (Goodman, 1991). Epitrochlearis muscles were dissected 24 hr after injection under anesthesia. Rats were killed by CO_2 asphyxiation after muscle dissection. Muscle tissue for membrane preparation was also collected from healthy mice and pigs after humane killing.

Cytokine treatment of L6 cells Fully differentiated L6 cells were treated with or without a). murine TNFα (10 ng/ml); b). murine TNFα (10 ng/ml)+ rat IFNγ (100U/ml) + endotoxin (10 μg/ml) for 24 h before the RNA isolation.

Characterization of membrane cytokine receptors in skeletal muscle by radio-ligand binding assay Plasma membranes from skeletal muscle and the control cell lines were used in both direct and competitive binding assay. Four radio-labeled cytokines were used: human I^{125}-TNFα, IL-6, and IFNγ and murine I^{125}-IL-1β. Bmax and Kd value were determined.

Detection of mRNAs of cytokine receptors in skeletal muscle cell by RT-PCR Total RNA was extracted from both differentiated rat muscle tissue and L6 cell line and used for RT-PCR. The presence of all six receptors: IL-1RI, IL-1RII, IL-6R, IFNR and TNFRI, II was tested. The PCR products were confirmed by restriction enzyme digestion assay.

Measuring the mRNA levels of cytokine receptors by semi-quantitative RT-PCR

Total RNA was extracted from skeletal muscles of experimental animals and L6 cells in cytokine treatment experiment. The relative abundance of mRNAs of all six cytokine receptors (IL-1RI, IL-1RII, IL-6R, IFNR and TNFRI, II) was measured by semi-quantitative RT-PCR assay with starting total RNA of 0.25 μg and GAPDH gene used as internal control.

3.3 Results

Presence of cytokine receptor in skeletal muscle Traditional radioligand binding assay was first employed on purified sarcolemma. RT-PCR was also used to detect mRNA encoding cytokine receptors. Since binding assays require large amounts of muscle membrane, the binding assays were first conducted on porcine sarcolemma and later were confirmed on murine sarcolemma as it is well established that human cytokines bind to murine receptors.

Evidence for presence of TNFα receptors in sarcolemma I^{125}-TNFα bound specifically to porcine sarcolemma and membrane from a control TNF responsive cell line L929 (see Figure III-1A). (see Figure 1A). The typical non-specific binding was ~40% of total binding. Since even at the highest concentration of I^{125}-TNFα (900 μM) specific binding was not saturated, competition binding assay was employed to determine the Kd and Bmax value of TNF binding sites on both porcine sarcolemma and L929 cell membranes. Competition studies employing unlabeled highly purified hTNFα is shown in Figure III-1B. hTNFα competed with the binding of I^{125}-TNFα in a dose-dependent manner. Scatchard plots were generated from the competition binding data (see Figure III-1C,D). The Kd and Bmax values, respectively, were: $39\pm4.7\times10^{-9}$ M, $3.5\pm0.23\times10^{-12}$ mol binding sites/mg protein for porcine sarcolemma, $20\pm2.7\times10^{-9}$ M, $3.7\pm0.19\times10^{-12}$ mol binding sites/mg protein for L929 cell membrane. Only human I^{125}-TNFα is commercially available, and human TNFα can not bind to murine or porcine TNFRII

(Lewis et al., 1991). Therefore, determination of the TNF receptor isoforms in other species by ligand blotting after SDS PAGE is precluded. There is also no commercially available specific anti-rat-TNFR for western-blotting. However, two TNFα receptor mRNA encoding the Type I and type II receptors were observed in both skeletal muscle and L6 myotubes (Figure III-3).

Evidence for the presence of IL-1, IL-6, IFNγ receptors There was no specific binding of I^{125}- labeled murine IL-1, human IL-6 and IFN-γ to either porcine (data not shown) or murine sarcolemma (Fig 2). All three I^{125}-labeled cytokines did specifically bind to control cell membrane (NOB-1: IL-1β; OKT4: IL-6; Hela: IFNγ), therefore, the failure of detection of these receptors is not due to technical problems. Direct binding assay was effective in detecting ≥1 fmol binding sites / mg plasma membrane protein, so the cytokine receptors, if present on sarcolemma, may be beyond the limit of sensitivity of ligand binding assay.

The ligand binding assays suggest that skeletal muscle either does not express receptors for IL-1, IL-6, and IFNγ, or to express very low levels of these receptors. To explore the latter possibility, we employed a highly sensitive method: RT-PCR technique to detect cytokine receptor mRNAs and also to determine whether receptor expression on skeletal muscle was subjected to upregulation by cytokines as shown in other cell types. Four transcripts of expected size were identified by RT-PCR: IL-1 receptor type I and II, IL-6 receptor, and IFNγ receptor on mature rat skeletal tissue (Figure III-3A). The PCR products were further confirmed by digestion using appropriate restriction enzymes and digested products of the predicted lengths appeared (Figure III-3A). Cultured cells are free of contaminating fibroblasts and other cell types that may express cytokine receptors.

and this source of RNA was used to determine that cytokine receptors can be expressed by muscle cells *per se*. L6 cells express mRNA encoding both TNF receptor isoforms, both IL-1 receptor isoforms as well as Il-6 and IFNγ receptors (Figure III-3B).

Validity of semi-quantitative RT-PCR To evaluate the ability of this method to measure the relative abundance of receptor mRNA, a group of concentration curves were generated for each of the cytokine receptor genes that we measured (Figure III-4) under the RT-PCR conditions defined in *Materials and Methods*. The linear range of starting RNA content for each cytokine receptor was evaluated. The intensity of the bands was related linearly with the initial RNA concentration in the range of 0.1-0.5 μg. A concentration within the linear range (0.25 μg) was chosen for further analysis. Glyceraldehydephosphate dehydrogenase (GAPDH) gene was used as an internal control and its level was evaluated in the same RNA samples as described (Bedard et al., 1997).

Regulation of muscle cytokine receptors in endotoxemia and the role of TNFα Eight rats were randomly divided into two groups to receive a saline or endotoxin (400 μg/kg body weight) injection. This dose is well below lethal levels for the rat, but could induce certain well-characterized symptoms including anorexia, lethargy, weight loss and muscle wasting (Goodman, 1991). As significant loss of body weight was observed in the endotoxic rats compared to healthy group at 24 h after injection (-15.6 ± 2.5% initial body weight v.s. -4.7 ± 0.5%, n=4, $p< 0.001$). Muscle samples were collected 24 hr after injection, and the levels of six cytokine receptor mRNAs were measured by semi-quantitative RT-PCR. The densitometric quantifications of the specific cytokine receptor

signals obtained, corrected for the constitutively expressed GAPDH gene, are represented in Figure III-5C. There was a significant increase of IL-6R and TNFRII mRNA in the endotoxin – treated group ($P<0.05$), while the expression of other receptor genes was unchanged. After injection of endotoxin or bacteria *in vivo*, TNFα is usually the first cytokine to appear in plasma, followed by IL-1 and IL-6 (Tracey and Cerami, 1993, Fong et al., 1989). TNFα is viewed as an initiator for production of other cytokines. Since TNFRs were the only abundant cytokine receptors found in skeletal muscle, we suspected that the changes observed in the endotoxin – injected animals might have been mediated by TNF. To evaluate the role of TNFα in such regulation, L6 myotubes were treated with TNFα alone for 24 hr. Southern analysis (Figure III-5B, 5D) revealed that TNF-stimulated myotubes showed an identical pattern of cytokine receptor expression as muscles from endotoxin – injected rats: expression of IL-6R and TNFRII, but the other cytokine receptors were not increased.

Regulation of cytokine receptors by cytokines and endotoxin treatment in skeletal muscle cell line It has been established that synergism among cytokines exists for specific biological responses: stimulation of iNOS acitivity by skeletal muscle requires an interaction between IFNγ and TNFα or IL-1β (Bédard et al., 1997, Williams et al., 1994), and combination of cytokines with endotoxin further enhanced the induction (Bédard et al., 1997). To investigate the synergy between cytokines and endotoxin, L6 cells were treated with the combination of TNFα, IFNγ and endotoxin for 24 hrs. All six pro-inflammatory cytokine receptors were examined: IL-1RI, IL-1RII, IL-6R, IFNR and TNFRI, II by semi-quantitative RT-PCR. As determined by quantitation of the signal in a

PhosphorImager, and corrected for the constitutively expressed GAPDH mRNA, southern analysis (Figure III-6) revealed that expression of all six receptors were clearly increased by this treatment, whereas TNF alone had induced expression of only IL-6R and TNFR II (Figure III-5B,D).

3.4 Discussion:

A full consideration of the cytokine axis of skeletal muscle in any physiologic or pathologic state must include local and systemic levels of cytokine concentration, cytokine receptor expression and its modulation. In this chapter I have focused specifically on receptor expression and its modulation.

TNFα was previously reported to bind specifically to myotubes (Beutler, 1985). Our ligand binding studies here show that I^{125}-TNFα bound specifically to porcine skeletal muscle plasma membrane, suggesting that differentiated skeletal muscle as well as transformed muscle cell types in culture express cytokine receptors. mRNAs for two types of TNFα receptors were reported in skeletal muscle (Hofmann et al., 1994). We confirmed the result by RT-PCR in both muscle cell lines and differentiated muscle tissue. The Kd value from our binding assay falls in the range of the Kd values for L929 TNF receptors from 10^{-9} to 10^{-11} M (Tsujimoto et al., 1985). The TNFα binding sites on sarcolemma (3.5×10^{-12}) are about ten – fold less abundant than those of insulin (Bmax=$1\sim5\times10^{-11}$ mol binding sites/mg sarcolemma protein) (Liu et al., 1994). The Bmax values cannot be compared with L929 tumor cell lines since only a crude cell membrane preparation was used, as compared to the highly purified porcine sarcolemma. Our RT-PCR results showed the presence of mRNAs encoding four other pro-inflammatory cytokine receptors (IL-1RI, IL-1RII, IL-6R and IFNγR) in skeletal muscle, however, the levels of these receptors were below the limits of detection of radioligand binding assay.

The relative abundance of TNFα receptors on sarcolemma suggests that TNFα plays a primary role in regulating muscle's biological function. Most or all of the injurious sequelae of septic shock syndrome are attributed to the effects of TNFα and the cytokine cascade triggered by TNFα (Tracey and Cerami, 1993, Fong et al., 1989). TNFα appears early in the plasma after infection (Tracey and Cerami, 1993, Fong et al., 1989) and early blockade of TNFα inhibited the production of IL-1, IL-6 (Fong et al., 1989). At the same time, IFNγ, not TNFα, was essential to formulate a functional cytokine cocktail inducing iNOS from skeletal muscle cells (Williams et al., 1994). IL-6, not TNFα, was found to shorten the half-life of long-lived proteins in C2C12 myotubes (Ebisui, et al., 1995). These results point to a high degree of complexity in cytokine responses by skeletal muscle. Cytokines were capable of activating a signal transduction pathway even at very low receptor level (Dinarello, 1996). IL-1 signal transduction has been observed in cells expressing less than ten type I receptors per cell. The contribution of individual cytokines may not be proportional to the density of their membrane receptors, so the significance of the discrepancy between the receptor density of different cytokines remains unclear.

Our studies showed that mRNA levels for TNFRII (p75) and IL-6R were increased in skeletal muscle samples from endotoxic animals, suggesting a regulatory role of these two cytokines. TNFα is able to regulate its own receptors as well as other cytokine receptors and the regulation pattern is cell type specific (Tsujimoto and Vilcek, 1987, Kalthoff et al., 1993, Rubio, 1994). Our work showed that TNFα alone was capable of upregulating the expression of TNFRII and IL-6R mRNA in L6 cells after 24hr incubation, implying that this cytokine is likely to be the mediator of such

adaptation *in vivo*. No response to TNFα was seen at earlier time points (within 12h) (data not shown). Compared to other cell type, skeletal muscle is a tissue that responds to cytokines slowly (Williams et al., 1994, Bader and Nettesheim, 1996). Inducible nitric oxide synthase (iNOS) mRNA expression in myotubes was seen after at least 6h incubation with cytokines and was still detectable after 24 h (Okuda et al., 1997). The protein product was detectable only after 12h stimulation by cytokines (Okuda et al., 1997). However, such induction can happen within 2hr in macrophages (Williams et al., 1994). Additionally, TNFα down-regulated TNFRI mRNA in 30 min and increased the expression of IFNγ and TNFα mRNA within 4hr in rat tracheal epithelial cells (Bader and Nettesheim, 1996). Therefore, it may take a longer time to observe muscle's response to cytokines, compared with fast-reacting cell types.

The various biologic activities of TNFα are thought to be mediated by two isotypes of receptors. It is not clear why only the expression of TNFR II, not TNFR I, is modified in response to TNFα stimulation and endotoxin challenge. Most of the known TNF responses are mediated via TNFRI (p55) and this notion has been confirmed *in vivo*. Mice deficient for the p55 TNFR were resistant to endotoxic shock (Heller and Kronke, 1994) and tumor - induced protein breakdown in skeletal muscle (Llovera et al., 1998). However, TNFRII is associated with thymocyte proliferation, and cytotoxity may be a function of TNFRII alone or together with TNFRI (Heller and Kronke, 1994). Furthermore, TNF upregulated the expression of TNFRII but not p55 TNFR in human malignant epithelial cells, while both receptors are functionally independent of each other (Kalthoff et al., 1993). Based on the present results, it is hard to draw any conclusion on the participation of individual cytokines in modulating muscle metabolism. The up-regulation of IL-6 receptor is not surprising because TNFα induces the production of IL-

6 (Tracey and Cerami, 1993, Fong et al., 1989). It can be speculated that some of the effects assumed to be caused by TNF are mediated by IL-6.

Additionally, soluble receptors of the TNFR and IL-6R families are known to exist and the circulating levels of soluble receptors were reported to be increased in various pathophysiological conditions (Tracey and Cerami, 1993, Heinrich et al., 1995). Soluble receptors may act as agonists or antagonists of cytokine action (Sehgal, 1996, Tracey and Cerami, 1993). Taking all of these factors into consideration, the significance of the mRNA expression for cytokine receptors described in the present study should be interpreted with caution, since no evidence has been provided for an increase in the corresponding protein levels or any correlation between receptor mRNA level and net biological effects.

TNFRI, IL-1RI, II, and IFNγR did not responded to TNFα within 24h incubation. As skeletal muscle may respond to cytokines slowly, it is possible the induction of those cytokine receptors may happen at a later time. Additionally, it remains unknown how long the induction of TNFRII and IL-6R is sustained and when the peak response occurs. Further experiments on time course may elucidate these points. The concentration of TNFα (10 ng/ml) used in the experiment is common for *in vitro* studies. The level used here is higher than physiological concentrations but relatively low compared to other published studies (Frost et al., 1997). Peak serum tumor necrosis factor (TNF) levels were approximately 2ng/ml in patients injected with endotoxin (Boujoukos et al., 1993), while TNFα exerted its highest inhibitory effect on protein synthesis in human myoblasts at the concentration of 100 ng/ml (Frost et al., 1997). Further dose response experiments may reveal the threshold concentration of TNFα necessary for *in vitro* stimulation of cytokine receptor gene expression in L6 cells and the concentration for maximal

stimulation. Based on the data from dose and time response experiments on cell lines, it is possible to have an optimized experimental condition to observe the maximized upregulation of cytokine receptor mRNA level by cytokines, and thereafter to study the protein level by ligand binding. Finally, in order to fully evaluate the significance of cytokine receptor modulation in skeletal muscle, the upregulation of gene expression of cytokine receptors has to be associated with the modification of specific biological activities.

We noted that the mRNA level of all six cytokine receptors was stimulated by the combination of cytokines + endotoxin. There are extensive interactions between the cytokines and other factors and these may agonize or antagonize each other depending on the system studied. The synergy between cytokines and other factors such as endotoxin may be necessary to see the full scope of a biological response, as in the stimulation of NO release through iNOS induction (Williams et al., 1994, Bédard et al., 1997). The synergy seems to be operative at the level of cytokine receptor expression and may provide a mechanism for sensitization of cytokine responses at the tissue level. The exact role of each cytokine in muscle is intriguing and difficult to elucidate. Further study with gene-knock-out animals may be helpful in revealing the relations between the regulation of muscle biologic activity with specific cytokine(s).

