ABSTRACT

Mitchell Douglas Lee. MONOKINE REGULATION OF HEXOSE METABOLISM IN L6 MYOTUBES. Department of Biology, May, 1986.

Exposure of fully differentiated L6 myotubes to a crude monokine preparation from endotoxin-stimulated RAW 264.7 cells resulted in a rapid and substantial (70%) increase in fructose 2,6-bisphosphate concentration coincident with depletion of cellular glycogen and an increased lactate production. During the time frame required for glycogen depletion (3 hrs), stimulation of 2-deoxyglucose uptake was observed reaching a maximum enhancement of 200% at 12-15 hrs after exposure to the monokine. The monokine had no effect on the Km value for 2-deoxy-D-glucose uptake (1.1 mM) while Vmax was increased 160% from 912-2400 pmol/min/mg protein. The increase was cytochalasin B inhibitable and dependent on protein synthesis. Photoaffinity labeling, as well as equilibrium binding studies with [^{3}H]-cytochalasin B, support the hypothesis that this increase in hexose transport is due to an increase in hexose transporters present in the plasma membrane. Neither purified recombinant interleukin-1 nor γ-interferon had any effect on hexose transport whereas purified recombinant tumor necrosis factor (rTNF) did stimulate hexose uptake 85%, with half-maximal stimulation occurring at 3000 pM. These data suggest a major role for the regulation of hexose transport in muscle cells by endotoxin-induced monokines. The inability of rTNF to achieve the maximum effect observed with the crude preparation suggests the presence of a second monokine acting alone or synergistically with TNF. Preliminary purification by gel filtration and isoelectric focusing indicates that this monokine is a protein of molecular weight 75,000 daltons and a pI = 6.3.

MONOKINE REGULATION OF HEXOSE METABOLISM IN L6 MYOTUBES

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MONOKINE REGULATION OF HEXOSE METABOLISM IN L6 MYOTUBES

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INTRODUCTION

Glucose is considered to play a fundamental role in the overall response of the host to infection due to the integration between glucose, amino acid and fat metabolism (Wolfe, 1981). Glucose metabolism plays an important role during sepsis and endotoxicosis in that failure to treat these conditions can lead to severe hypoglycemia and eventual death (Filkins, 1982). In order to elucidate the biochemical events which contribute to the severe hypoglycemia associated with late sepsis and endotoxemia, a thorough understanding of the host's immune response during infection is necessary.

Of primary importance in the initial response of the host to generalized infection are the cells of the mononuclear phagocyte system (Gans, 1984). These cells are commonly referred to as macrophages and originate from pluripotent hematopoietic stem cells in the bone marrow. After partial development, macrophages migrate via the blood to various tissues to become resident cells. Once the macrophages are established in the tissues, instances of altered homeostasis such as inflammation and infection can modify their morphology, metabolism and physiology (Van Furth, 1980). These macrophages are now classified as "activated" and have the capacity to perform many of the functions necessary for the host's normal immune response. Characteristics of "activated" macrophages include: (1) increases in both cell size and the number of lysosomes within the cell (Kastello and Canonico, 1981; Adams and Hamilton, 1984), (2) increased chemotactic and phagocytic efficiency (Cohn, 1983), (3) capability for presentation of antigens to T lymphocytes (Kastello

and Canonico, 1981; Unanue, 1984), and (4) an increased secretion of various proteins and other metabolites (Cohn, 1983; Adams and Hamilton, 1984).

Much of the previous work concerning macrophages has dealt with the classic Metschnikoff concept of the macrophage as the key cell in the host's defense due to its phagocytic and satyriasistic actions (Metschnikoff, 1884; Bibel, 1982). Consequently, studies concerning the mechanism of the macrophage system function during pathogenesis initially concentrated on the endocytic activities of the "activated" macrophage (Filkins, 1985). Research over the past few years has revealed new insights into the role of the macrophage as a major secretory cell (Cohn, 1983; Takemura, 1984). It is now well understood that macrophages synthesize and secrete a large number of mediators associated with chronic infection (Takemura, 1984; Filkins, 1985). Many of the substances and their functions are summarized in Table I. Among the vast number of macrophage secretory proteins are the signal molecules that regulate the growth and function of other cells. These proteins are termed monokines.

The concept of the monokine has evolved from three separate, yet related, areas of research (Filkins, 1985). These areas of study include: (1) the mediators secreted in response to infection, (2) the mediators associated with the host's metabolic response to infection, and (3) the mediators directly responsible for macrophage-lymphocyte interactions in regulation of immune responses. Monokines, which may be defined as regulatory proteins secreted by activated monocytes or macrophages, function in either a paracrine or endocrine fashion to provide

Table I. Secretory Products of "Activated" Macrophages and their Functions.

Product	Function	References
Proteins that Regulate Function or Growth of Other Cells		
Interferon	Antiviral	Fleit and Rabinovitch, 1981
Interleukin-1	Immunoregulatory, Induction of Acute Phase Proteins Fever Production, Metabolic Regulation	Oppenheim et al., 1978; Kampschmidt, 1981
Glucocorticoid-Antagonizing	Regulation of Glucose Metabolism	Moore et al., 1978; Berry et al., 1980
Macrophage Insulin-like Activity	Regulation of Glucose Metabolism	Filkins, 1984

Table I. (cont.)

Product	Function	References
Low Molecular Weight Substances		
Oxygen Metabolites H_2O_2, O_2^-, OH	Microbicidal, Tumoricidal	Nathan and Cohn, 1980; Murray et al., 1980
Polyunsaturated Fatty Acid Metabolites Prostaglandin E_1 6-Ketoprostaglandin $F_{1\alpha}$ Thromboxane B_2 Leukotriene C 5-Hydroxyeicosatetraenoic acid 12-Hydroxyeicosatetraenoic acid	Inflammatory, Immunoregulatory	Gemsa et al., 1978; Rouzer et al., 1980
Platelet-Activating Factor	Inflammatory, Activates Platelets	Demopoulous et al., 1979; Mencia-Huerta and Benveniste, 1979

Table I. (cont.)

Product	Function	References
Enzymes		
Plasminogen Activators	Inflammatory, Tissue Repair	Unkeless et al., 1974
Collagenase I-V	Inflammatory	Werb and Gordon, 1975; Mainardi, et al., 1980
Elastase	Inflammatory	Cammer et al., 1978; Banda and Werb, 1981
Angiotensin-Converting Enzyme	Activation of Angiotensin	Silverstein et al., 1979
Arginase	Antimicrobial, Tumoricidal, Immunoregulatory	Currie, 1978
Acid Hydrolases	Inflammatory	Allison et al., 1978
Lysozyme	Antimicrobial	Gordon et al., 1974
Lipoprotein Lipase	Metabolism of Lipoproteins	Khoo et al., 1981

Table I. (cont.)

Product	Function	References
Plasma Proteins		
Complement Components C_1, C_2, C_3, C_4, C_5 Factors B, D, I, H	Antimicrobial, Inflammatory, Opsonic	Fey and Colten, 1981
Coagulation Factors V, VII, IX, X	Coagulation, Tissue Repair	Osterud et al., 1981
Apolipoprotein E	Transport of Cholesterol from Macrophage to Liver, Immuno-regulatory	Basu et al., 1981
Transcobalamin II	Transport of Vitamin B_{12}	Rachmilewitz, 1978
α_2-Macroglobulin	Regulation of Plasma Enzyme Activities	Hovi et al., 1977

both nonspecific local and systemic host defense mechanisms (Filkins, 1985).

The classification of monokines into functionally discrete categories presents difficulties due to the fact that some monokines may fall into one or more categories. Nonetheless, monokines may be classified into two functionally distinct categories:

(1) Those monokines with an immunoregulatory function, and

(2) Monokines that regulate metabolism.

Despite the important function that immunoregulatory monokines play in the host's immune response, recent studies involving the metabolic regulatory monokines have provided a greater understanding of the overall role monokines perform in the host's response to infection (Filkins, 1982; Pekala et al., 1983b).

During infection, macrophages produce tissue specific monokines that interact with peripheral tissue to mobilize the body's energy reserves that are needed to meet the excess energy demands of the host (Filkins, 1985). These excess energy needs are a direct result of activation and production of cells involved in the host's immune response, as well as the increased production of many serum protein factors.

Alterations in fat metabolism are one of the common metabolic responses that occur during sepsis and endotoxicosis. Studies in both experimental animals and cell culture support the view that during chronic infection, the adipose cell switches from an anabolic storage state to a catabolic supply mode (Bagby and Spitzer, 1980; Kawakami and Cerami, 1981; Pekala et al., 1984). Hypertriglyceridemia, elevated levels of free fatty acids, increased lipolysis, and decreased fatty

acid uptake and oxidation are among the changes that occur during infection in adipose tissue (Rouzer and Cerami, 1980; Pekala et al., 1983a; Pekala et al., 1984). Kawakami and coworkers demonstrated the ability of an endotoxin-induced mediator from mouse peritoneal macrophages to suppress by greater than 90% the activity of lipoprotein lipase in 3T3-L1 preadipocytes (Kawakami et al., 1982). Recent studies involving the purification of this mediator indicate it to be the monokine tumor necrosis factor (Beutler et al., 1985). Thus, tumor necrosis factor may play a primary role in the etiology of septic hypertriglyceridemia. Pekala et al. (1983a) have demonstrated that crude conditioned medium from endotoxin-induced macrophages contains one or more monokines that suppress the key enzymes for de novo fatty acid biosynthesis in differentiating preadipocytes. Furthermore, recent studies indicate that interleukin-1 may be the monokine responsible for the activation of hormone sensitive lipase, the enzyme responsible for the breakdown of triacylglycerol in adipose tissue (Price, S. R. and Pekala, P. H., manuscript in preparation). Thus, monokines have been demonstrated to play a pivotal role in the change of status of the fat cell from an anabolic supply to a catabolic energy mode.

While adipose tissue is undergoing important functional changes during infection, the liver is involved in the synthesis of a variety of proteins (Filkins, 1985). Evidence indicates that interleukin-1 mediates the production of hepatic acute phase reactants such as serum amyloid A protein, fibrinogen, haptoglobin, and C-reactive protein (Rupp and Fuller, 1979; Sipe et al., 1979; Powanda and Moyer, 1981).

Decreased hepatic ketogenesis during sepsis and endotoxicosis can be related to increased levels of insulin or insulin-like monokines (Filkins, 1985).

Due to decreases in hepatic ketogenesis during chronic infection, peripheral tissues like muscle must rely on glucose and amino acid oxidation for cellular functions. Thus, numerous changes in carbohydrate metabolism in peripheral tissues are necessary in order to make up for impaired hepatic ketogenesis. Since muscle tissue occupies a significant percentage of total body mass, it is important that glucose homeostasis in this tissue be maintained during an infection. The focus of these studies presented herein has been to clarify the mechanism of action of various monokine(s) involved in carbohydrate metabolism in skeletal muscle during endotoxicosis.

HISTORICAL REVIEW

Menten and Manning (1923) are generally credited with the primary experimental description of blood glucose changes during endotoxicosis. In rabbits given i.v. heat-killed organisms of the enteritidis-paratyphoid B group, they reported an early hyperglycemic response followed by an ensuing hypoglycemia. This biphasic response was confirmed by Zeckwer and Goodell (1925 a,b) using a variety of heat-killed gram-negative bacteria. Since these initial studies, there has been extensive literature verifying that disturbances in blood glucose regulation are among the most characteristic and consistently observed alterations in endotoxicosis. The familiar pattern of early hyperglycemia which progresses to a late hypoglycemia has been documented in a wide range of species including chicks, mice, rabbits, rats, guinea pigs, cats, cows, horses, baboons, monkeys, and man (Berry, 1971; Agarwal, 1975; Hinshaw, 1976; Filkins, 1979a).

Blood glucose levels are the net result of regulated inputs as compared with outputs from the circulating glucose pool (Filkins, 1984). Inputs are of three primary types: (1) alimentation - either via the intestinal route or parenterally as in the clinical setting; (2) glycogen mobilization resulting in direct glucose release in the liver or muscle and in subsequent lactate release via glycolysis in muscle; and (3) gluconeogenesis, which is the production of glucose in the liver from amino acids, lactate, and glycerol. Outputs are also of three primary types: (1) renal excretion due to renal damage or insufficient renal tubular transport; (2) utilization of glucose for the synthesis of

glycogen, lipid, or protein; and (3) peripheral utilization of glucose through both aerobic oxidation to CO_2 and H_2O as well as anaerobic production of lactate.

Endotoxicosis affects inputs to the glucose pool in several ways. Dietary intake of glucose is impaired due to loss of appetite as well as dysfunction of gastrointestinal motor function (Berry, 1971). Massive glycogen depletion, especially in the liver, and a reduction in total body carbohydrate content are constant findings in endotoxic shock (Berry, 1971, 1975). In vitro, glycogen is lost more rapidly from endotoxin-treated homogenized liver cells than from normal cells (Zwadyk and Snyder, 1973). The enhanced glycogenolysis observed after injection of endotoxin has been implicated in the early hyperglycemia associated with endotoxicosis. The hypoglycemia associated with late endotoxemia has been attributed to impaired gluconeogenesis in the liver. Isolated hepatocytes from endotoxin-treated donors were shown to have impaired rates of gluconeogenesis from various precursors (Filkins and Cornell, 1974).

Endotoxicosis effects on outputs from the glucose pool may also contribute to the hypoglycemia associated with chronic infection. Increased peripheral utilization and hypercatabolism of glucose are dominant factors in endotoxic hypoglycemia (Holtzman et al., 1974; Hinshaw, 1976; Romanosky et al., 1980; Leach and Spitzer, 1981). Thus, the impaired gluconeogenic capacity of the liver, in the wake of increased peripheral glucose utilization, may explain the hypoglycemia associated with late sepsis and endotoxicosis.

Secretory proteins of the mononuclear phagocyte system (monokines)

have been implicated in the regulation of these events in both hepatic and peripheral tissues. Filkins (1979b) implicated an endotoxin induced glucoregulatory monokine in the stimulation of glucose oxidation in isolated fat pads. Since this was a characteristic of insulin, the term macrophage insulin-like activity (MILA) was proposed and the macrophage suggested to be functioning as an endocrine tissue in this situation (Filkins, 1984). This concept is further supported by the work of Berry et al. (1971, 1975, 1977) on the monokine known as the glucocorticoid-antagonizing factor, suggested to be responsible for the suppression of the synthesis of phosphoenolpyruvate carboxykinase and subsequent failure of gluconeogenesis.