This study is the first to have characterized the cytokine receptors in skeletal muscle cells. Further studies will lead to understanding the role of individual cytokines and their receptors in muscle function and metabolism.

Figure III-1. Binding curve of recombinant human I^{125}-TNFα to sarcolemma and murine L929 cell membranes.

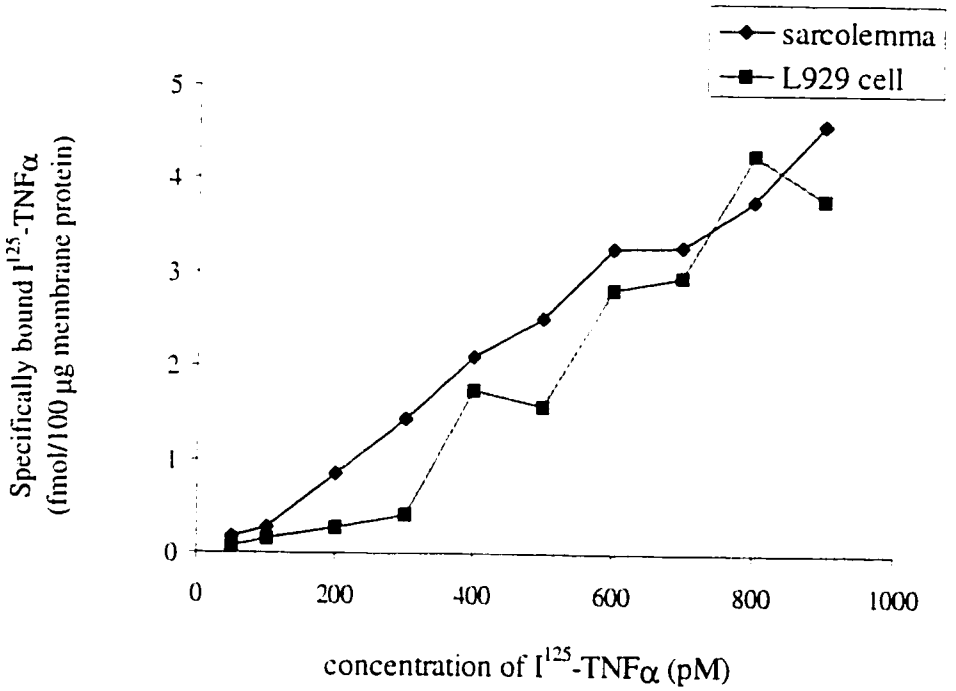


Figure III-1A. Binding curve of I^{125}-TNFα . L929 cell membranes, which were known to express TNFRs and used as positive controls, and porcine skeletal muscle sarcolemma were incubated with the increasing concentration of I^{125}-labeled cytokines at 25 °C for 4 hr. The data represent the amount of specifically bound I^{125}-labeled cytokine.

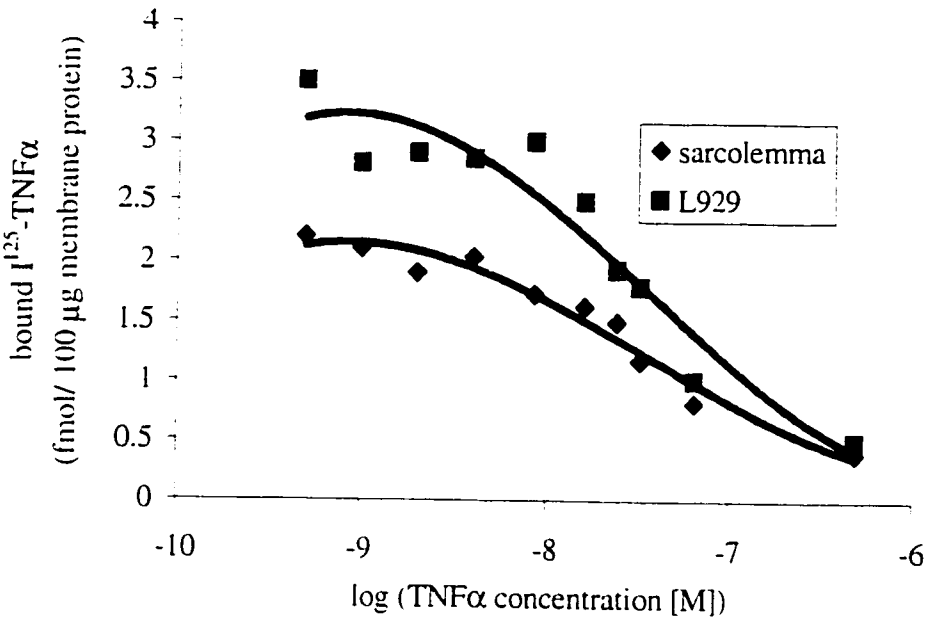
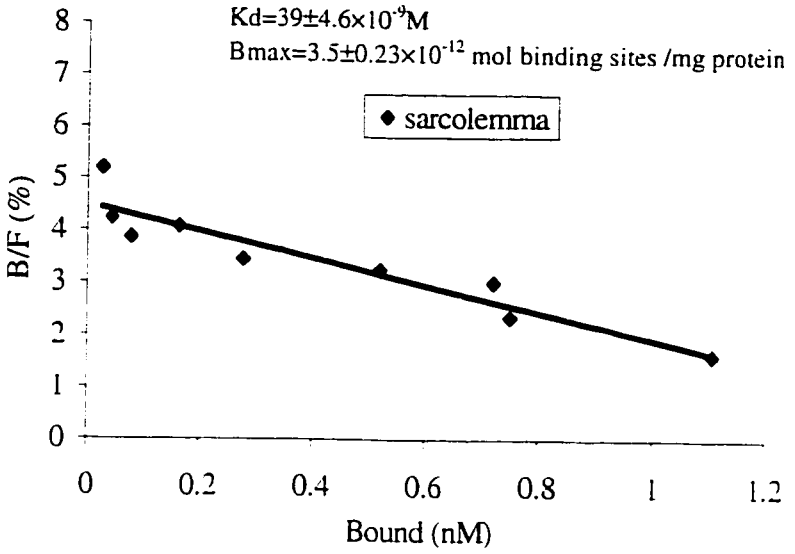
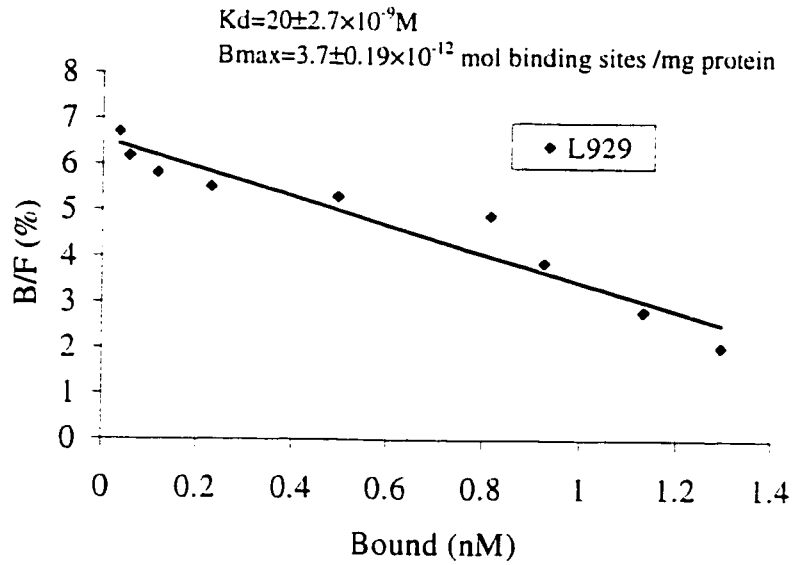


Figure III-1B. Binding sites competition between hI^{125}-TNFα and unlabeled TNFα. 500 pM I^{125}-TNFα was incubated with porcine sarcolemma and L929 cell membrane in the presence of the indicated concentrations of unlabeled TNFα at 25 °C for 4 hr. The data represent the amount of specifically bound I^{125}-labeled cytokine to membranes. The competition binding data obtained were analyzed by GraphPAD program. Scatchard analysis of competition data with unlabeled hTNFα from Figure 1B are shown in C-D.



$h I^{125}$-TNFα to

Figure III-1C. The binding results of sarcolemma in the Scatchard format.



$h^{125}I$-TNFα to

Figure III-1D The binding results of L929 cell membrane in the Scatchard format.

Figure III-2. Binding of I^{125}-labeled cytokines to sarcolemma and control cell membranes. Cell membranes, which were known to express specific cytokine receptors and used as positive controls, and murine skeletal muscle sarcolemma were incubated with the indicated concentration of I^{125}-labeled cytokines at 25 °C for 4 hr. The data represent the amount of specifically bound I^{125}-labeled cytokines.

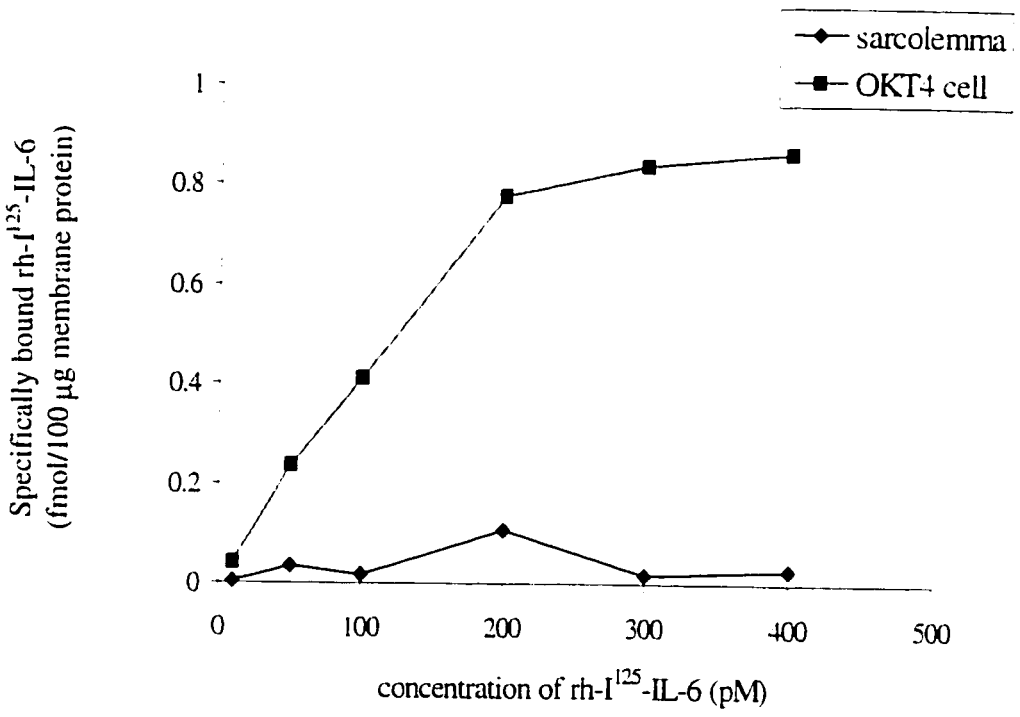


Figure III-2A Recombinant human IL-6 (rh-IL-6) binding curve.

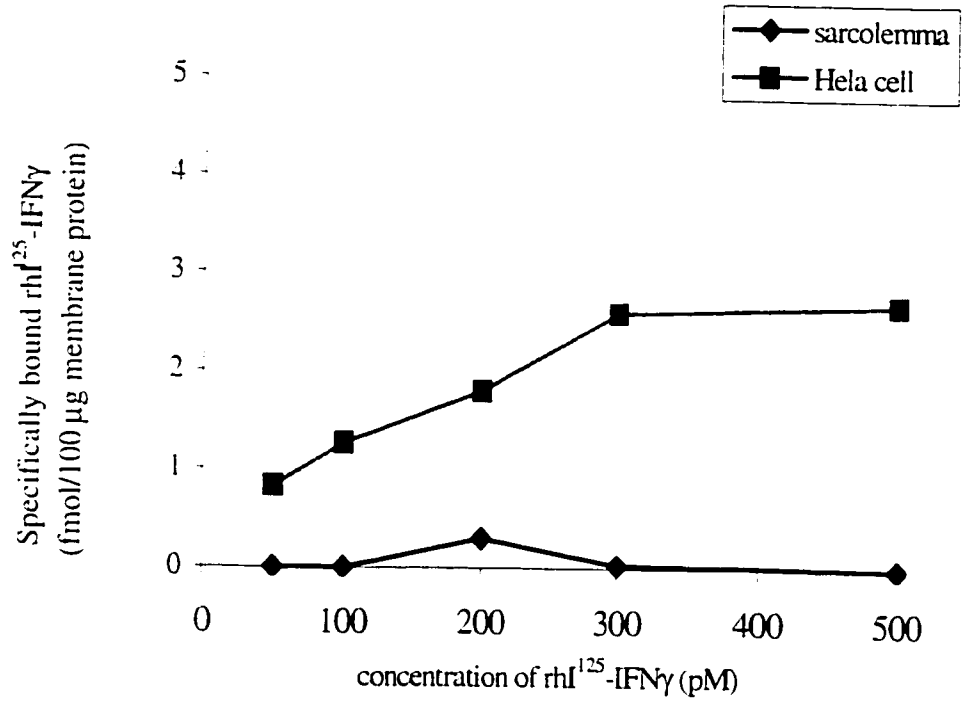


Figure III-2B. Recombinant human IFN-γ (rh-IFNγ) binding curve.

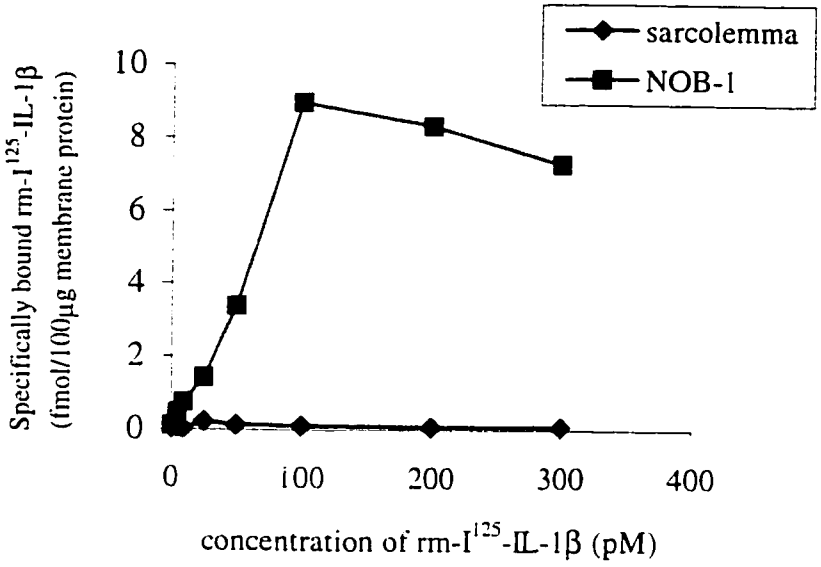
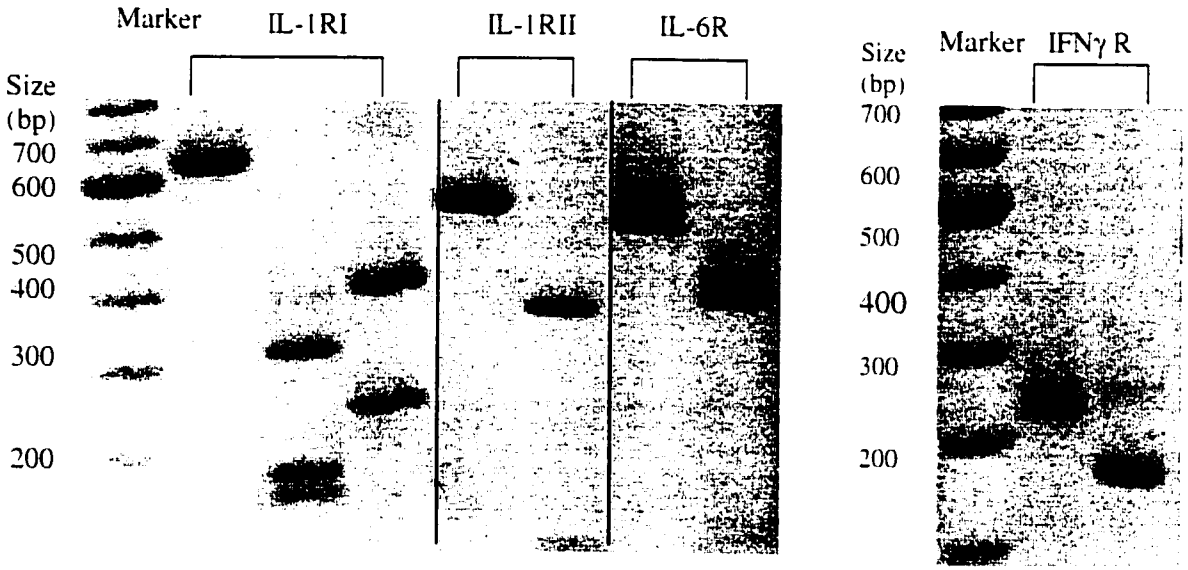


Figure III-2C. Recombinant murine IL-1β (rm-IL-1β) binding curve.