Previous work in our laboratory has demonstrated the presence of several monokines in the culture supernatant of RAW 264.7 macrophages that appear to play a role in the induction of the catabolic state (Pekala et al., 1983a; Pekala et al., 1984; Price et al., 1986) in adipose tissue. As a major function of the catabolic state is to provide peripheral (muscle) tissue with glucose, we questioned as to whether these, and/or other, monokines might modulate hexose metabolism in muscle tissue. Filkins (1984) has proposed that these monokines act at two levels, first by stimulation of insulin secretion by the pancreas, and secondly by acting directly at the level of specific tissue to stimulate glucose oxidation.

The first step in glucose oxidation is the utilization of plasma glucose after transport into the cell. Since glucose is a polar molecule, it diffuses across the hydrophobic core of lipid bilayers very

slowly. Therefore, transport systems that facilitate its passage are found in the plasma membrane of most cell types (Elbrink and Bihler, 1975). Movement of hexose across the membrane occurs by facilitated diffusion using the hexose transporter, a heterogeneously glycosylated polypeptide with an apparent molecular weight of about 55,000 daltons (Baldwin and Lienhard, 1980; Wheeler and Hinkle, 1985). The isolated transporter contains about 15% carbohydrate by weight (Sogin and Hinkle, 1978) and its galactose residues can be radiolabeled by treatment of the intact cell with the impermeable enzyme, galactose oxidase, followed by reduction with sodium borotritide (Gorga et al., 1979). Therefore, the carbohydrate portion must be exposed on the outside of the cell. Heterogeneity in the carbohydrate moiety is evidenced by the fact that much of the radiolabel can be removed by treatment with an endo-β-galactosidase from Escherichia freundii that specifically cleaves galactose β1--->4N-acetylglucosamine residues (Gorga et al., 1979). Heterogeneity is also shown by the finding that after treatment with endo-β-galactosidase the broadness of the band exhibited by the transporter on SDS PAGE is supplanted by a much sharper band with an apparent molecular weight of 46,000 daltons (Gorga et al., 1979). Further studies concerning the position of the transporter in the membrane have dealt with the use of proteases which are membrane-impermeable. Treatment of the intact cell with trypsin has no effect on the ability of the cell to transport glucose unless inside-out vesicles are prepared from the membrane (Baldwin et al., 1980). This evidence indicates that the transporter must be exposed to the cytoplasmic face of the membrane, and since the carbohydrate portion of the transporter is exposed to the exterior of the cell,

the protein must span the membrane (Baldwin et al., 1980).

Elucidation of the mechanism of action of glucose transport has been aided by the use of the compound cytochalasin B, which is a member of the class of cytochalasins produced from several mold species. Cytochalasin B reversibly inhibits glucose transport by binding specifically to the glucose transporter. In addition, cytochalasin B also prevents actin polymerization by binding to the actin microfilaments. The cytochalasins D and E also bind to the actin filaments, but fail to have any affinity for the glucose transporter. Therefore, high concentrations of cytochalasin D or E can be used to limit the binding of cytochalasin B to sites other than the glucose transporter during binding studies.

Numerous studies using [^{3}H]-cytochalasin B have enabled investigators to clarify the mechanism of action of the glucose transporter in several different tissues (Cushman and Wardzala, 1980; Baldwin et al., 1981; Klip et al., 1982; Carter-Su et al., 1982; Klip and Walker, 1983). Adipocytes and muscle cells possess transport systems for glucose that can be stimulated as much as ten fold by insulin in both a rapid and reversible fashion (Whitesell and Gliemann, 1979). Kinetic studies have shown that in the rat adipocyte this phenomena results from an increase in the Vmax for transport, with no change in the Km (Whitesell and Gliemann, 1979). The increase in Vmax could result from either an increase in glucose transporters available at the plasma membrane, or from an increase in the rate of the existing transport system.

By using [^{3}H]-cytochalasin B binding techniques, Wardzala et al. (1978) found that the number of transporters in the plasma membranes of

adipocytes increased upon prior exposure of the cells to insulin. Further studies by Cushman and Wardzala (1980) indicated that the increased number of plasma membrane associated transporters were recruited from an intracellular membrane pool. Their findings, supported by those of Suzuki and Kono (1980), indicate that in cells exposed to insulin, the number of D-glucose specific transporters available at the plasma membrane increases by the same amount as the number of transporters decreases in the microsomal fraction. These studies and their interpretations represent an important advance in our understanding of the mechanism of insulin action, and most investigators now favor what has been termed the "recruitment" hypothesis.

An untested assumption of the recruitment hypothesis is that the hexose transporters contained in the low-density microsomal fractions are functioning organelles in the intact cell. In order to obtain low density microsomes, the cells must first be homogenized. Therefore, with the exception of the quantitative enzyme-marker data, there is no direct evidence that the transporters in the plasma membrane fraction actually have a different cellular location from those in the microsomal fraction. Recently, the laboratory of Michael Czech has developed a method of specifically labeling hexose transporters in numerous cell types so that the cellular location of the transporters can be monitored directly (Carter-Su et al., 1982; Pessin et al., 1982; Oka and Czech, 1984). The method involves using ultraviolet light to photoaffinity label [^{3}H]-cytochalasin B to the glucose transporter. The peak of the action spectrum for labeling was at 280 nm, where cytochalasin B does not have significant absorbance, indicating that the residue of the

transporter itself becomes activated by the light and reacts with the ligand. Czech's studies were able to confirm that the erythrocyte transporter has a molecular weight of about 55,000 and to identify the fibroblast glucose-transport systems (Rubin et al., 1977; Pessin et al., 1982). They were also able to confirm Kono's and Cushman's results regarding the recruitment hypothesis. The methods described above have enabled researchers to test the hypothesis that in response to insulin, latent transporters located in the microsomal fraction are translocated to the plasma membrane. The mechanism of this insulin-mediated conversion is still unclear but it is known that the mode of insulin action is energy-dependent and protein synthesis-independent (Kono et al., 1981).

The evidence that hexose transport is regulated both acutely and chronically by hormonal, nutritional and developmental signals indicates that regulation may affect transporter activity directly (Gliemann and Rees, 1983) or modify the localization and/or synthesis of the transport protein itself (Cushman and Wardzala, 1980; Pessin et al., 1982; Kono et al., 1982; Toyoda et al., 1986).

As peripheral tissues such as muscle must rely on glucose oxidation for most of its energy needs during infection, we are interested in studying how various monokines might regulate hexose metabolism in muscle during sepsis or endotoxicosis. The L6 muscle cell line cloned from the thigh of a 3 day old rat provides a convenient model for studying muscle tissue under controlled conditions. These cells are plated as myoblasts and undergo several cell divisions before fusing to form multinucleated fibers which continue to differentiate without fur-

ther nuclear replication (Yaffe, 1968; Shainberg et al., 1971). These multinucleated fibers are termed myotubes and exhibit characteristics indicative of skeletal muscle (Podelski et al., 1979). These characteristics include the ability to form myo-neural junctions when grown with explants of rat spinal cord (Steinbach et al., 1973), as well as possession of specific plasma membrane acetylcholine receptors (Seto et al., 1977). The L6 cells also engage in the synthesis of actin, myosin and creatine phosphokinase (type mm), which are specific marker proteins for muscle (Konieczny et al., 1982; Matsumura et al., 1983). As the L6 cells differentiate into myotubes, the synthesis of these proteins rises dramatically (Whalen et al., 1979; Feldman and Benoff, 1981; Beguinot et al., 1986). The development of insulin receptors and insulin responsiveness have also been demonstrated to correlate well with the onset of the fully differentiated myotube morphology (Beguinot et al., 1986).

The study of endotoxin-induced macrophage secretory proteins has been greatly aided by the utilization of the RAW 264.7 mouse monocyte-macrophage cell line. These cells can be grown in large quantities and secrete a number of regulatory monokines in response to E. coli lipopolysaccharide (Kawakami et al., 1982; Pekala et al., 1984; Beutler et al., 1985; Filkins, 1985; Mahoney et al., 1985). This cell line was established from the ascites of a tumor induced in a male mouse by the intraperitoneal injection of Abelson leukemia virus. Utilizing the RAW 264.7 cell culture system in conjunction with the 3T3-L1 cells, these studies will investigate the regulation of hexose transport in cultured murine L6 myotubes by a variety of monokines including preparations of cachectin, tumor necrosis factor, interleukin-1, and gamma-interferon.

EXPERIMENTAL PROCEDURES

Materials - Dulbecco's Modified Eagle's Medium was purchased from Gibco Laboratories, Grand Island, N.Y. Calf and fetal calf serum were purchased from Sterile Systems, Logan, Utah. Nuserum was obtained from Collaborative Research, Lexington, Mass. Insulin was provided by Eli Lilly Co. Lipopolysaccharide B (endotoxin) from E. coli 0127:B8 was obtained from Difco Laboratories, Detroit, Mich. Trioleate was obtained from Nu Chek Prep, Elysian, Mn. [^{3}H]triolein (19.9 Ci/mmol), [^{3}H]2-deoxy-D-glucose (30.2 Ci/mmol), [^{3}H]cytochalasin B (15.5 Ci/mmol), D[U-^{14}C]glucose-6-phosphate, sodium salt (125 mCi/mmol) and [^{35}S]-methionine (1200 Ci/mmol) were ordered from New England Nuclear, Boston, Massachusetts. Recombinant tumor necrosis factor and gamma interferon were supplied by Biogen. Purified recombinant interleukin-1 was the generous gift of Dr. Stephen B. Mizel, Department of Microbiology and Immunology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina. 3T3-L1 preadipocytes, L6 myoblasts, and RAW 264.7 macrophages were obtained from American Type Culture Collection. 2-Deoxy-D-glucose, cytochalasin B, enzymes, and all other biochemical reagents, unless otherwise specified, were obtained from Sigma Chemical Co., St. Louis, Missouri.

L6 Cell Culture - This continuously cultured muscle cell line are plated as myoblasts and eventually fuse to form multinucleated myotubes. These myotubes show several structural features indicative of skeletal muscle (Yaffe, 1968; Podelski et al., 1979). Cells were cultured at 37°C in

Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum in a humid atmosphere of CO_2, 90% air. Stock cultures were maintained by subculture every 5 days. Cells were detached with 0.05% trypsin, placed in serum-supplemented Dulbecco's Modified Eagle's Medium, and plated at an initial density of 10^4 cells/cm^2 in 0.5% gelatin-coated Corning 35-mm tissue culture plates and cultured for 18-20 days. Media was changed every other day until the cells were fully differentiated. In this study, myotubes refer to L6 cells grown for 18-20 days at which time creatine phosphokinase levels were at maximum levels and greater than 90% of the cells were multinucleated as determined by histological staining and microscopic examination.

3T3-L1 Preadipocyte Cell Culture - 3T3-L1 preadipocytes were cultured as described by Mackall *et al.* (1976) in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum in a humid atmosphere of 10% CO_2 at 37°C. Differentiation was induced using a modification of the method of Rubin *et al.* (1978). Two days post confluence the cells were placed in medium supplemented with 10% fetal calf serum, 0.5 mM 3-isobutyl-1-methylxanthine (MIX), 1 µM dexamethasone (DEX) and 10 µg insulin per ml of medium. After 48 hrs, the medium was removed and replaced with medium supplemented with fetal calf serum and insulin alone. Cells were used 3-5 days after the removal of MIX and DEX.

RAW 264.7 Macrophage Cell Culture and Incubation of Monolayers with Endotoxin - RAW 264.7 macrophages, obtained from the American Type Culture Collection, Bethesda, MD, were grown to confluence in Dulbecco's Modified Eagle's Medium supplemented with 10% Nuserum. After conflu-

ence, the medium was replaced with serum-free medium and the monolayers incubated in the presence of 0.5 μg endotoxin per ml of culture medium. After 20 hrs, the medium was removed and assayed for lipoprotein lipase suppressive activity.

Bioassay for Lipoprotein Lipase Suppressive Activity - Samples to be tested for monokine activity were added to fully differentiated 3T3-L1 cells and incubated under normal growth conditions for 17 hrs. At that time the medium was aspirated and the lipoprotein lipase associated with the plasma membrane was released by incubation for 1 hr in medium supplemented with 10 units per ml heparin. Previous studies have shown that the amount of lipoprotein lipase released into the medium on heparin treatment is the most sensitive assay for the lipoprotein lipase suppressive factor (Kawakami et al., 1982). Moreover, it accurately reflects total cell lipoprotein lipase activity and its suppression in response to the monokine (Kawakami et al., 1982). After heparin treatment, lipoprotein lipase activity was assayed according to the method of Nilsson-Ehle and Schotz (1976) with minor modification. Briefly, 75 μl of the enzyme preparation was combined with 25 μl of substrate consisting of 22.6 mM [^{3}H]triolein, 2.5 mg/ml lecithin, 40 mg/ml bovine serum albumin, 33% human serum (v/v), and 33% glycerol (v/v) and incubated at 37°C for 90 min. Reactions were stopped by addition of 3.25 ml of methanol-chloroform-heptane, 1.41:1.25:1 (v/v/v), followed by 1.05 ml of 0.1 M potassium carbonate-borate, pH 10.5. The free fatty acid released during the incubation was extracted by vigorous vortexing. A 0.3 ml aliquot of the upper aqueous phase was counted by liquid scintillation

spectroscopy. One mU of lipoprotein lipase activity is defined as the release of 1 nmole of oleic acid per minute. By definition, one unit of lipoprotein lipase suppressive activity is defined as the amount of crude conditioned medium required to achieve 50% suppression of lipoprotein lipase activity after a 17 hrs incubation. All assays were performed in triplicate.

Creatine Phosphokinase Assay - Creatine phosphokinase levels were determined by the method of Oscai and Holloszy (1971). Cells were scraped in 175 mM KCl containing 0.1 mM EDTA and sonicated on ice for 15 sec. Creatine phosphokinase activity was measured in a system coupled with pyruvate kinase and lactate dehydrogenase. The assay mixture contained in a final volume of 1 ml: 50 µmoles of Tris-HCl, pH 9.0; 5 µmoles of $MgCl_2$; 4 µmoles of ATP; 30 µmoles of creatine; 2.4 µmoles of phosphoenolpyruvate; 0.15 µmole of NADH; 50 µg of pyruvate kinase; and 50 µg of lactate dehydrogenase. After a 5 min incubation period, during which time contaminating ADP and pyruvate were used up, the reaction was started by the addition of creatine. The oxidation of NADH was then followed at 340 nM. All assays were performed in triplicate. A unit of enzymatic activity is defined as the amount of enzyme that catalyzes the formation of 1 µmole of product per min at 30°C.