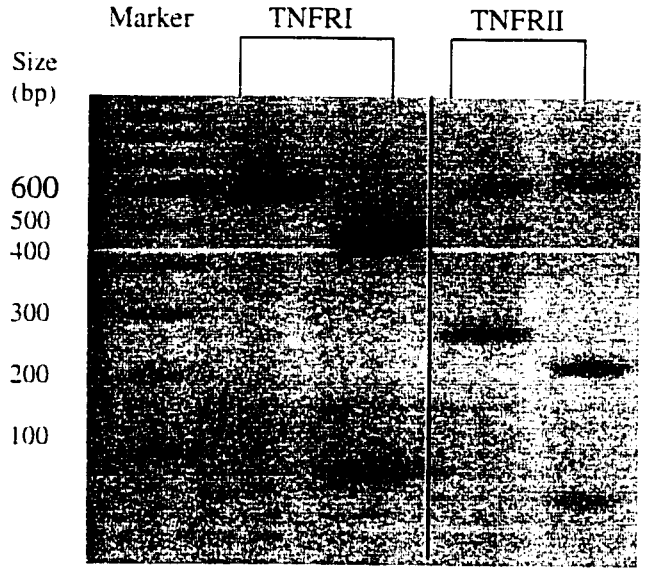
Figure III-3: RT-PCR products of cytokine receptors

Figure III-3A: Identification of PCR products by digestion using adequate restriction enzymes. Restriction enzymes used (nd=not digested) and predicted length of each major digested product are shown under the each lane. Low DNA Mass™ Ladder (GIBCOBRL) was used for a molecular size marker. Six receptor genes were amplified and verified: IL-1RI, IL-1RII, IL-6R, IFNR and TNFI, II.



restriction enzymes	nd	Taq I	Hind III		nd	Bcl I		nd	Taq I
predicted length	641	318	403		529	363		499	391
		171	238						
		152							

nd	Taq I	
323	264	



Restriction enzyme	nd	AluI	nd	NcoI
Predicted length (bp)	594	435 84 75	256	202 54

Figure III-3B: RT-PCR products from L6 cell Lines. The size of the amplified products was as expected for cytokine receptors: IL-1RI, IL-1RII, IL-6R, IFNR.

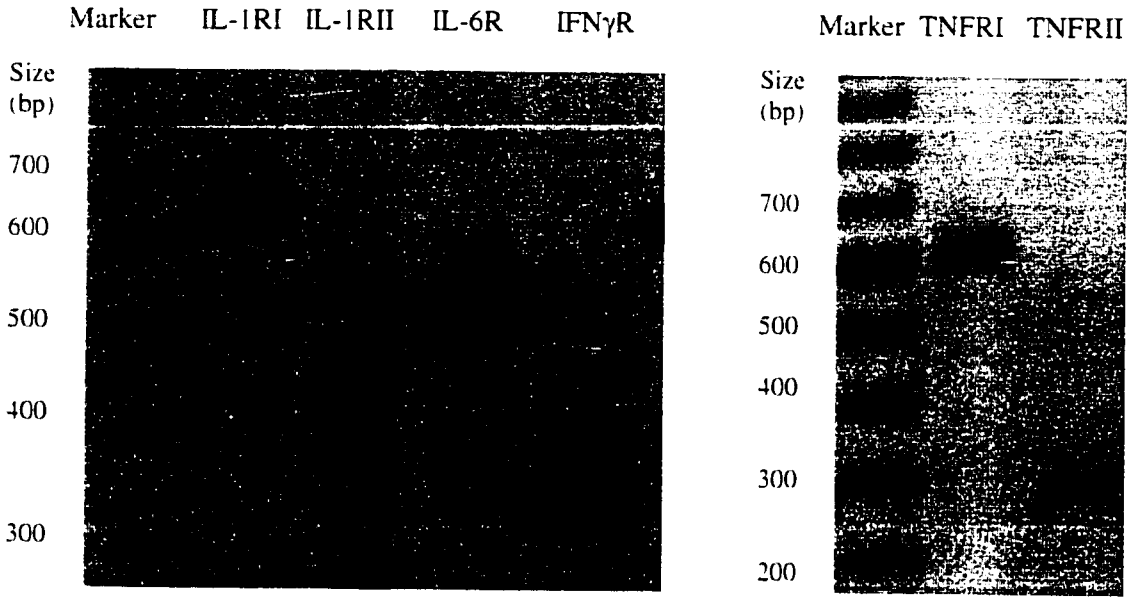
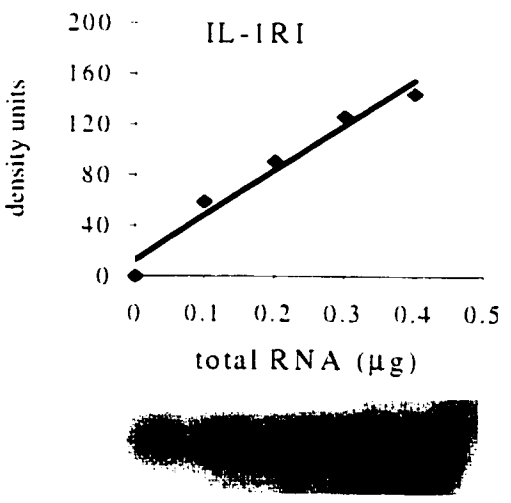
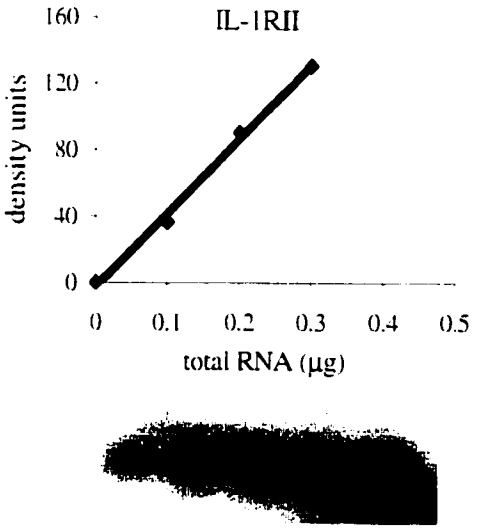
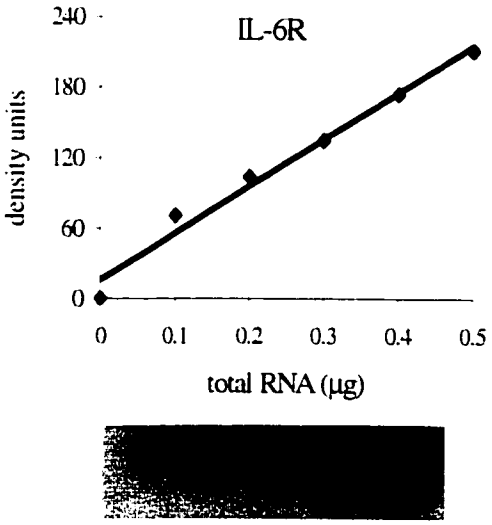
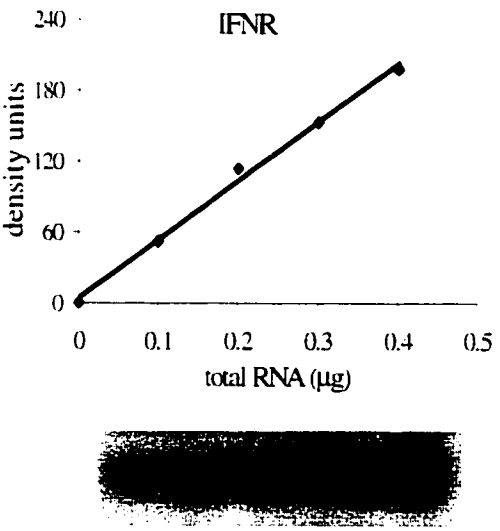


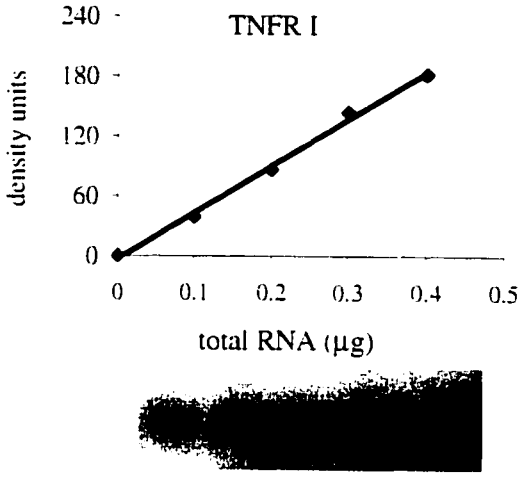
Figure III-4 Quantification of mRNA levels of cytokine receptors by RT-PCR method. Samples of different RNA content (0.1-0.5 µg) were analyzed for each cytokine receptor: IL-1RI, IL-1RII, IL-6R, IFNR, TNFRI, II and GAPDH. Data are expressed as arbitrary units obtained by quantitative densitometry. Only the linear part is shown in the figure.

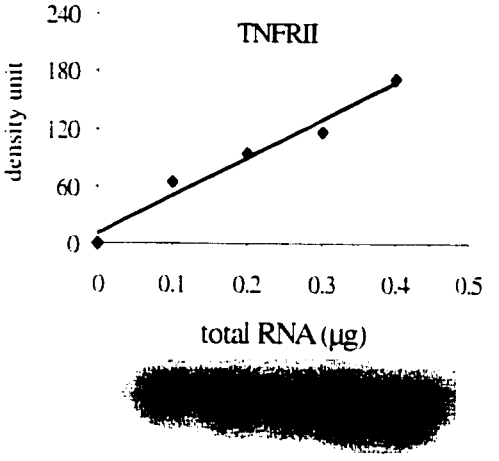









TNFR I
density units
240
180
120
60
0
0
0.1
0.2
0.3
0.4
0.5
total RNA (µg)


TNFRII
density unit
240
180
120
60
0
0
0.1
0.2
0.3
0.4
0.5
total RNA (µg)

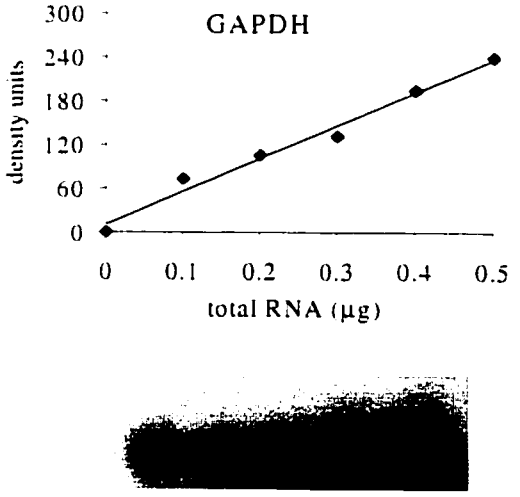

GAPDH
density units
300
240
180
120
60
0
0
0.1
0.2
0.3
0.4
0.5
total RNA (µg)

Figure III-5 (A-D). Effects of endotoxin injection or TNFα alone on the expression level of cytokine receptor mRNA in skeletal muscle tissues and L6 myotubes, respectively. Endotoxin or saline was injected in to rats and epitrochlearis muscle samples were collected 24h after injection. L6 cells were incubated with the TNFα (10 ng/ml) for 24hr and collected for RNA isolation. mRNA levels of six cytokine receptors: IL-1RI, IL-1RII, IL-6R, IFNR and TNFRI, II were measured. All RT-PCR reactions were done in triplicate. A: Southern blot of RT-PCR signal of cytokine receptors from endotoxin treatment; B: Southern blot of RT-PCR signal of cytokine receptors from TNFα treatment (they are representative of three replicates); C: Densitometric analysis of specific RT-PCR signals shown in A. Data are corrected for the GAPDH mRNA signal and expressed as percentage of the controls (n=4/group). D: Densitometric scan result of RT-PCR signals shown in B. Data have been corrected for GAPDH mRNA signal and expressed as percentage of the controls. *$P<0.05$ compared with control.

Figure III-5 A:

Figure III-5B

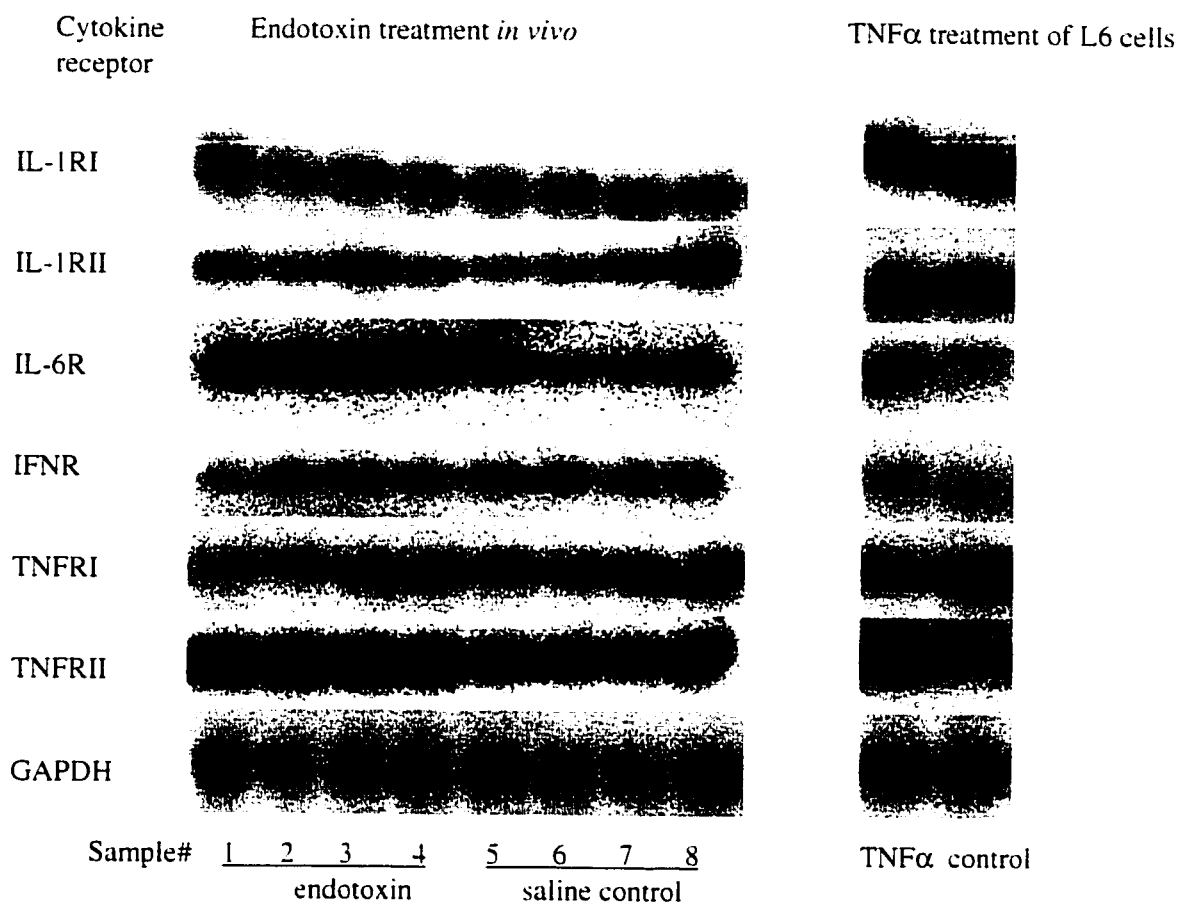


Figure III-5C

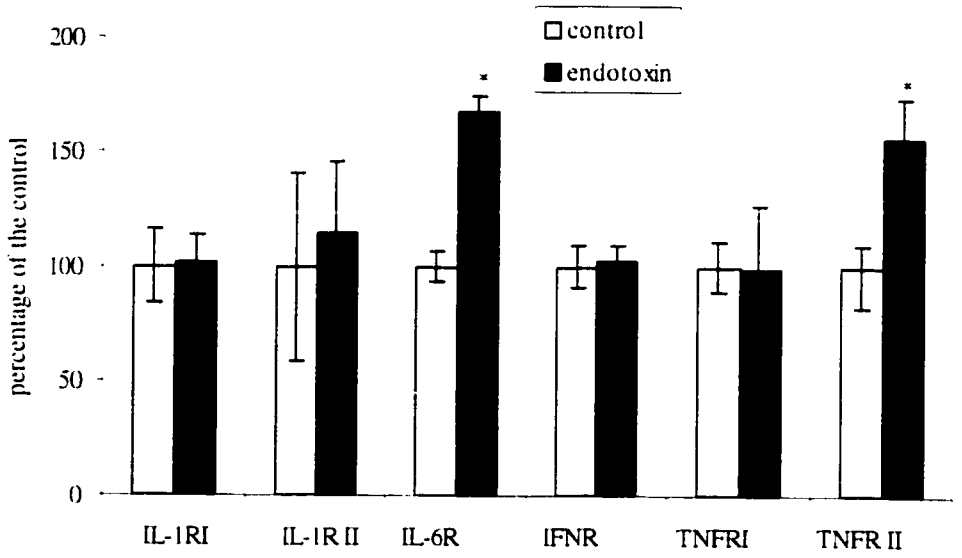


Figure III-5D

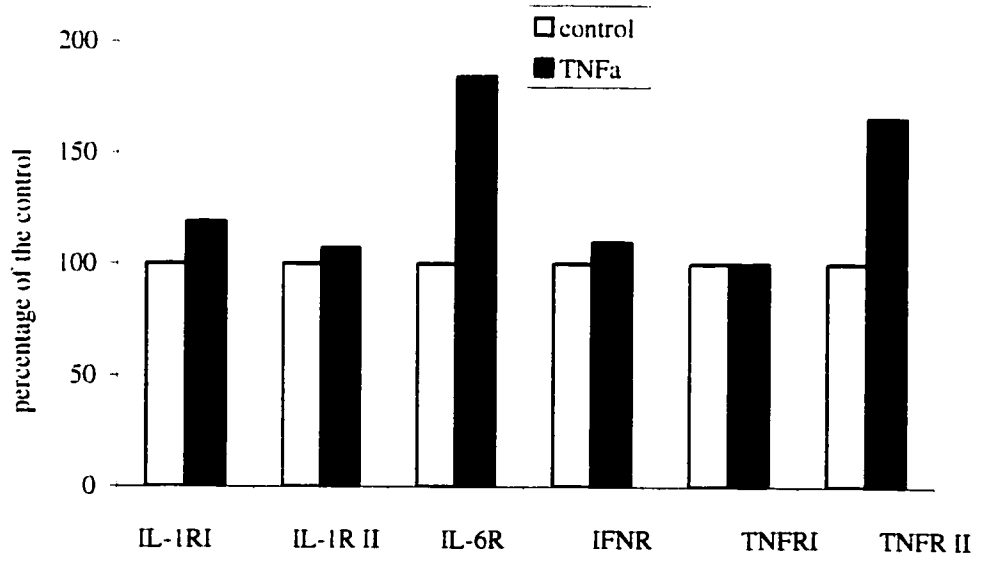
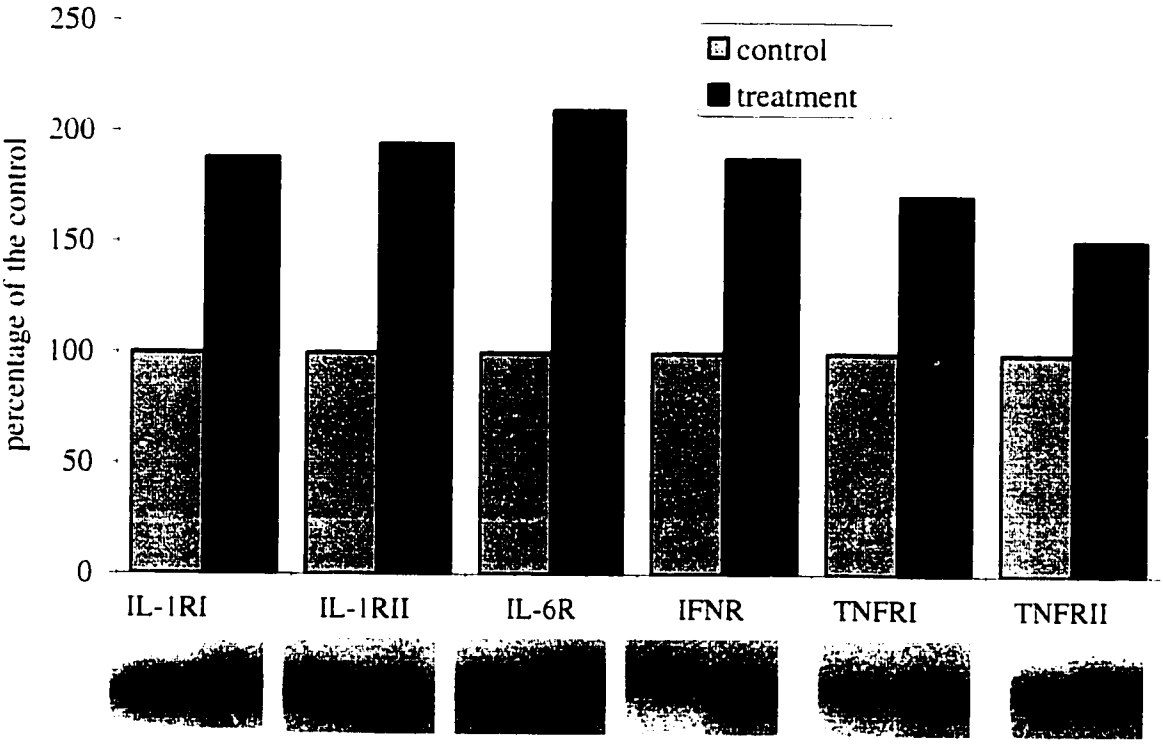


Figure III-6. TNFα+IFNγ+endotoxin induce multiple cytokine receptor mRNAs in L6 myotubes.



L6 myotubes were incubated with the cytokines and endotoxin (TNFα: 10ng/ml, IFNγ: 100U/ml, endotoxin: 10 μg/ml) for 24hr and mRNA level of six cytokine receptors: IL-1RI, IL-1RII, IL-6R, IFNR and TNFRI, II were measured. All RT-PCR reactions were done in triplicate. The illustration at the bottom shows southern blot of RT-PCR signal of cytokine receptors and GAPDH. The pictures are representative of three independent experiments. The figure represents the data from densitometric analysis of specific RT-PCR signals. Data are corrected for the GAPDH mRNA signal and expressed as percentage of the controls.

Chapter 4 Experiment II: The mechanism of cytokine-induced muscle wasting: modified gene expression and role of nitric oxide.

4.1 Introduction to study design

This group of experiments were conducted to test the second and third hypotheses: Cytokines may induce proteolysis in skeletal muscle directly via upregulation of genes of ubiquitin-proteasome pathway, and this might be influenced by the nutritional state of the animals. Alternatively, cytokines may exert their proteolysis-inducing activity via stimulation of iNOS gene expression and nitric oxide release. The regulation by NO may partly inhibit insulin's action on protein syntheis and degradation.

The identification of cytokine receptors and their modulation in response to various stimuli implies a direct action of cytokines on skeletal muscle. However, incubation of muscles with cytokines often gives negative results when protein turnover is determined and whether there is a direct action on protein turnover remains to be clarified. If the mechanism of action of cytokines on protein turnover is at the level of gene expression or synthesis of new mediators, a problem encountered in previous studies was probably insufficient incubation time since it thus takes time to exert biological activities of cytokines. In order to study the mechanism of cytokine induced protein breakdown on skeletal muscle tissue, the incubation time needs to be increased without negatively affecting the viability of the muscle tissue.

Compared with the other muscle types (EDL, soleus), epitrochlearis (EPI) is thin, which may allow easier diffusion of O_2 into the center of muscle and decrease the central hypoxic core usually seen in incubated soleus and EDL muscles (Bonen et al., 1994, Wallberg-Henriksson, 1987). In 120g rats the epitrochlearis muscle is about 0.75mm thick and has about 21-24 fibers at its widest point (Wallberg-Henriksson, 1987). This type of muscle was suitable for long-time incubation and was used in the study.

To assess the viability of skeletal muscle and integrity of membrane cytokine receptors in skeletal muscle during the 12h incubation period, a number of markers of tissue viability and positive controls of receptor function must be measured including: the rate of protein turnover (Baracos et al., 1989, Gulve et al., 1991, Wallberg-Henriksson, 1987), tissue content of ATP/phosphocreatine (PC), glycogen and biological response of skeletal muscle to cytokine treatment. Cytokines are known to induce the expression of iNOS in myotubes and further combination with endotoxin enhanced the reaction (Okuda et al., 1997, Williams et al., 1994, Bedard et al., 1997). This property was verified in differentiated skeletal muscle tissue and was exploited to examine the integrity of cytokine receptors during long term (12hr) incubation. Muscles were treated with TNFα+IFNγ+endotoxin at concentrations known to induce expression of iNOS in L6 myotubes (Bedard et al., 1997)) for 12 hr *in vitro*, and total RNA was extracted and sent to Dr. Marette's lab for detection of iNOS expression.

Three different cytokine treatments were investigated: TNFα (1500 U/ml), IFNγ (1000U/ml), and TNFα (1500 U/ml) + IFNγ (1000U/ml) to compare the individual cytokine treatments and their potential synergism. The combination of these two cytokines was essential to stimulate the expression of iNOS in skeletal muscle cells (Bedard et al., 1997). The concentration of cytokines was within the range that was used

by other investigators. Endotoxin (10 μg/ml) was found to enhance the cytokines action on iNOS expression *in vitro* (Bedard et al., 1997) and was tested in our study as well. To examine the effect of caloric depletion on cytokine function, in one experiment, epitrochlearis muscles were collected from both fed and fasted animals and compared. The rate of proteolysis was measured by the traditional tyrosine release assay and by measuring the mRNA level of key genes of the main proteolytic pathway responsible for muscle wasting: ubiquitin and E2-14D genes, as in previous studies (Baracos et al., 1995, Llovera et al., 1997, Ebisui et al., 1995).

I also tested whether NO is capable of modulating protein turnover in skeletal muscle directly and whether that modulation is insulin-dependent as shown for muscle glucose metabolism. NO is released from resting skeletal muscle preparations *in vitro*, and the endogenous NO was reported to affect basal glucose uptake in skeletal muscle (Balon and Nadler, 1994, 1997). The role of endogenous and exogenous NO may be tested by the use of NO donor compounds and NOS inhibitors.

Sodium nitroprusside (SNP) within the concentration range of 0.1 mM to 25 mM has been used to generate exogenous nitric oxide in various studies (Kapur et al., 1997, Etgen et al., 1997, Young et al., 1997). SNP generates cyanide ions (Smith and Kruszyna, 1976), which has cytotoxic effects on muscle cells. In Kapur's study, SNP (1 mM) was able to stimulate basal glucose uptake, inhibit insulin-dependent glucose uptake and to release a comparable amount of NO as seen in cytokine-stimulated L6 myotubes and skeletal muscle from endotoxic rats. Additionally, SNP failed to affect L6 cell membrane integrity and soleus maximal force production at this concentration. Insulin maximally stimulates protein synthesis at 10 nmol/L (Dardevet et al., 1994) and L-NAME (a NOS

inhibitor) fully inhibited NOS activity in muscle extracts and basal muscle NO production at a concentration of 2 mmol/L (Kapur et al., 1997). Therefore, epitrochlearis muscle was incubated with 1 mM SNP in the presence or absence of insulin (10nM) or L-NAME (2 mM).

The duration of incubation of skeletal muscle with NO donor or inhibitor in my study is short as NO exerts its effects on glucose metabolism in skeletal muscle very quickly (within 2-3hr) (Etgen et al., 1997, Ranganathan et al., 1996, Roberts et al., 1997, Young et al., 1997). Protein synthesis and breakdown were assessed by [^{3}H]-phe incorporation and tyr release, respectively, and two separate experiments were performed to study the regulatory role of NO alone on protein metabolism and its interaction with insulin-dependent changes in protein metabolism. Epitrochlearis muscle received six treatments in each experiment: 1) control, 2) +insulin, 3) +L-NAME, 4) +SNP, 5)+insulin+L-NAME, 6) +insulin+SNP. Since there are only two epitrochlearis muscles per rat, paired comparisons may be made within animals and unpaired comparisons between animals. The treatments are paired differently in the two experiments (see Table IV-3 and IV-4).

4.2 Experiments

Experimental animals Female and male Sprague-Dawley rats were used in the study. Some of the rats were deprived of food overnight before the experiment as indicated in the text.

Evaluating the viability of skeletal muscle during a 12h incubation period Immediately after dissection, epitrochlearis muscles were incubated in 3.0 ml of Krebs-Ringer bicarbonate (KRB) buffer, containing 2 mM HEPES/NaOH (pH7.4), supplemented with 8 mM glucose, 20 nM insulin and amino acids at concentrations similar to those found in plasma (Watkins and Rannels, 1980) (Table II-2). Muscles were routinely pre-incubated at 34°C for 1 hr, then transferred to fresh media every 2h for a total 12h period. The rates of protein synthesis, net tyr release, and the tissue content of ATP/phosphocreatine, glycogen were measured. We did not measure rate of protein degradation directly because the presence of cycloheximide in the medium may harm the muscle tissue over the longer incubation period.

Assessing the integrity of cytokine receptors and stimulation of iNOS expression in skeletal muscle during a 12h incubation period Epitrochlearis muscles were pre-incubated in 3.0 ml incubation medium supplemented with 8 mM glucose, 20 nM insulin, and amino acids at concentrations similar to those found in plasma (Watkins and Rannels, 1980) (Table II-2) for 0.5h. Muscles were then transferred to fresh media with or without rat TNFα (1500 U/ml) + rat IFNγ (1000 U/ml) + endotoxin (10 μg/ml) every 4h for a total 12h period (n=8/per group). The RNA samples were used for detection of iNOS expression.

Measuring the proteolytic activity of cytokines on skeletal muscle Epitrochlearis muscles were dissected from both fed and fasted animals. Muscles from each group were pre-incubated in 3.0 ml incubation medium supplemented with 8 mM glucose, 20 nM

insulin, and amino acids at concentrations similar to those found in plasma (Watkins and Rannels, 1980) (Table II-2) for 1h, and were then transferred to fresh media with or without a) rat TNFα (1500 U/ml), b) rat IFNγ (1000 U/ml), c) rat TNFα (1500 U/ml) + rat IFNγ (1000 U/ml) every 4h for a total 12h period (n=8/per treatment). In another experiment, the direct effect of rat TNFα (1500 U/ml) + rat IFNγ (1000 U/ml) + endotoxin (10 μg/ml) on muscle protein breakdown were tested. The skeletal muscle was transferred to fresh media every 2h for a total 12 h period (n=8/per treatment) in that study. After incubation, the incubation medium was used for tyr assay and muscles were frozen immediately in liquid nitrogen and stored at -80°C for RNA extraction. The expression level of ubiquitin and/or E2-14KD gene were assessed by Northern blotting.

Muscle incubations with NO donor and NO synthesis inhibitor Epitrochlearis muscles from fasted rats were pre-incubated in 3.0 ml of Krebs-Ringer bicarbonate (KRB) buffer, supplemented with 8 mM glucose, with or without the presence of SNP (1mM), or L-NAME (2mM) at 34°C for 0.5 hr. After the initial incubation, muscles were transferred to fresh media containing [^{3}H]-phe (0.1 μCi/ml) and 1 mM phe in the presence or absence of insulin (10nM) and incubated for 2hr. The NO donor and inhibitor were present throughout the 2h incubation if they were present in the previous incubation. Rates of protein synthesis and net protein degradation were measured.