Fructose-2,6-bisphosphate - Fructose-2,6-bisphosphate levels were determined as described by Van Schaftingen et al. (1982). Briefly, myotubes grown on 35 mm plates were scraped in 0.5 ml of 0.1 M NaOH and the resulting mixture heated for 5 min at 80°C. After cooling, the samples

were centrifuged and the supernatant neutralized with acetic acid in the presence of 20 mM Hepes. The mixture was again centrifuged and $Fru(2,6)P_2$ measured in the supernatant by its ability to stimulate pyrophosphate:fructose-6-phosphate phosphotransferase (PPi:PFK). PPi:PFK activity was assayed by the production of $Fru(1,6)P_2$. The assay mixture contained in a final volume of 3 ml: 50 mM Tris/HCl, pH 8; 5 mM $MgCl_2$; 0.15 mM NADH; 50 µg/ml aldolase; 10 µg/ml glycerol-3-phosphate dehydrogenase; and 1 µg/ml of triosephosphate isomerase. All assays were performed in triplicate.

Lactate - Culture medium was removed from plates and lactate measured by the method of Gutmann and Wahlefeld (1974). The assay mixture contained in a final volume of 3 ml: 0.43 M glycine, pH 9.0; 0.34 M hydrazine; 2.75 mM NAD; and 34 µg LDH. After an initial measurement at 340 nM (E_1), the reaction was started by the addition of the LDH suspension. After 60 min in a water bath at 25°C the change in absorbance was measured and the concentration of lactate in the mixture calculated. All assays were performed in triplicate.

Glycogen - L6 myotubes were scraped in 1 N ice cold perchloric acid and glycogen determined by the method of Keppler and Decker (1974). The assay mixture contained in a final volume of 1 ml: 0.3 M Triethanolamine hydrochloride, pH 7.5; 1 mM ATP; 0.9 mM NADP; 4.0 mM $MgSO_4$; 5.0 µg G6P-DH; and 9 µg hexokinase. After addition of hexokinase to the mixture, extinction was followed at 340 nM until constant.

Determination of 2-Deoxy-D-glucose Uptake - Fully differentiated L6 myo-

tubes were incubated in the presence of increasing concentrations of lipoprotein lipase suppressive activity for the indicated times prior to the assay. 2-Deoxy-D-glucose uptake was then measured by a modification of the method of Pekala et al. (1983b). The cells were rinsed 3 times with Krebs-Ringer-Hepes buffer supplemented with 1% (wt/vol) BSA (122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$-$7H_2O$, 0.3 mM $CaCl_2$, and 12 mM Hepes, pH 7.4). Uptake was measured by the addition of buffer containing various concentrations of 2-deoxy-D-glucose and 2-deoxy-D-[^{3}H]glucose (30.2 Ci/mmol) to the monolayer followed by incubation for 1-5 min at 37°C. At concentrations of 2-deoxy-D-[^{3}H]-glucose, 1 mM and above incubations were carried out for 1 min. At all other concentrations the length of incubation was 5 min. Specific activity of the reaction mixture varied from 0.3-5 μCi/μmole to insure adequate uptake of radiolabel. Uptake was terminated by aspiration of the medium and 4 washes with cold Krebs-Ringer-Hepes buffer containing 25 mM D-glucose. The cells were air-dried and lysed with 1 ml of 0.4 N NaOH, followed by sonication on ice for 15 sec. A sample of 700 μl was then neutralized with 0.4 N HCl and the radioactivity determined by liquid scintillation. The remainder of the sample was used for protein determination.

In order to avoid the problem of nonlinearity which has been ascribed to insufficient ATP levels (Graff et al., 1978), assays were routinely performed in the presence of 0.1 mM 2-deoxy-D-glucose for a maximum of 5 min. For the kinetic studies involving higher 2-deoxy-D-glucose concentrations, assay times were shortened to 1-3 min in order to reduce nonlinearity to negligible levels. The data obtained were fitted to the Michaelis-Menten equation using a non-linear curve-fitting

routine employing the simplex algorithm (Nelder and Mead, 1965; Deming and Morgan, 1973) as suggested previously by Dowd and Riggs (1965). Cytochalasin B (10 μM) was added to duplicate monolayers in order to estimate carrier-independent uptake.

2-Deoxy-D-Glucose Phosphorylation - Determination of intracellular 2-deoxy-D-glucose phosphorylation was performed by the method of Standaert et al. (1984). After 5 min of the uptake assay, 1 ml of 6% trichloroacetic acid was added immediately after the 4 cold termination washes. After 5 min, the cells were scraped in the acid and extracted 4 times with 1 ml of diethyl ether in glass tubes (13 X 100 mm). Residual ether was removed with a stream of air, and 100 μl of the aqueous layer was used to determine total 2-deoxy-D-glucose uptake. Aliquots of the aqueous portion were then placed on a 0.5 ml column of AG1-X8, 200-400 mesh, chloride ion exchange resin equilibrated with H_2O. Free 2-deoxy-D-glucose was determined by collecting the initial fractions along with two 1 ml H_2O washes. Phosphorylated 2-deoxy-D-glucose was eluted from the column with three 1 ml washes of 1 N HCl. The HCl and H_2O eluates were scintillation counted after neutralization of the HCl fractions with 1 N NaOH. Recovery of radioactivity of the columns was 95-100%.

Photoaffinity Labeling - Photoaffinity labeling of L6 cells was performed by a modification of Oka and Czech (1984). Cells were plated in thirty 100 mm gelatin coated Corning tissue culture plates at 10^4 cells/cm^2 and cultured for 20 days in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. The myotubes were then incu-

bated in the presence or absence of 20 U/ml of lipoprotein lipase suppressive activity for 17 hrs. The medium was aspirated off and the cells incubated in the presence of [^{3}H]cytochalasin B (15.5 Ci/mmol) and unlabeled cytochalasin B at a final concentration of 300 nM and cytochalasin E at a final concentration of 2 µM. D-glucose was added to one half of the plates at a concentration of 500 mM for determination of nonspecific binding. For the photolabeling procedure 1 µCi/ml of [^{3}H]-cytochalasin B was added to the plates and incubated at 37°C for 10 min. The cells were then irradiated (in a final volume of 5 ml) for three 10 s intervals with 10 sec rest periods with a 1000-watt Porta-cure lamp (American Ultraviolet Co.) through a glass color filter (no. 7-54, Farrand Optical Co.) which transmits light from 260 to 400 nM, at a distance of 26 cm. After irradiation the medium was aspirated off and the cells rinsed 2 x with warm Krebs-Ringer-Hepes, pH 7.4, and the cells subsequently scraped in homogenization medium consisting of 250 mM sucrose and 20 mM Hepes, pH 7.4, at 37°C.

Preparation of Plasma Membranes and Microsomes - Membrane fractions were prepared by a modification of Grimditch et al. (1985). After combining cell scrapes from seven 100 mm plates, the cells were further disrupted by polytron homogenization for 15 sec, at a setting of 7 (Model PT-10, Brinkman Instruments) in an ice bath. A volume of KCl medium (3 M KCl, 250 mM sodium pyrophosphate) equal to 10% of the homogenate volume was added to solubilize contractile proteins and the resulting mixture was vortexed vigorously. DNAase (10 µg) was added and the mixture incubated for 60 min at 30°C. The membranes were then mixed vigorously and spun

at 750 x g for 15 min. The supernatant was removed and spun at 200,000 x g for 60 min. The resulting pellet was resuspended in 1 ml of 45% sucrose and placed on the bottom of an ultracentrifuge tube, and a discontinuous gradient constructed by carefully adding 1.5 ml each of 38, 32, 30, 27 and 12% sucrose in layers. The gradient was centrifuged at 64,000 x g for 24 hrs. The gradient was fractionated into six 1.5 ml fractions, and the activity of the plasma membrane marker enzyme, γ-glutamyl transpeptidase, was determined in each by the method of Tate and Meister (1974). This assay involves the use of L-γ-glutamyl-p-nitroanilide where the formation of p-nitroaniline is determined from the increase in absorbance at 410 nm. The standard assay solutions contained in a final volume of 1 ml: 0.05 M Tris-HCl buffer, pH 8.0; 2.5 mM L-γ-glutamyl-p-nitroanilide; 20 mM glycylglycine, pH 8.0; and membrane preparation (0.2 ml). The rate of release of p-nitroaniline was followed at 410 nM in an IBM model 9410 spectrophotometer. The peak enzyme activity was located in the top (low density) 2 fractions. These fractions were combined and considered to be the plasma membrane fraction. The microsomal marker enzyme, glucose-6-phosphatase, was assayed across the fractionated gradient by the method of Kitcher et al. (1978). A 0.1 ml aliquot of each fraction was incubated in the presence of 10 mM glucose-6-phosphate, 50 mM PIPES, pH 6.5, and 1 μCi/ml [^{14}C]-glucose-6-phosphate. After 3-4 hrs the reaction was terminated by the addition of 0.7 ml BaOH followed by 0.5 ml of $ZnSO_4$, the mixture was centrifuged and radioactivity in 0.3 ml of the supernatant was determined by liquid scintillation techniques. The peak of glucose-6-phosphatase activity was determined to reside in fractions 4 and 5 of the gradient. These

fractions were combined and designated as a microsomal membrane fraction.

The amount of D-glucose competable [^{3}H]cytochalasin B binding activity in the isolated fractions was then determined by scintillation counting. Aliquots of the fractions were saved for protein determination.

Equilibrium Binding of [^{3}H]Cytochalasin B to Membrane Preparations of Fully Differentiated L6 Cells - Membrane preparations were prepared and characterized as described above from both control cells and those that had been exposed to 20 U/ml of lipoprotein lipase suppressive activity for 17 hrs. The individual membrane fractions (plasma and microsomal) were diluted 1:3 with 20 mM Hepes, pH 7.4, and concentrated by sedimentation at 200,000 x g for 24 hrs. The pellet was resuspended in 250 µl of cold 50 mM Tris-HCl, pH 7.0. Membrane protein (10-20 µg) was incubated overnight at 4°C in 50 mM Tris-HCl, pH 7.0, containing 76 nM [^{3}H]cytochalasin B and 5 µM cytochalasin E. Identical incubations were carried out in the presence of 500 mM D-glucose for determination of nonspecific binding. After the overnight incubation, membranes were sedimented at 100,000 x g and bound [^{3}H]cytochalasin B determined by liquid scintillation techniques.

Labeling of Cellular Protein - L6 myotubes were incubated in the presence or absence of 15 U/ml of lipoprotein lipase suppressive activity for 17 hrs. The monolayers were then washed once with phosphate buffered saline (PBS), pH 7.4, and incubated for 30 min in methionine-free

Dulbecco's Modified Eagle's medium under normal growth conditions. Afterwards, the medium was replaced with 1 ml of methionine-free medium containing 0.3 mCi [^{35}S]-methionine (1200 Ci/mmol) and incubated for 30 min. The medium was then removed, the cell monolayers washed twice with PBS, pH 7.4, and the soluble cytosolic proteins were released by the digitonin method of Mackall et al. (1979). The remainder of the cell monolayer containing the membranous fraction was then scraped in 1 ml of 100 mM Hepes buffer, pH 7.5, containing 0.5% Nonidet P-40 nonionic detergent and 1 mM phenylmethylsulfonyl fluoride. After trituration with a Pasteur pipette, the suspension was centrifuged at 10,000 x g for 10 min at 4°C, and the supernatant was saved. [^{35}S]-Methionine incorporation into acid-insoluble material was determined according to Pekala et al. (1983a). Aliquots of [^{35}S]-methionine-labeled samples were added to 0.5 ml ice cold 20% trichloroacetic acid (TCA) with 25 µl of 0.5% bovine serum albumin (BSA) added as carrier. After sitting at 4°C for 1 hr, the mixture was centrifuged at 2,000 x g for 5 min. The pellet was solubilized by incubation in 0.5 ml of 1 M NH_4OH at 37°C for 30 min. The protein was reprecipitated by addition of 5 ml of cold 10% TCA and filtered onto Whatman GF/C filters. The filters were extracted with diethyl ether for 20 min, dried, and counted by liquid scintillation spectroscopy.

SDS Polyacrylamide Gel Electrophoresis and Fluorography - SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) using a standard 95 linear polyacrylamide gradient resolving gel with a 3% polyacrylamide stacking gel. Protein molecular weights were deter-

mined using the following standards: myosin H chain (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa). Gels were fixed and stained according to Fairbanks et al. (1971) in 0.025% coomassie blue, 25% isopropanol, and 10% acetic acid and destained in 10% isopropanol, 10% acetic acid and 1% glycerol.

Gels were prepared for fluorography according to Chamberlain (1979) by soaking in H_2O for 30 min followed by a 1 M sodium salicylate solution for an additional 30 min. The gels were dried and exposed to Kodak XAR-5 film at -80°C.

Partial Characterization of Monokine - The RAW 264.7 cultured supernatant containing the macrophage mediator was harvested, phenylmethylsulfonyl fluoride (PMSF) added to a final concentration of 0.1 mM, and centrifuged at 9500 x g for 15 min at 4°C. The supernatant was concentrated using an Amicon YM-10 membrane hollow fiber concentrator in combination with an Amicon stirred cell with a PM-10 membrane.

The concentrated supernatant was applied to a TSK-3000 SW high performance liquid chromatography (HPLC) gel filtration column equilibrated in 50 mM Tris-Cl, pH 7.4, containing 150 mM NaCl and 10^{-4} M dithiothreitol. Fractions of 0.5 ml were collected. All even-numbered fractions (0.5 ml) were assayed for monokine activity as previously described and the fractions containing monokine activity pooled. Standards used to calibrate the column were bovine serum albumin (64 kDa), ovalbumin (45 kDa), myoglobin (17.2 kDa), and cytochrome C (12.4 kDa). The 0.5 ml fractions were collected with a flow rate of 0.2 ml/min at a pressure of

400 psi and a chart speed of 0.25 cm/min (full scale 1.0).

Isoelectric Focusing - The isoelectric point of the protein responsible for stimulation of glucose transport was determined using a LKB preparative electrofocusing column. Fractions 15-25 from the HPLC column were dialyzed against water for 6 hrs and the proteins then separated on a 120 ml, 0-40% glycerol gradient containing 2% Bio-Rad 3-10 ampholytes. The glycerol gradient was formed using a 500 ml BRL gradient former by mixing 60 ml of a "dense" solution (40% glycerol containing 1.875 ml ampholytes) and 60 ml of a "light" solution (the dialyzed protein sample, H_2O, and 0.625 ml ampholytes). The column was run with the anode solution (0.01 M H_2SO_4 in 45% glycerol) at the bottom of the column and the cathode solution (0.01 M NaOH) at the top. The column was electrofocused for 20 hrs at 4°C at a constant voltage of 500 volts. Afterwards, 2 ml fractions were collected and the pH of each fraction determined. Phenol red was added to each fraction and the pH adjusted to neutrality with either saturated potassium triphosphate or 30% phosphoric acid (5 µl increments). Even-numbered fractions were then assayed for the ability to suppress lipoprotein lipase in 3T3-L1 cells or enhance glucose transport in the L6 cells.