4.3 Results

Evaluation of the validity of isolated muscle incubation experiment The rate of protein turnover was linear during the whole 12h incubation (Figure IV-1). Tissue ATP, phosphocreatine, and glycogen content was determined after preincubation (T0) and at time points indicated in Figure IV 2-3. The concentration of ATP (Figure IV-3) was constant during the whole incubation period. The average ATP content was 7.8±0.9 μmol/g muscle. The level of glycogen and phosphocreatine showed a gradual but significant ($P \leq 0.05$) decrease through the whole incubation. The overall concentrations of ATP, phosphocreatine and glycogen concentrations all fell within the ranges in previous studies (Huijing, 1970, Baracos and Goldberg, 1986).

Cytokines induced the expression level of the iNOS mRNA After 12 hr incubation, iNOS mRNA was detectable in both control and the cytokine treated muscles (Figure IV-4). There was a moderate but significant increase of iNOS mRNA level in cytokine treated muscles ($n=8$, $p<0.05$). The expression of iNOS in the controls was unusual because fresh skeletal muscle does not express iNOS under resting condition (Bedard et al., 1997, Kapur et al., 1997, Thompson et al., 1996, Williams et al., 1994). It was suspected that iNOS expression may be induced by *in vitro* incubation itself. The iNOS mRNA level was then tested on the samples from both incubated muscles (12 h incubation) and freshly dissected muscles (Figure IV-5). iNOS mRNA was not detectable in the freshly dissected muscle samples, confirming our suspicion that *in vitro* incubation induces iNOS expression. There is substantial amount of NO_2 in the incubation medium during the whole 12 h incubation, suggesting that the release of NO by incubated skeletal muscles occurred at very early time points (Table IV-5). Since the time of pre-incubation

is only half of an hour, the rate of NO release at pre-incubation is much higher than that of later time points.

Net protein catabolism in response to cytokine treatment Three different cytokine treatments were used on samples from both fed and fasted animals (Table IV-1). The samples from same time points in the same fed or fasted group were assayed at the same time and compared. There were no significant effects of cytokine treatments on net protein breakdown. Endotoxin was supplemented to the incubation media in addition to TNFα and IFNγ in an experiment. However, this combination had no effect on net catabolism (Table IV-2). The conclusion is that cytokines are unable to increase the rate of tyrosine release during a 12hr incubation period.

The responses of ubiquitin-proteasome pathway to cytokine treatments Equal amounts of RNA (7.5 μg) from tissues of control and cytokine-treated groups were compared. Northern hybridization analysis showed two distinct polyubiquitin mRNA bands (2.6 and 1.2 kb) (Figure IV-6A,B) and two E2-14kD mRNA bands (1.8 and 1.2 kb) (Figure IV-6C). The 2.6 kb ubiquitin transcript and the 1.8 kb E2-14KD transcripts were relatively abundant, which is consistent with previous reports on rat muscle samples (Baracos et al., 1995, Voisin et al., 1996, reviewed by Attaix et al., 1998). The auto-radiographic signals were corrected for 18S rRNA abundance, and there was no significant difference among treatments (Figure IV-6), which is contrast to a previous report (Llovera et al., 1997) that the expression of ubiquitin gene increased in rat soleus muscle incubated with TNFα. This experiment was repeated several times and the same results were obtained.

The role of nitric oxide in protein metabolism in skeletal muscle The total rate of protein degradation was not measured directly because the presence of cycloheximide in the medium inhibits protein synthesis. The experiment was designed to measure the two aspects of protein turnover (protein synthesis and protein degradation) in the same muscle sample. The rate of total tyrosine release was calculated from net catabolism and protein synthesis as described in Table IV-3. SNP did not alter the rates of net or total protein degradation in rat epitrochlearis muscles in either experiments, while it decreased the rate of phe incorporation in the absence of insulin ($P<0.05$) (Table IV-3,4). SNP, in the presence of insulin, inhibited the synthetic activity significantly in one experiment (Table IV-3) and tended to decrease synthetic activity in the second experiment (Table IV-4). L-NAME had no effect on either basal or insulin-stimulated protein synthesis or degradation under any of the studied conditions.

The inter-relationship between insulin and nitric oxide in protein metabolism in skeletal muscle Insulin significantly increased protein synthesis in all groups. Insulin decreased the rate of total protein breakdown ($p<0.001$) but not as large a degree as for protein synthesis. Similar results have been documented by others (Baracos et al., 1989). As a net result, insulin partly restored the nitrogen balance. However, the modulation of protein metabolism by insulin was not modified by either SNP, or L-NAME (Table IV-4).

4.4 Discussion

Our studies indicates that metabolically, epitrochlearis muscle stays relatively viable after a 12hr incubation (the constant ATP content and linear protein turnover rate) (Figure IV 1-2), and the cytokine receptors are functional and intact because iNOS expression was further increased in response to cytokine treatment (Figure IV-4). This stimulation was in consistent with previous reports and suggests a direct action through cytokine receptors (Kapur et al., 1997, Okuda et al., 1997). However, *in vitro* incubation indeed modified muscle subcellularly: muscle glycogen/phosphocreatine content decreased, which may be evidence of hypoxia (Van Breda et al., 1990), and the increase of iNOS mRNA level in incubated muscles indicated activation of certain signal transduction pathways.

The modulation of the proteolytic process by cytokines was assessed by the tyrosine assay and by measurement of transcripts of genes of ubiquitin-proteasome pathway. There was no detectable change in any of these parameters in response to cytokine treatment during the 12h incubation in muscle tissue from either fed or fasted animals, which is contrary to a previous study by Llovera et al. (1997). Ubiquitin mRNA level increased in incubated soleus muscle after 1.5h exposure to TNFα (10,000 U/ml) treatment in their study. The dose, time course and muscle type was different in two studies. However, as there is only one published study in the literature, the real cause of such discrepancy is unclear. In my experiment with L6 cells, TNFα alone failed to induce the expression of TNFRII and IL-6 before 12h, and skeletal muscle was known to respond to cytokines slowly. Longer incubation time may be required for showing cytokine's effect on protein metabolism and gene regulation. However, 12h is the upper

limit of our incubation experiment, it will be difficult to further increase the incubation time without damaging the muscle as hypoxia already developed within 12h. The skeletal muscle cell line can be used to circumvent the problem encountered in tissue incubation since the duration of incubation can be easily increased up to 48h as in many studies (Bartoccioni et al., 1994, Ebisui et al., 1995, Okuda et al., 1997).

iNOS expression was stimulated by the *in vitro* incubation condition, most likely hypoxia, as the expression of iNOS gene was induced in rat cultured glial cells after hypoxia (Kawase et al., 1996). The induction of iNOS mRNA is associated with substantial release of NO into incubation medium by incubated muscles in my study (Table IV-5). It was proposed that many bioactivities of cytokines are carried out by NO (Kapur et al., 1997, Bedard et al., 1997, Vara et al., 1996, Ahsan et al., 1997, Frandson et al., 1996) and NO has a role in muscle wasting, at least *in vivo* (Buck et al., 1996). If the regulation of proteolytic process by cytokines is via synthesis of this mediator, that may account for the failure to detect direct effect of cytokines on protein turnover, since the presence of NO in controls may minimize the difference in between. The question can be elucidated by evaluating the role of NO in skeletal muscle protein metabolism.

SNP inhibited the rate of protein synthesis in skeletal muscle in an insulin-independent manner, but did not modulate protein catabolism in the presence or absence of insulin in skeletal muscle in our study. Endogenous NO has no effects either, as L-NAME did not modulate any aspect of protein metabolism in every aspect we studied. Therefore, the failure to see increased protein breakdown in cytokine treated muscles is not confounded by the fact that NO release is stimulated by *in vitro* incubation.

SNP activates basal glucose transport in skeletal muscle and suppresses insulin dependent glucose transport (Kapur et al., 1997). However, SNP had no effect on insulin-dependent protein synthesis or degradation. The NO concentration released by SNP (1mM) was in the same range as that released by rat muscles in endotoxemia or L6 myotubes in response to cytokine treatments (Kapur et al., 1997), and SNP was found to alter insulin function on glucose metabolism at the same concentration within a shorter time (Kapur et al., 1997, Young et al., 1997). NO may simply act on the part of the insulin pathway that is divergent in regulation of glucose and protein metabolism in skeletal muscle.

The inhibition of SNP on protein synthesis seen in the experiment should be interpreted with caution. SNP might give rise to cyanide ions or other compounds by contact with skeletal muscle (Smith and Kruszyna, 1976) and their activities need to be clarified. Rhodanese and sodium thiosulphate can be added to dissipate the cyanide ions (Smith and Kruszyna, 1976), while hemoglobin can be used as a scavenger of NO because it binds avidly to NO and rapidly nullify the effects of NO (Tsuura et al., 1994). Furthermore, there are other NO donors commercially available. The role of NO needs to be confirmed in carefully designed experiments with the methods described above.

The interactions between the cytokines and other factors may be necessary to see the full scope of a biological response of cytokines (Foex and Shelly, 1996). This notion was confirmed by my results showing that the mRNA level of six cytokine receptors was stimulated by the combination of cytokines+endotoxin while TNFα alone only increased TNFRII and IL-6R mRNA levels in L6 cells. The interactions between TNFα, IFNγ and endotox in were tested and all failed to induce any detectable changes in protein turnover. The number of possible combinations between the four cytokines and other invasive

stimuli, such as endotoxin, PGE_2, and catabolic hormones is enormous and can not be all tested, however the weight of evidence presented here is in favor of the absence of a response of protein turnover to cytokines.

Overall, a growing list of cytokines has been demonstrated to modify metabolism of protein in a way that is consistent with their participation in wasting associated with disease states. Data suggesting that cytokines are indeed causal have been obtained in studies employing neutralizing antibodies, specific inhibitors or gene-knock out mice. The mechanism of cytokine induced muscle wasting still remains elusive. The direct role of cytokines on skeletal muscle's protein metabolism can not yet be definitively excluded yet due to the current technical limitation. However, the weight of evidence produced here do not favor a direct action of cytokines or one of their intracellular mediators (NO) on muscle protein metabolism.

Table IV-1 Rates of protein turnover in cytokine treatments at different incubation time points in fed and fasted animals.

Cytokine treatments	Net protein degradation (pmol tyr/mg muscle/2hr)					
	Fed animals			Fasted animals		
Time points	0-4 h	4-8 h	8-12 h	0-4 h	4-8 h	8-12 h
Control	118±10[a]	169±13[a]	142±7[a]	221±16[a]	219±12[a]	186±8[a]
rat-TNFα(1500U/ml)	120±15[a]	168±18[a]	154±13[a]	204±14[a]	210±12[a]	170±6[a]
rat-IFNγ (1000 U/ml)	142±15[a]	187±16[a]	160±14[a]	207±12[a]	207±9[a]	183±5[a]
rat-TNFα(1500 /ml)+ rat-IFNγ(1000 U/ml)	149±17[a]	196±17[a]	161±14[a]	231±14[a]	213±11[a]	168±6[a]

Values are means±SE. Numbers with different superscripts in the same column are different compared with control group, $P<0.05$, n=8. Epitrochlearis muscles were preincubated for 60 min in incubation media defined in the *Experiments*, and were then transferred to fresh media of the same composition with or without cytokines. The media were collected and replaced with fresh media every 4hr. The rate of net protein degradation was determined by the rates of tyrosine release.

Table IV-2 Effect of cytokines+endotoxin on protein turnover in skeletal muscle.

Treatments	Net protein degradation (pmol tyr/mg muscle/2hr)					
Time points	0-2 h	2-4 h	4-6 h	6-8 h	8-10 h	10-12 h
Control	45±2[a]	48±6[a]	37±4[a]	51±6[a]	52±8[a]	65±9[a]
rat-TNFα(1500 U/ml)+ rat-IFNγ (1000U/ml) +endotoxin(10 μg/ml)	52±5[a]	48±3[a]	39±5[a]	55±7[a]	60±6[a]	59±9[a]

Values are means±SE. Numbers with different superscripts in the same column are different compared with control group, $P<0.05$, n=8. Epitrochlearis muscles from fed animals were preincubated for 30 min in incubation media defined in the *Materials and Method*, and were then transferred to fresh media of the same composition with or without cytokines plus endotoxin. The media were collected and replaced with fresh media every 2hr. The rate of net protein degradation was determined by the rates of tyrosine release.

Table IV-3 Effect of a nitric oxide donor (SNP) and a NOS inhibitor (L-NAME) on protein turnover in skeletal muscle.

Treatment	Protein synthesis (pmol phe/mg muscle/2hr)	
	control	+insulin
Control	90±5 [b]	133±7 [b] *
+L-NAME	87±6 [b]	129±11 [b] *
+SNP	73±5 [a]	114±6 [a] *

Treatment	Net protein degradation (pmol tyr/mg muscle/2hr)	
	control	+insulin
Control	273±19 [a]	198±13 [a]*
+L-NAME	285±14 [a]	185±14 [a]*
+SNP	267±9 [a]	207±11 [a]*

Treatment	Total protein degradation (pmol tyr/mg muscle/2hr)	
	control	+insulin
Control	339±21 [a]	298±11 [a*]
+L-NAME	349±15 [a]	282±13 [a]*
+SNP	320±8 [a]	292±8 [a]*

Values are means±SE. Numbers with different superscripts in the same column are significantly different compared with control group. *Significant effect of insulin, $P<0.05$, n=7. There were six treatments in the experiment: G1: control, G2: +insulin, G3: +L-NAME, G4: +SNP, G5: +L-NAME+insulin, G6: +SNP+insulin. Since there are only two epitrochlearis muscles per rat, paired comparisons may be made within animals and unpaired comparisons between other group. G1 was paired with G2, G3 paired with G4, G5 paired with G6. The rates of protein synthesis and degradation were determined by the rates of phe incorporation and tyrosine release respectively. Rate of total tyrosine release was obtained by adding the measured rates of protein synthesis and of net protein degradation as described above.

Table IV-4. The interrelationship between insulin and nitric oxide on protein turnover in skeletal muscle.

Treatment	Protein synthesis (pmol phe / mg muscle/2hr)		
	control	+insulin	Difference
Control	130±12 [b]	199±14 [a]*	53±13 [a]
+L-NAME	128±8 [b]	185±11 [a]*	57±6 [a]
+SNP	103±5 [a]	168±8 [a]*	65±6 [a]

Treatment	Net protein degradation (pmol tyrosine / mg muscle/2hr)		
	control	+insulin	Difference
Control	407±26 [a]	325±37 [a]*	82±32 [a]
+L-NAME	425±27 [a]	311±18 [a]*	114±24 [a]
+SNP	405±14 [a]	303±13 [a]*	101±17 [a]

Treatment	Total protein degradation (pmol tyrosine / mg muscle/2hr)		
	control	+insulin	Difference
Control	507±30 [a]	466±34 [a]	42±23 [a]
+L-NAME	524±29 [a]	454±21 [a]*	70±22 [a]
+SNP	484±16 [a]	434±11 [a]*	51±15 [a]

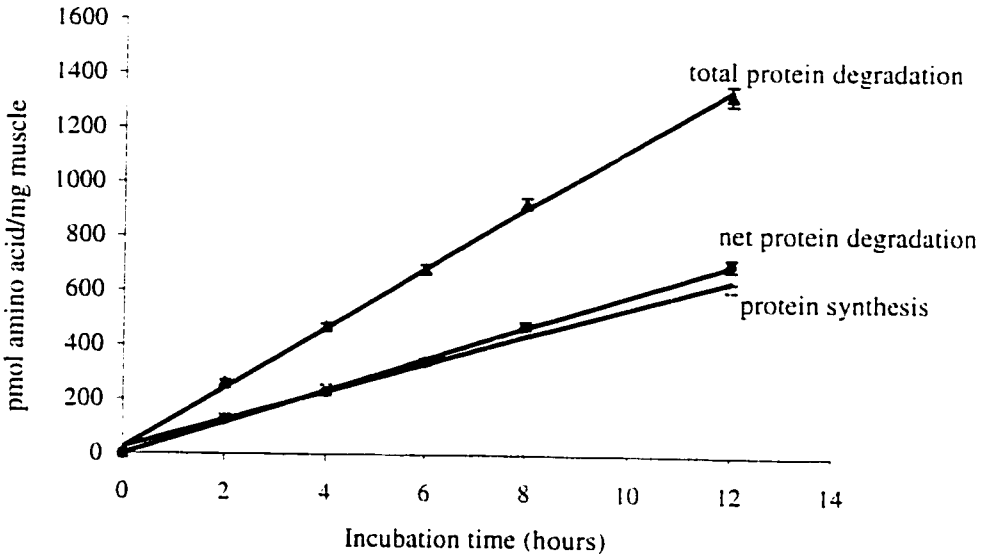
Values are means±SE. Numbers with different superscripts in the same column are different compared with control group. * Significant effect of insulin, $P<0.05$, n=8. There were six treatments in the experiment: G1: control, G2: +insulin, G3: +L-NAME, G4: +L-NAME+insulin, G5: +SNP, G6: +SNP+insulin. In this experiment the stimulation by insulin was studied within animals, so the comparison between control and insulin-stimulated muscles is a paired comparison. The rates of protein synthesis and degradation were determined by the rates of phe incorporation and tyrosine release respectively. Rates of total tyrosine release were obtained by adding the measured rates of protein synthesis and of net protein degradation as described above.