Protein Determination - All proteins were determined by either the method of Lowry *et al.* (1951) or Bradford (1976) using the BioRad reagent. Bovine serum albumin was used as standard.

RESULTS

Characterization of L-6 Cell Differentiation

Undifferentiated L-6 myoblasts plated at a density of 2000 cells/cm^2 of culture dish grow logarithmically, reach confluence in approximately 5 days, orient in a directional manner and begin fusing to form myotubes (Figs. 1-3). These results are in agreement with previous studies done with the L6 cell line (Yaffe, 1968; Podelski et al., 1979). Differentiation of the L6 cells to myotube morphology has also coincided with attainment of maximal levels of creatine phosphokinase (Konieczny et al., 1982; Beguinot et al., 1986). The activity of this enzyme reaches a maximum after 12-20 days in culture. The results in Figure 4 indicate that increases in creatine phosphokinase (CPK) levels began after the cells reached confluence (day 4) and continued to rise concomitantly with increases in cell protein. Maximum CPK levels (135 mU/mg protein) were obtained when cells were almost exclusively multinucleated myotubes as determined by cytological staining and microscopic examination (Figs. 1-3). Termination of differentiation, based on cell morphology and maximal CPK activity, was determined to be 16-18 days after plating (Fig. 3). Thus, all subsequent studies using the L6 cells were done at this time point. Effects of the various treatments on L6 myotube viability were examined by monitoring both lactate dehydrogenase leakage into the culture medium and trypan blue exclusion by the cells. By these methods, even after prolonged exposure of the cells to various monokines, no more than 2% of the myotubes exhibited loss of membrane integrity.

Fig. 1. L6 Myoblasts 24 Hours after Plating.

L6 cells were plated as described under "Experimental Procedures" and allowed to grow for 24 hrs. Cells were then rinsed 2 x with phosphate buffered saline, pH 7.4, and stained using a standard Papinacolau stain.

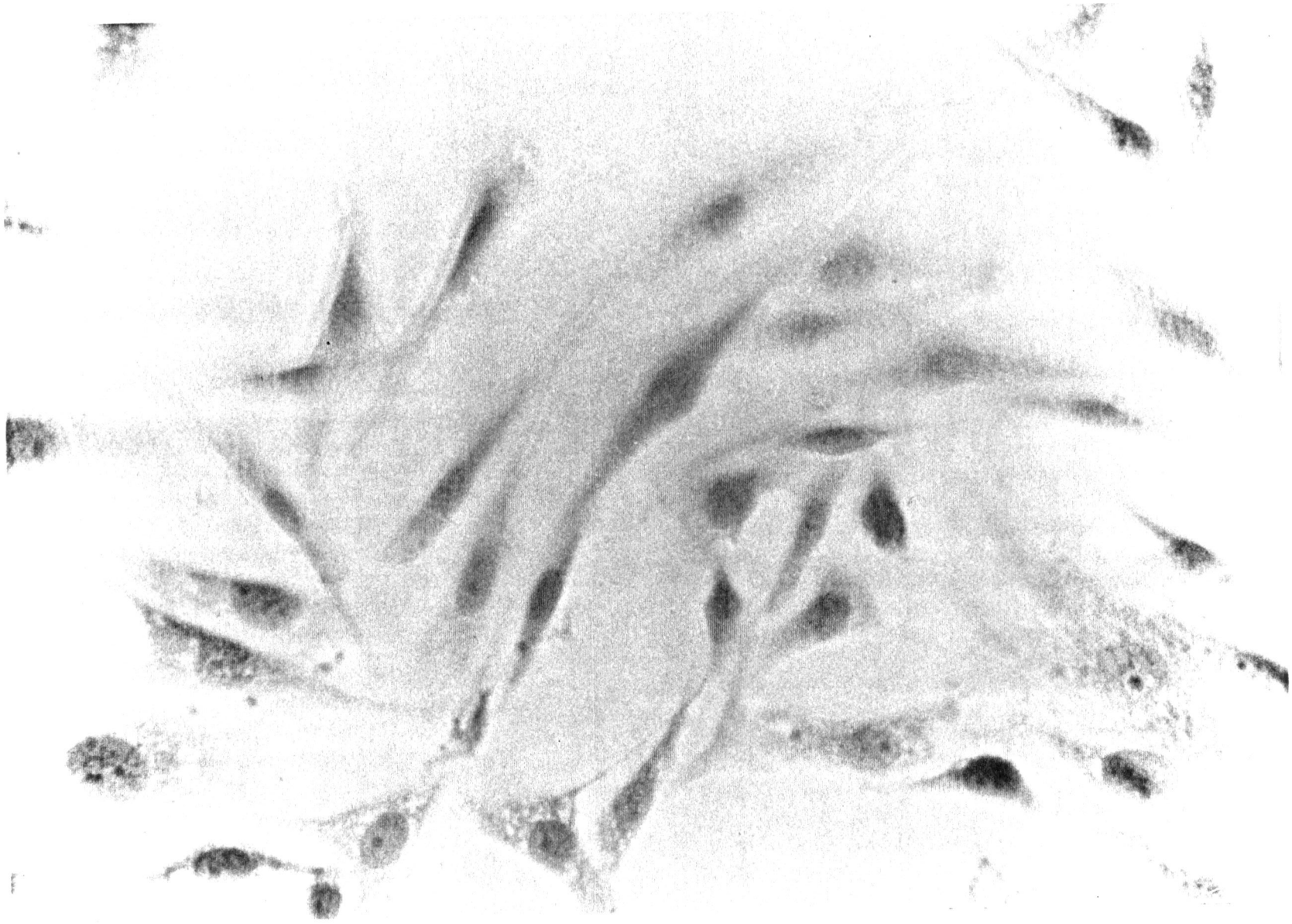


Fig. 2. L6 Cells Two Days Post-Confluence.

L6 cells were plated as described under "Experimental Procedures" and allowed to grow for 8 days. Cells were then rinsed 2 x with phosphate buffered saline, pH 7.4, and stained using a standard Papinacolau stain.