Table IV-5. NO_2 concentration in the incubation medium during different incubation time points (n=8).

Time points	Pre incubation	0-2 h	2-4 h	4-6 h	6-8 h	8-10 h	10-12 h
NO_2 (μM)	6.30±0.32	5.26±0.38	5.11±0.13	4.38±0.18	4.75±0.21	5.07±0.44	4.60±0.29

Values are means±SE. Epitrochlearis muscles were preincubated for 30 min in incubation media and were then transferred to fresh media of the same composition. The media were collected and replaced with fresh media every 2hr. NO_2 was measured by the flurometric method as described in Chapter 2. The blank medium (without muscle) was used to make standards for the assay.

Figure IV-1 Linearity of protein turnover rate during 12h incubation.



Each point represents the mean value of the same time group (n=8). Rates of total and net protein degradation are expressed as pmol tyr/mg muscle. Protein synthesis rate is expressed as pmol phe/mg muscle. Epitrochlearis muscles were preincubated for 60 min in incubation media defined in the *Experiments*, and were then transferred to fresh media of the same composition. The media were collected and replaced with fresh media every 2hr. Measures of synthesis/degradation were made for each muscle during the final 2hr of incubation. The rates of protein synthesis and net degradation were determined directly by the rates of phe incorporation and tyrosine release respectively. Rate of total tyrosine release was obtained by adding the measured rates of protein synthesis and of net protein degradation. Rates of phe incorporation were multiplied by a value of 0.77, i.e., the molar ratio of tyrosine to phe in rat muscle proteins, to obtain tyrosine equivalents (Tischler et al., 1982).

Figure IV-2. Epitrochlearis glycogen content during 12 hr of incubation. Epitrochlearis muscles were preincubated for 60 min in incubation media defined in the *Materials and Method*, and were then transferred to fresh media of the same composition. The media were collected and replaced with fresh media every 2hr. Each point represents an individual muscle sample

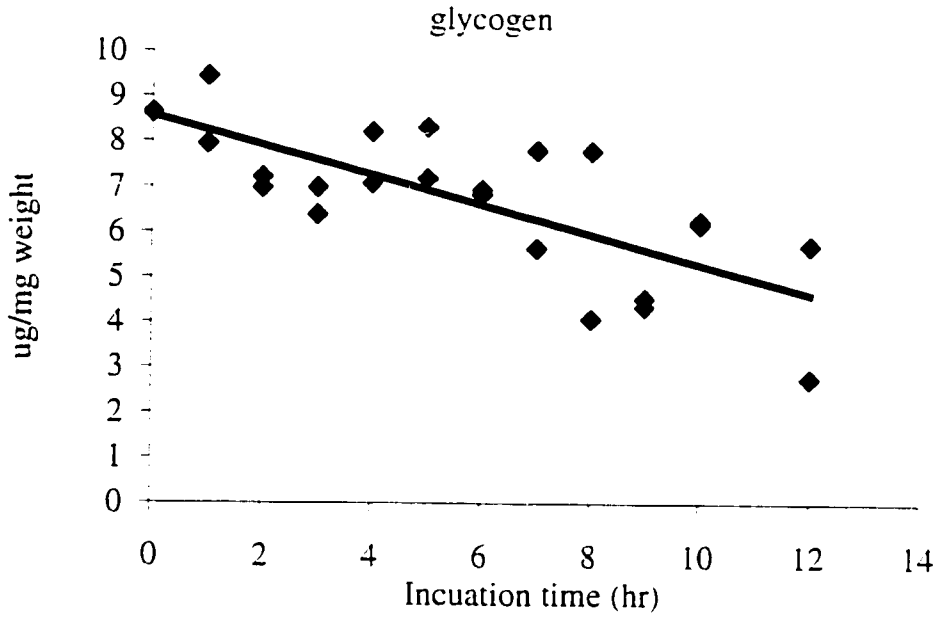


Figure IV-3. Epitrochlearis ATP and phospho-creatine content during 12 hr of incubation. Epitrochlearis muscles were preincubated for 60 min in incubation media defined in the *Materials and Method*, and were then transferred to fresh media of the same composition. The media were collected and replaced with fresh media every 2hr. Each point represents an individual muscle sample

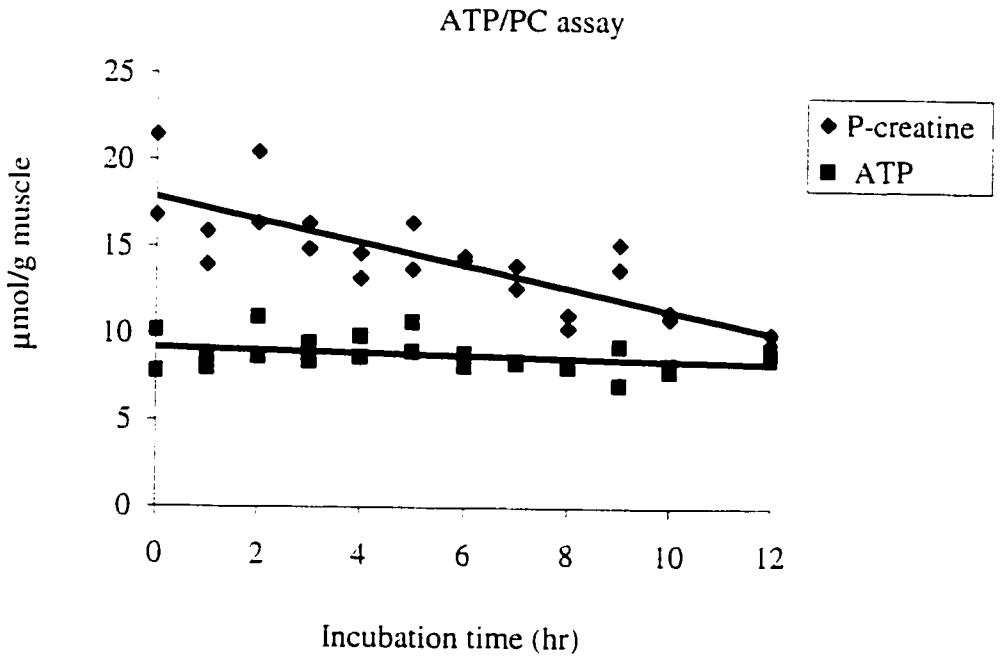
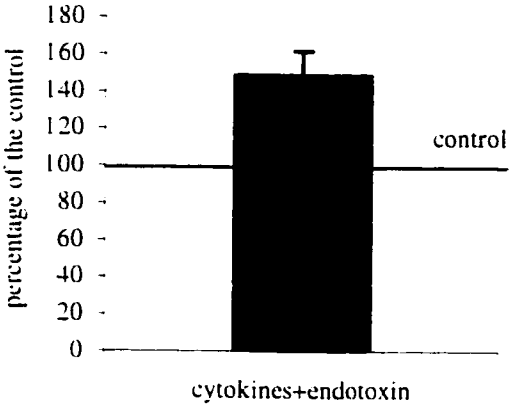
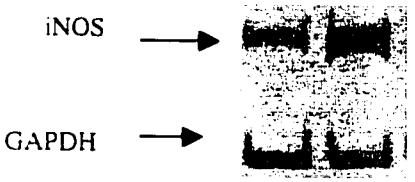


Figure IV-4. Effect of cytokine+endotoxin treatment on the expression of iNOS mRNA. Epitrochlearis muscles were preincubated for 30 min in incubation media defined in the *Materials and Method* plus 2% of fetal bovine serum retenate filtered by Millipore Ultrafree-MC centrifugal M.W. 10,000 filter units (Bedford, MA), and were then transferred to fresh media of the same composition with the presence of cytokines+endotoxin (1500 U/ml rat TNFα + 1000 U/ml rat IFNγ + 10 μg/ml endotoxin) or not for 12 h. The media were collected and replaced with fresh media every 2hr. At the end of 12h incubation, muscle samples were frozen into liquid nitrogen immediately and stored at -80C for further RNA extraction. iNOS mRNA and GAPDH mRNA levels were evaluated in the same RNA samples by RT-PCR. Auto-radiographic signals were corrected for GAPDH abundance and expressed as percentage of the controls. Data are means±SEM (n=8, vertical bars), *P<0.05. The image shown here represents the eight samples in each group.





Treatment control cytokine+endotoxin

Figure IV-5. Effect of incubation on the expression of iNOS in skeletal muscles. Total RNA was collected from both freshly dissected muscles (Sample 1,3,5,7,9,11) and muscles incubated for 12 hrs (Sample 2,4,6,8,10,12) under the conditions detailed in *Experiments*. iNOS mRNA and GAPDH mRNA levels were evaluated in the same RNA samples by RT-PCR.

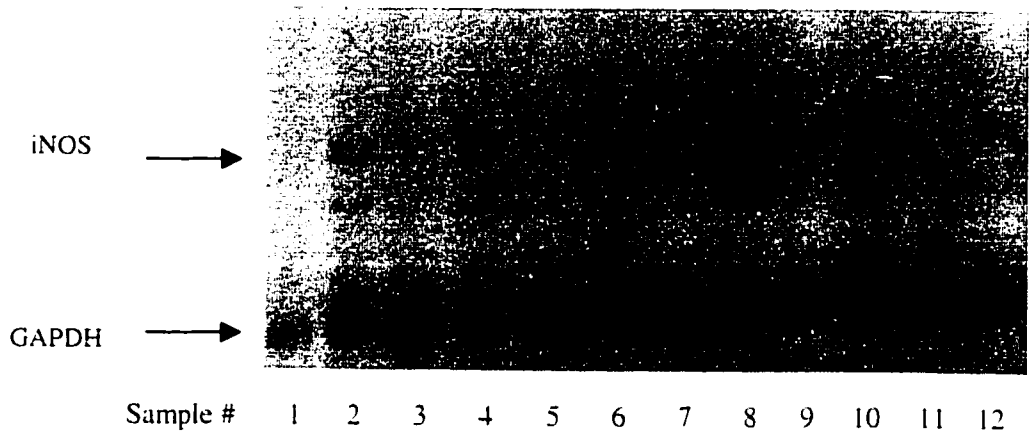
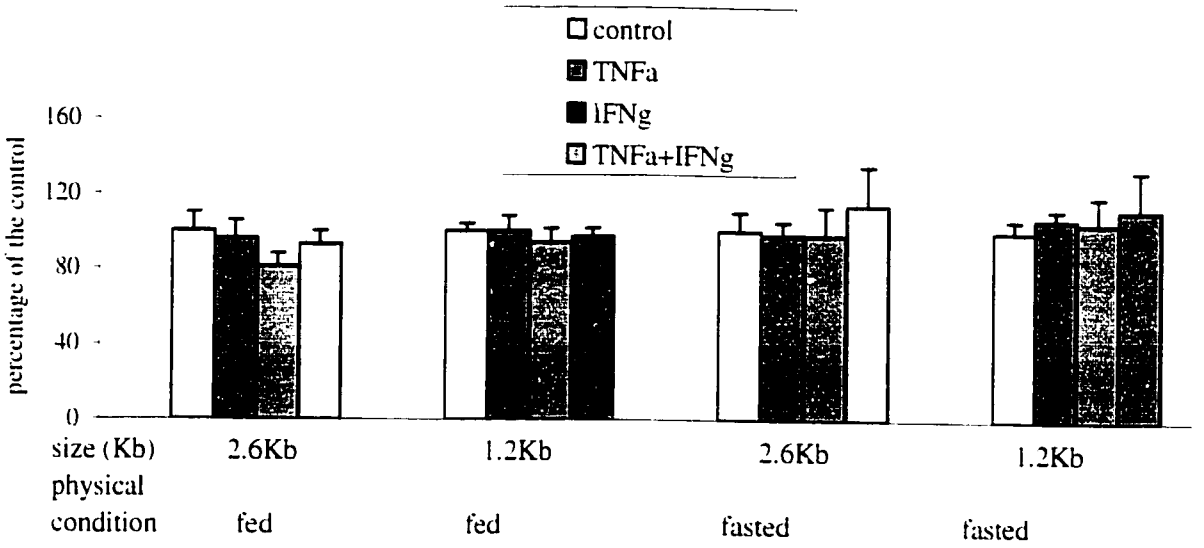


Figure IV-6. Quantification of mRNA levels for ubiquitin, 14-kDa E2 in epitrochlearis muscle from control and cytokine treatment groups. Epitrochlearis muscles were preincubated for 30-60 min in incubation media and were then transferred to fresh media of the same composition with the presence of cytokines or not for 12 h. The media were collected and replaced with fresh media every 2hr. At the end of incubation, muscle samples were frozen in liquid nitrogen immediately and stored at -80°C for RNA extraction. Auto-radiographic signals were corrected for 18S rRNA abundance and expressed as percentage of the controls. Data are means±SEM (n=8, vertical bars). Representative Northern blots are also shown.

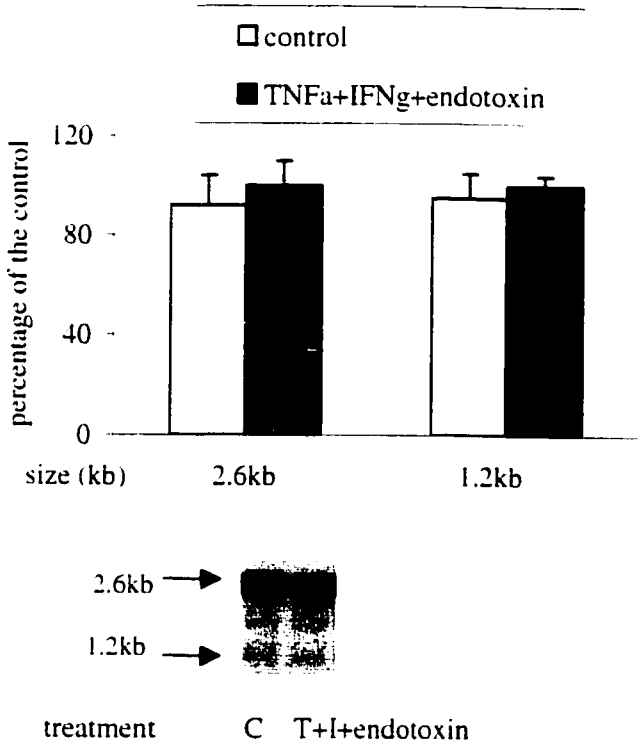
Figure IV-6A. The effect of cytokine treatment on the expression level of ubiquitin mRNA in skeletal muscle (n=8).



C: control, T: TNFα (1500U/ml), I: IFNγ (1000U/ml),

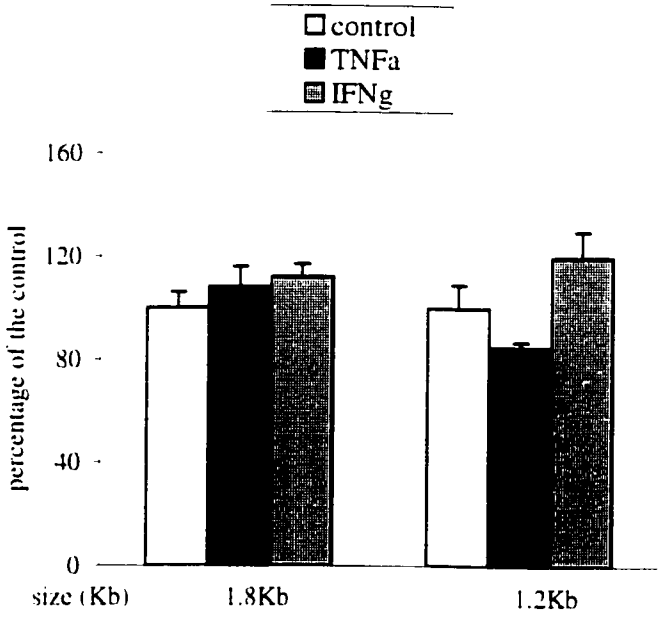
T+I: TNFα (1500 U/ml) + IFNγ (1000U/ml)

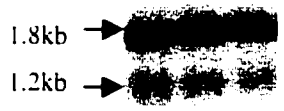
Figure IV-6B. The effect of cytokines+endotoxin on the mRNA level of ubiquitin in skeletal muscle (n=8)



C: control, T+I+endotoxin: TNFα (1500 /ml)+IFNγ (1000U/ml) +endotoxin (10μg/ml)

Figure IV-6C. The effect of cytokines on the expression of E2 (14KD) gene in skeletal muscle (n=8)





treatment C T I

C: control, T: TNFα (1500U/ml), I: IFNγ (1000U/ml)

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Appendix 1: Skeletal muscle membrane preparation

The receptors for neurotransmitters, hormones, cytokines and drugs on skeletal muscle can be monitored using purified skeletal muscle membrane. The major problems encountered in skeletal muscle membrane preparations are the extensive connective tissue network that make the cells relatively resistant to disruption, the high concentration of insoluble contractile protein, and the presence of other extensive membrane systems including the sarcoplasmic reticulum and T-tubular network (Barchi et al., 1977).

Homogenization disrupts the cells and the connective tissue. The solublized cell fragments are recovered by filtering the homogenate through cheesecloth. Potassium chloride is added to the crude homogenate to solublize the contractile proteins and further purification is achieved by density gradient centrifugation. Myofibrillar and sarcoplasmic reticular materials, are eliminated through these steps (Grimditch et al., 1985, Festoff and Engel, 1974). A further difficulty arises in distinguishing between the true sarcolemma and membranes arising from the T-tubular system. Both membrane are continuous, and T-tubular membrane fragments band between 22%-26% on a sucrose density gradient (Barchi et al., 1977). The crude membrane isolated obtained from sucrose gradient centrifugation is a mixture of T-system and sarcolemmal elements. Since the carbohydrate chain of membrane glycoprotein and glycolipids are extracellular, sealed right-side-out membrane vesicles should bind to lectin, whereas sealed inside-out vesicles and T-tubule membrane fail to bind, a result confirmed by Charuk (1989) and Ohlendieck et al. (1991) through immunohistological method. Through the wheat germ agglutination step, highly purified sarcolemma can be collected.

Materials and Methods (as described by Ohlendieck et al., 1991)

Buffer A: 20 mM sodium pyrophosphate, 20 mM sodium phosphate monohydrate, 1 mM $MgCl_2$, 0.303 M sucrose, 0.5M EDTA, 76.8 nM aprotinin, 1.1 μM leupeptin, 0.7μM pepstatinA, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH7.0

Buffer B: 0.303 M sucrose, 20 mM Tris-maleate, 0.6 M KCl 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.0

Buffer C: 50 mM sodium phosphate, 0.160 M NaCl, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.4

Buffer D: 20 mM Tris-HCl, 0.303 M sucrose, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH 7.4

Buffer E: 0.878 M sucrose, 0.6M KCl, 20 mM Tris-maleate, 0.83 mM benzamidine, 1 mM iodoacetamide, 0.23 mM PMSF, pH7.0

Part I: Isolation of crude muscle membranes: All subsequent steps are carried out at 0-4°C.

1. Cut skeletal muscle (~150g) into small pieces and grind them in a meat grinder. Weigh the ground meat.
2. Homogenize the tissue in a blender three times for 30s in 7.5 vol. of buffer A.

3. Centrifuge the homogenate in a Beckman JA-10 rotor at 14,000g (10,000 rpm) for 15 min and filter the supernatant through six layers of cheesecloth.
4. Resuspend the pellet from step 3 in 0.7 vol. of the original volume buffer A and repeat step 2 and 3.
5. Combine the supernatant from steps 3 and 4. Centrifuge the supernatant in a Beckman JA-14 rotor at 30,000 g (15,000 rpm) for 30 min.
6. Discard the pellet. Add solid KCl to a final concentration of 0.6 M to the supernatant.
7. Centrifuge the supernatant in a Sorvall (Du-Pont) T647.5 rotor at 142,000g (43,500 rpm) for 35 min.
8. Resuspend the pellet in 400 ml buffer B and repeat step 7.
9. Resuspend the pellet in 30 ml buffer B.
10. Add 35 ml of buffer E into ultraclear centrifuge tubes and load 5 ml aliquots from step 9 on top of the buffer E and centrifuge in a Sorvall AH629 swing rotor at 112,000g (25,000 rpm) for 17h.
11. Collect the crude surface membrane fraction at the 0.303/0.878 M sucrose interface and dilute it with buffer B.
12. Repeat step 7.
13. Resuspend the pellet in to buffer C and store the membrane at -80°C.

Part II: Isolation of purified sarcolemma by wheat germ agglutination.

1. Resuspend the crude membrane vesicles at a protein concentration of 1mg/ml in buffer C and mix it with an equal volume of 1 mg/ml wheat germ agglutinin (WGA).
2. Incubate the mixture on ice for 10 min.

3. Centrifuge the mixture for 90s at 14,000g in an Eppendorf model 5402 centrifuge.
4. Resuspend the lectin-agglutinated vesicles in buffer D and centrifuge as step 3.
5. Repeat step 4.
6. Resuspend the pellets from step 5 in buffer D to deglutinate the pellets.
7. Centrifuge the suspension from step 6 for 90s at 14,000g.
8. Centrifuge the supernatant from step 7 in a TL100.2 rotor (Beckman) for 20 min at 150,000g (58,000 rpm).
9. Resuspend the pellet in buffer D and repeat step 8.
10. Resuspend the pellet in buffer C and repeat all the steps from 1-9.
11. Finally, resuspend the pellet in buffer C and store at -80°C for future use.

Appendix 2: Radiolabeled ligand binding assay.

The principle of the ligand binding assay is very simple and straightforward (Rosenthal, 1967):

Ligand ([u]) + membrane Receptor ([m]) → [Ligand-Receptor] complex ([b])

where [u] is the concentration of free ligand; [m] is the concentration of free binding sites; [b] is the concentration of bound ligand. The moieties in the system are finally in mutual equilibrium. κ is the association constant. In the simplest case of one ligand and one group of binding sites: $\kappa=[b]/([u]\times[m])$ (1)

or $[b]/[u]=\kappa\times[m]$ (2)

The ligand is usually labeled with radioisotope. After separating the ligand-receptor complex from free ligand by dialysis, ultrafiltration or ultracentrifugation, the presence and amount of membrane receptor can be detected by measuring the radioactivity. Basic information on the association of small molecules (ligand) with macromolecules (membrane) can also be obtained from such assay. It is customary to conduct a series of such experiments by varying the concentration of the small molecule or ligand while the concentration of the binding macromolecules (membrane) is held constant. Different graphic methods have been used to plot the experimental results but all these methods yield a straight line on a graph with rectangular coordinates in the simplest case of one ligand and one group of mutually non-interacting binding sites. The two parameters for binding: the number of binding sites per unit of membrane protein (n), and the intrinsic association constant (κ), are obtained from the geometry of these graphs.

In the Scatchard plot, [b]/[u] is plotted against [b]. [M] is the concentration of membrane protein.

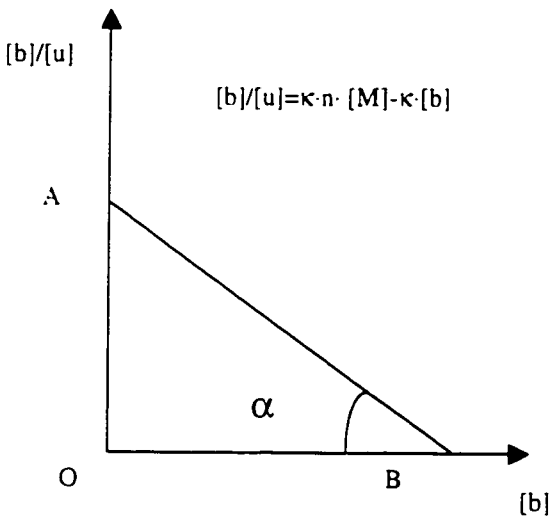
$[m]=n\times[M]-[b]$ (3)

Upon replacing the concentration of free binding sites in expression (3) by the difference in concentration of available binding sites and the concentration of occupied binding sites, the following equation is obtained:

$$[b]/[u]=\kappa(n\times[M]-[b]) \quad (4)$$

Equation (4) is a straight line with a slope of –k, and intercept on the ordinate of k×n[M], and an intercept on the abscissa of n[M] (Figure A-1). The product n[M] is the concentration of binding sites in this coordinate system, therefore if [M] is known, n can be calculated.

Figure A-1: Binding curve for one ligand and one group of binding sites



$\kappa = OA/OB = -tag(\alpha)$;
$OB = n\cdot[M]$

In a system with more than one groups of non-interacting binding sites, where ni[Mi]≠nj[Mj], ki≠kj, a plot of [b]/[u] against [b] gives a curved line. The mathematics involved to calculate each binding site is very complex and beyond the scope of this thesis.

Materials and methods (as described by Bird et al., 1988 with some modification)

Recombinant cytokines: murine IL1β, human IL6, TNFα, and IFNγ were obtained from Peprotech (Peprotech Inc. Rocky Hill, NJ) and I^{125}-labeled cytokines: murine IL1β, human IL6, TNFα, and IFNγ were purchased from Du-Pont-New England Nuclear (Boston, MA).

Binding buffer A: 50 mM sodium phosphate, 1% BSA, pH7.4

Part I. Direct binding assay with filter separation.

1. Resuspend the I^{125}-labeled cytokines and non-radioactive cytokines in binding buffer A to the desired concentration, and dilute the membrane to the concentration of 1 mg /ml in buffer A.
2. Label one set of tubes for total binding detection, and another set for the non-specific binding assay.
3. Add 25 µl I^{125}-labeled cytokines into all tubes at the indicated concentration.
4. Add 25 µl cold cytokines into the tubes set aside for non-specific binding assay at a concentration of 200 fold greater than that of radioactive cytokines.

5. Add 25 µl binding buffer A into the tubes assigned for total binding detection.
6. Add 50 µl of membrane into all the tubes.
7. Incubate the binding mixture in a shaking water bath at 25 °C for 4 hr.
8. Stop the binding reaction by chilling binding buffer A (3ml/tube).
9. Vortex the tubes.
10. Soak Whatman GF-B filter papers (r=24 mm) in buffer A + 0.5% polyethylene (Sigma) for 10 min and place the filter papers into the wells on a Millipore vacuum filtration unit.
11. Pour the mixture into wells and separate the membrane-bound ligand from free ligand under vacuum.
12. Wash the tubes three times with 3 ml chilled binding buffer A and pour the washing buffer into corresponding wells.
13. Dry the filter paper under vacuum.
14. Take out the filter paper and place it at the bottom of a test tube.
15. Count the test tube in a Packard gamma-counter.
16. Calculate the specific binding. (Specific binding =total binding - non-specific binding).
17. Process the data with Graphpad prism program to obtain a binding curve.

Part II. Competitive binding with filter separation.

1. Repeat step-1 in Part I.
2. Label one set of tubes instead of two.

3. Add 25 µl of a constant amount of I^{125}-labeled cytokines and 25 µl of cold cytokines at an increasing concentration into all the test tubes.
4. Non-specific binding is determined in the presence of a 1000 fold excess of non-radioactive cytokine in the test tube.
5. Repeat steps 6-17 as in Part I.

Part III. Binding assay with WGA separation (described by Liu et al., 1994) with minor modifications).

The difference between the two separation methods occurs after the binding.

1. Repeat step 1-7 in Part I in microcentrifuge tubes instead of glass tubes.
2. Stop the binding reaction by adding ice-cold 100 µl WGA (1 mg /ml). Vortex the tubes.
3. Place the tubes on ice for 30 min.
4. Centrifuge the mixture for 7 min at 14,000g in an Eppendorf model 5402 centrifuge at 4°C.
5. Aspirate off the supernatant. Be careful not to touch the pellet.
6. Wash the pellet with cold 0.25M sucrose buffer and vortex the tube.
7. Repeat steps 4 and 5.
8. Repeat steps 6 and 7 twice.
9. Dry the pellet. Cut the tip of the centrifuge tube and put the tip into a testing tube.
10. Count the test tube in a Packard gamma counter, calculate the specific binding and process the data as described before.

The WGA separation method gave lower non-specific binding (~40% total binding) than filter separation method and was thus adopted in our study.

Appendix 3. Measurement of presence of mRNA by RT-PCR:

Sensitive techniques which detect mRNA molecules offer important insights into the expression systems of organisms, tissues, and cells that could othwise not be studied. In addition to conventional methods such as Northern blot analysis, S1 nuclease assays, RNase A protection assays, and *in situ* hybridization, PCR offers a new dimension to the detection of rare mRNAs by amplifying a single stranded cDNA after reverse transcription of the RNA. This combination is called RT-PCR (Hagen-Mann and Mann, 1995).

Briefly, the first step of reverse transcription is performed by RNA-dependent DNA polymerases. The two enzymes used as reverse transcriptases (RT) are AMV (from avian myeloblastosis virus) and MMuLV (from moloney murine leukemia virus). For longer fragments (>500 bp) MMuLV is the enzyme of choice since it has a low helicase activity and the recombinant protein is RNase H free (Bloch, 1991). The latter enzyme was employed in our experiments. RT enzymes need a 3'-OH group as part of a double stranded molecule. There are three different methods for priming (Kawasaki, 1991):

a) Oligo-dT is able to hybridize to the poly-A tail of eukaryotic mRNAs. All mRNAs will be reverse transcribed.

b) Random hexamers anneal along each single stranded nucleic acid and RT will produce a template mixture for the following amplification.

c) Specific antisense primers at the 3'-end of a mRNA prime the synthesis of the cDNA of the respective species of mRNA. In order to amplify the abundant transcripts, specific priming is always preferred.

Before any conclusions can be drawn from RT-PCR experiments based solely on the generation of a PCR product, verification must be made by a second method. This is typically achieved either by partial or complete nucleotide sequencing, restriction mapping, or sequence-specific probe hybridization. Obtaining a nucleotide sequence is the most convincing verification method, although it is technically the most demanding and time consuming. Restriction mapping is often the most convenient verification method, accomplished simply by noting the presence of one or more characteristic restriction sites situated between the primer templates. This method was adopted in our experiment. Verification of RT-PCR products can also be achieved by successful hybridization of a synthetic oligonucleotide probe that recognizes a unique sequence situated between the PCR primer templates. Typically, a 30-nucleotide antisense, synthetic oligonucleotide probes allows for stringent hybridization and washing conditions (Kawasaki, 1991, Bloch, 1991, Sambrook et al., 1989).

Materials and Methods:

Taq DNA polymerase (GIBCO) and Expand™ Reverse Transcriptase (Boehringer Mannheim).

1. First strand cDNA synthesis (RT step).
    - Add the reagents in the order listed in the table below to a sterile Rnase and Dnase free 0.2 ml thin-walled PCR tube on ice.

Component	Volume	Final concentration
Total RNA	10 µl	0.1-1.0 µg total RNA

Anti-sense primer (10 µM)	1 µl	1 µM

- Denature RNA and primer for 10 min at 65°C in a thermocycler.
- Immediately cool on ice.
- Add the reagents in the order listed in the table below to the same PCR tube.

Component	Volume	Final concentration
5X reverse transcriptase buffer	4 µl	1X
100 mM DTT	2 µl	10 mM
100 mM dNTP	2 µl	10 mM
Rnase inhibitor 40 U/ µl	0.5 µl	20U
Reverse transcriptase 50U/ml	1 µl	50 U
total	20.5 µl	

- Incubate the PCR tube at 42°C for 60 min.
- Stop reaction by placing on ice.

2. Polymerase chain reaction (PCR): standard protocol.

- Add the following components to a sterile 0.2 ml thin-walled PCR tube on ice.

components	Volume	Final concentration
Template DNA	5-10 µl	-----
10 X PCR buffer minus Mg	5 µl	1X
10 mM dNTP mixture	1µl	0.2 mM
50 mM MgCl2	1.5 µl	1.5 mM
Primer (10 µM each)	2 µl each	0.4 µM each
Taq DNA polymerase (5U/ µl)	0.25 µl	1.25 U
Sterile water	Adjust to total vol. of 50 µl	----

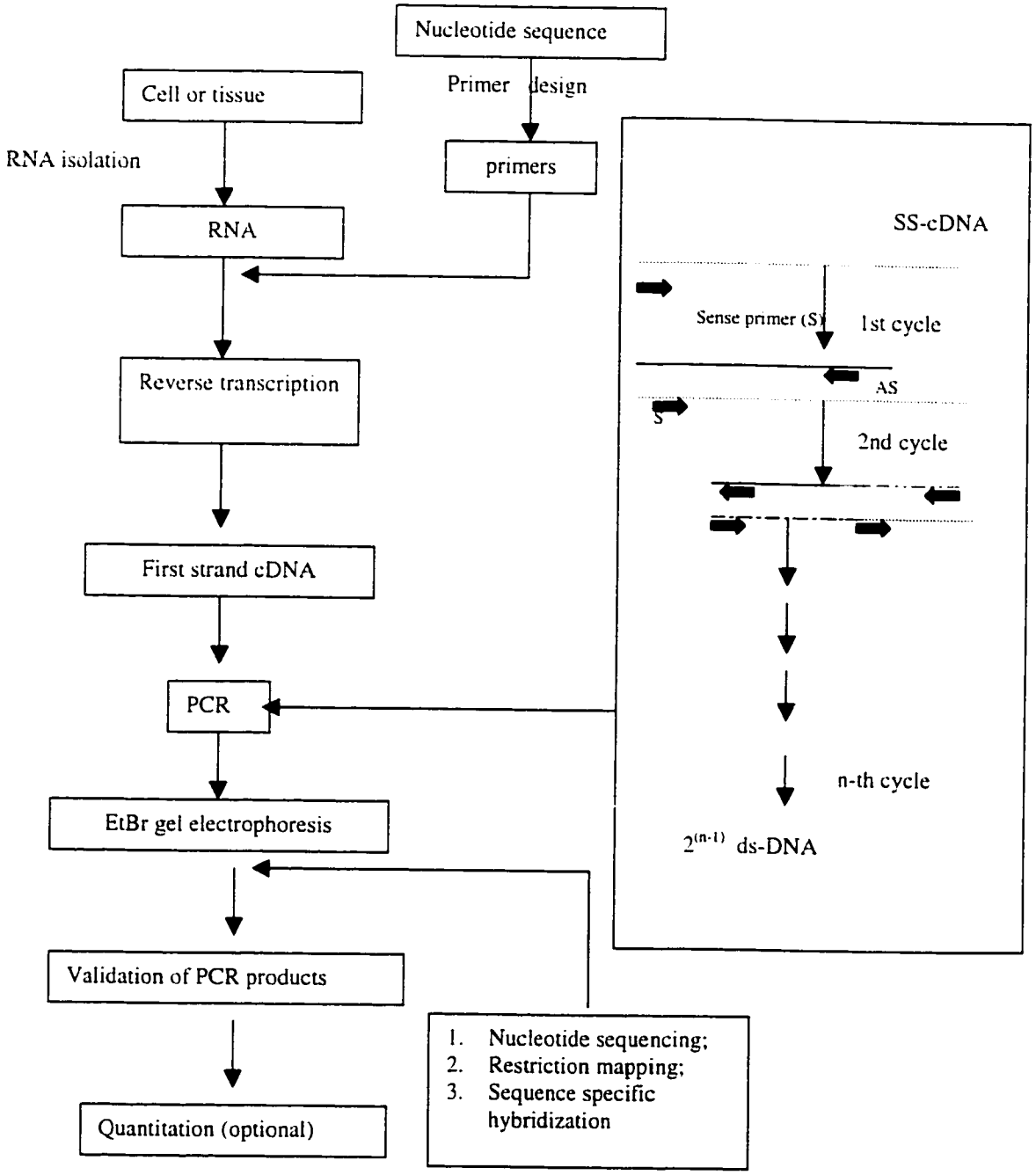
- Mix gently and thoroughly.
- Incubate the tubes in a GeneAmp PCR system 2400 thermal cycler (from Perkin Elmer) and set up the PCR programs as described in the Materials and Methods for each gene.
- At the end of the reaction, store the samples at –20°C.

3. "Hot-start" protocol.

- Add all components as described in the Standard protocol above, except for the Taq DNA polymerase.
- After initial denaturation at 94°C, maintain the reaction at 80°C.
- Add 1.25 U of Taq DNA polymerase to each tube.

- Continue the PCR cycles.
- At the end of the reaction, store the sample at –20 °C

Figure A-2. Flow chart of RT-PCR



Appendix 4. Measurement of ubiquitin mRNA level with Riboprobe.

Part I. Riboprobe labeling with *in vitro* Riboprobe System T7 labeling kit from Promega.

1. Linearize the plasmid for ubiquitin with EcoRI (5 µl plasmid+1 µl EcoRI + 3 µl 10×Buffer3+21 µl d.d. H_2O) for 2h at 37°C.

2. Load the digest on a 1% agrose gel and purify the cDNA fragment with the Gene-Clean Kit (Bio 101). The following picture (Figure A-3) shows the result after digest.

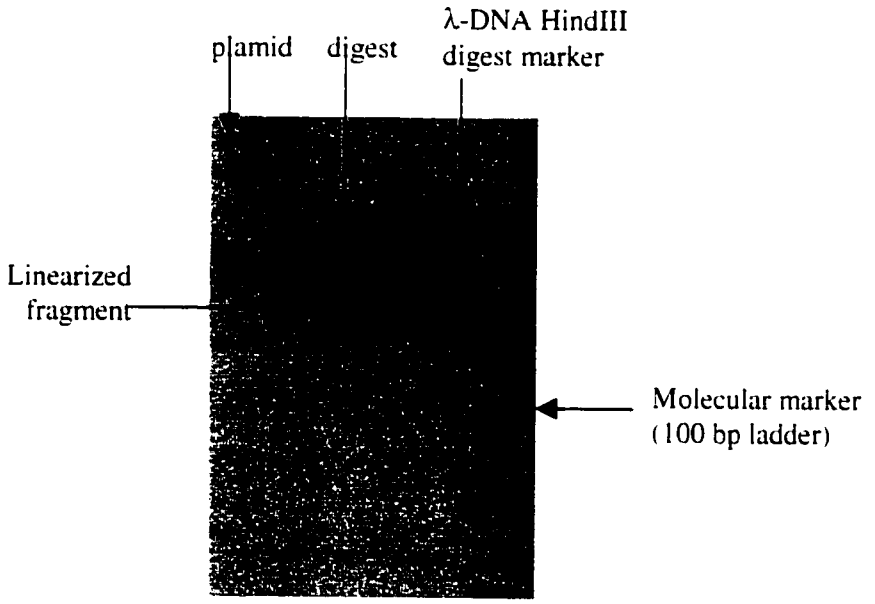


Figure A-3. Linearization of pGEM-4 plasmid transformed with ubiquitin gene. The λ-DNA HindIII digest marker contains 8 fragments (0.12-23.1kbp: 125, 564, 2027, 2322, 4361, 6557, 9416, 23130 bp).

3. Riboprobe transcription reaction mixture: all the reagents must be at room temperature before using except the enzyme and inhibitors which should be stored at -20°C.

4.5 µl linearized DNA
1.0 µl 10 mM ATP
1.0 µl 10 mM GTP
1.0 µl 10 mM UTP
2.0 µl 100 mM DTT
0.5 µl RNase inhibitor (40 units / µl stock) (store at -20°C until needed)
4.0 µl 5X Transcription buffer
5.0 µl [32P] CTP 200 µCi.
1.0 µl T7 RNA polymerase. (store at -20°C until needed)

20.0 µl
Mix well and allow to incubate for 1h at 37°C.

4. Remove DNA template:

-add 0.5 µl RNase inhibitor.
-add 1.0 µl RQ1 DNase.
-mix, centrifuge briefly and incubate at 37 °C for 15 min.
-add 4 µl 5M ammonium acetate
-add 40 µl 100% EtOH.
-precipitate RNA at -20°C overnight.

5. Centrifuge the mixture for 15 min at high speed of a Lab-top centrifuge.

6. Resuspend the pellet in 100 µl d.d. H2O.

7. Count the probe.

Part II. Hybridization and Prehybridization.

Prehybridization solution:

Final concentration	Stock solution	Volume
60%	100% deionized formamide	12 ml
1X	20X SSPE	1 ml
0.5%	10% blotto	1 ml
10%	50% dextran sulphate	4 ml
1%	20% SDS	1 ml
0.5 mg/ml	10 mg/ml ss DNA*	1 ml

*Denature ssDNA in a boiling water bath for 10 min before adding it.

Hybridization solution

Final concentration	Stock solution	Volume
60%	100% deionized formamide	11 ml
1X	20X SSPE	1 ml
0.5%	10% blotto	1 ml
10%	50% dextran sulphate	4 ml
1%	20% SDS	1 ml

- Mix hybridization solution well and preheat to 50°C
- Add an appropriate volume of purified probe solution (to achieve a final 2*106 dpm /ml hybridization solution) to a tube containing 1 ml ssDNA and 1 ml 100% deionized formamide, and mix.
- Heat probe/ssDNA/formamide solution at 70°C for 5 min in a water bath, and then add to preheated hybridization solution, and mix.

Place the Zeta-Probe membrane with RNA sample on it into a hybridization tube.

1. Add 0.15-0.25 ml Prehybridization solution / cm^2 of membrane and prehybridize the membrane for 1-2hrs at 50°C.
2. Drain Prehybridization solution.
3. Add hybridization solution into tube and hybridize overnight.

Part III. Membrane Washing

1. After hybridization, drain hybridization solution.
2. Briefly rinse the membrane in 2XSSC.
3. Wash membrane in 2XSSC/0.1%SDS vigorously at room temperature for 30 min on a shaker
4. Wash membrane in 0.2XSSC/1%SDS at 70°C for 30 min in a shaking water bath.
5. Briefly rinse membrane in 0.2XSSC.
6. Briefly blot membrane between Whatman 3MM filter paper and them immediately heat-seal membrane is a transparent plastic bag.

Appendix 5. Measurement of E2-14kD mRNA level with a cDNA probe

Part I. cDNA probe labeling with random Primer DNA labeling system from GIBCO.

1. The plasmid for E2-14Kd were digested with restriction endonucleases (5 μl plasmid+1 μl BamH1 + 1μl KpnI + 3μl 10×Buffer4+20 μl d.d. H_2O) for 2h at 37°C.
2. Load the digest on a 1% agrose gel and purify the cDNA fragment with Gene-Clean Kit (Bio 101). The following picture (Figure A-4) shows the result after digest.

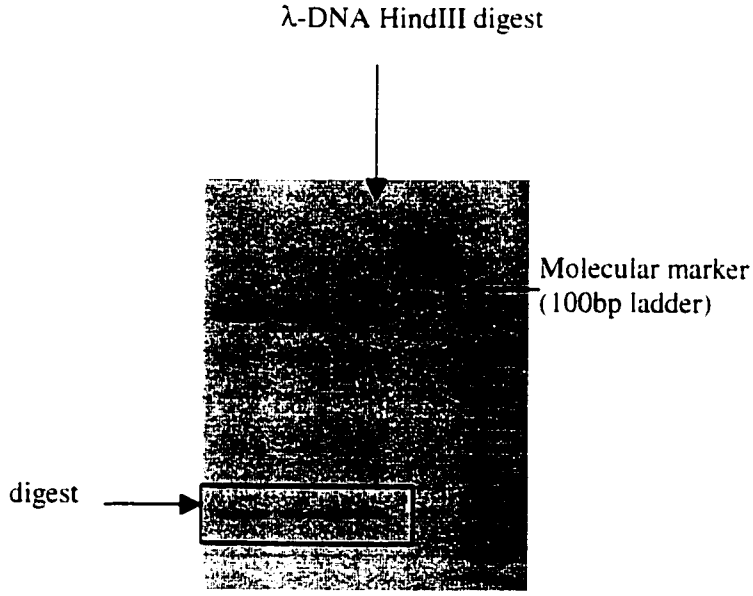


Figure A-4. The digest of pTZ18R plasmid transformed with E2-14kD gene. The λ-DNA HindIII digest marker contains 8 fragments (0.12-23.1kbp: 125, 564, 2027, 2322, 4361, 6557, 9416, 23130 bp.

3. Denature 5 µl DNA + 18 µl d.d.H2O in a microcentrifuge tube by heating for 5 min in a boiling water bath, then immediately cool on ice.

4. Perform the following additions on ice:

2 µl dCTP

2 µl dGTP

2 µl dTTP

15 µl random primer buffer mixture

5 µl P32 dATP

-mix briefly

Add 1 µl Klenow fragment.

Mix well and allow to incubate for 1h at 25°C.

Add 5 µl stop buffer.

Use this labeled probe for hybridization directly.

Part II. Hybridization and Prehybridization.

Prehybridization and Hybridization solution:

Final conc.	Stock solution	Volume
6X	20X SSPE	150 ml
0.5%	10% SDS	25 ml
5X	50X Denhardt's*	50 ml
	H2O	275 ml

*50X Denhardt's (1% Ficoll 400, 1%PVP, 1% BSA).

- Add 1 ml ssDNA and 1 ml 100% deionized formamide, mix.
- Heat probe/ssDNA/formamide solution at 70°C for 5 min in a water bath, and then add to preheated hybridization solution, and mix.

Prior to hybridization, briefly dip the membrane in deionized water to remove excess salt from the membrane

Place the baked nitrocellulose membrane with RNA samples into a hybridization tube.

Add 8 ml Prehybridization solution and prehybridize the membrane for 1-2hrs at 65°C.

Drain Prehybridization solution.

Prepare the probe: add 100 µl of tRNA (10mg/ml) to labeled probe, boil for 2 min and cool down on ice.

Add the probe to fresh prewarmed Prehybridization solution in the tube. Hybridize overnight at 65°C.

Part III. Membrane Washing

1. After hybridization, drain hybridization solution.
2. Briefly rinse the membrane in 2XSSPE, 0.1% SDS twice.
3. Wash membrane in 2XSSPE, 0.1% SDS vigorously at room temperature for 30 min on a shaker
4. Wash membrane in 0.1XSSC/0.1%SDS at 65°C for 30 min in a shaking water bath.
5. Briefly blot membrane between Whatman 3MM filter paper and then immediately heat-seal membrane in a transparent plastic bag.

Appendix VI. Determination of glycogen in small tissue samples. (according to Lo et al., 1970)

The present method depends on basic digestion of the sample and the measurement of the resulting glucose with phenol-sulfuric acid.

Materials and Methods

Buffer A (30% KOH saturated with sodium sulfate), 95% ethanol, 5% phenol, H_2SO_4

Standard glycogen solutions (20-400 µg/ml).

150×20 mm test tubes with screw caps

1. Put the frozen samples into ice-cold tubes on ice.
2. Add 1.5 ml Buffer A into tubes and cap them. Put the tubes in a boiling water bath for 20-30 min until a homogeneous solution is obtained.
3. Remove tubes from hot water and cool on ice.
4. Precipitate the glycogen with 1.1-1.2 vol. of ethanol. Incubate the mixture on ice for 30 min.
5. Centrifuge the samples at 840g for 30 min. Aspirate the supernatant.
6. Dissolve the pellet in 3.0 ml d.d. H_2O.

7. Aliquot 250 µl suspension from step 6 or 1 ml standard solution into a 150×20 mm test tube and bring the volume of suspension to 1ml by adding d.d. H_2O.

8. Add 1 ml 5% phenol solution.

9. Add 5 ml H_2SO_4 rapidly into liquid and incubate for 10 min

10. Shake the tubes for 20 min in a water bath at 25°C.

11. Read the absorbency on a spectrophotometer at 490 nM.

All tests are carried out in triplicate to minimize errors.

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