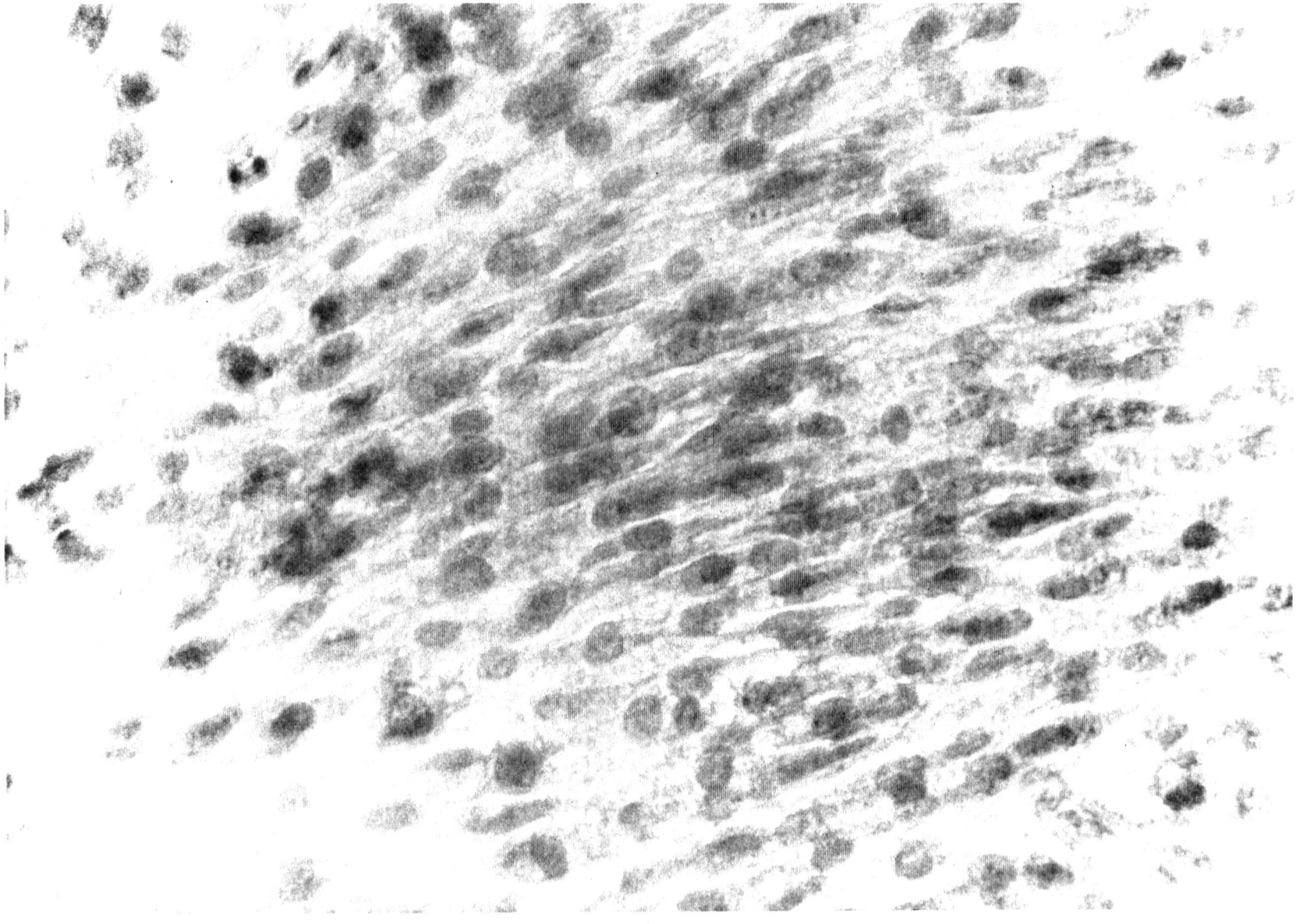
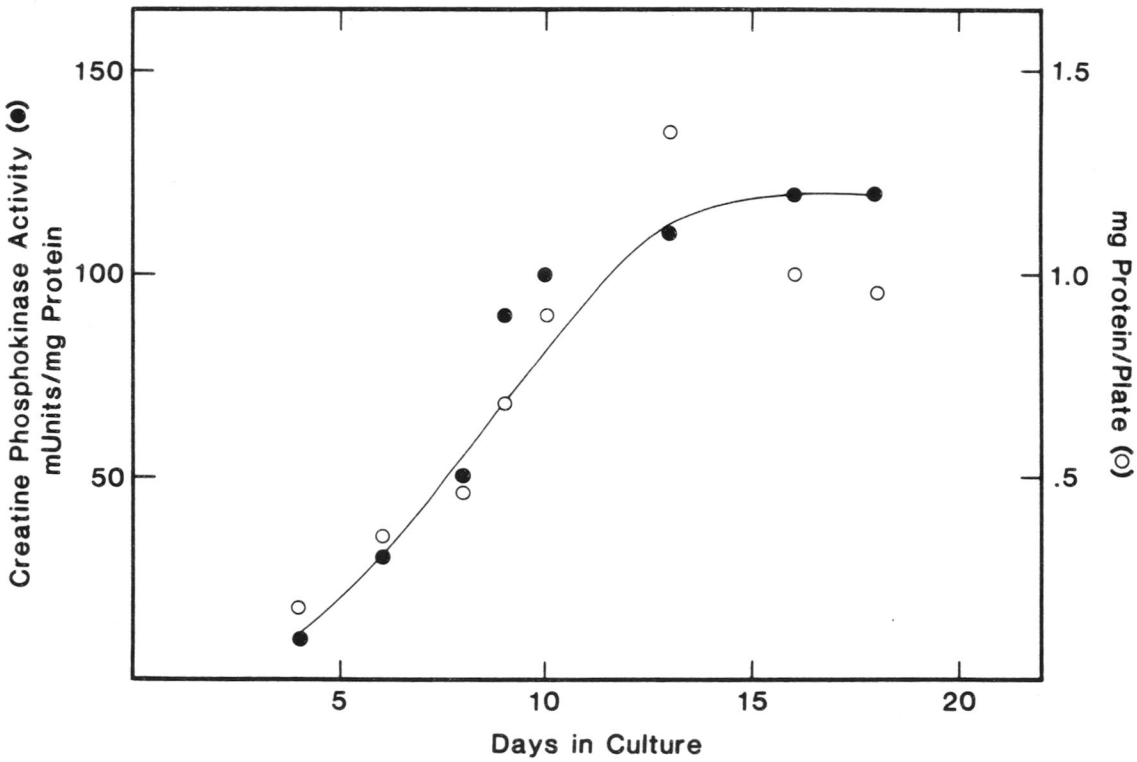


Fig. 3. L6 Cells Eighteen Days after Plating.

L6 cells were plated as described under "Experimental Procedures" and allowed to grow for 18 days. Cells were then rinsed 2 x with phosphate buffered saline, pH 7.4, and stained using a standard Papinacolau stain.

Fig. 4. Accumulation of Creatine Phosphokinase during Differentiation from Myoblasts to Myotubes.

L6 cells were plated and maintained; cell protein (O) and creatine phosphokinase (●) were determined as described in "Experimental Procedures". Each point is the mean of assays on duplicate monolayers in three separate experiments.


Creatine Phosphokinase Activity (●)
mUnits/mg Protein
150
100
50
1.5
1.0
.5
mg Protein/Plate (○)
5
10
15
20
Days in Culture

Standardization of the Monokine Preparation.

As a reference for monokine activity, conditioned medium from RAW 264.7 cells that had previously been stimulated with endotoxin was concentrated, dialyzed and tested for the ability to suppress the activity of lipoprotein lipase in 3T3-L1 fatty fibroblasts (Fig. 5). A unit of lipoprotein lipase activity is defined as the amount of conditioned medium required to suppress lipoprotein lipase to 50% of control cell values. From the experiment displayed in Figure 5 we were able to determine that 1 unit of lipoprotein lipase suppressive activity corresponded to 3 µl of conditioned medium from endotoxin stimulated RAW 264.7 cells.

Monokine Stimulation of 2-Deoxy-D-Glucose Uptake in L6 Myotubes.

When fully differentiated L6 myotubes were exposed to the monokine preparation, a dose dependent increase in 2-deoxy-D-glucose uptake was observed with 5 units of lipoprotein lipase suppressive activity (LLSA) required for half-maximal stimulation (Fig. 6). A maximum 200% increase over basal 2-deoxy-D-glucose uptake was observed, and separate experiments using as much as 100 units of LLSA failed to produce any greater increases in hexose uptake in the L6 cells.

In order to determine if the effect on hexose uptake was a time dependent process, L6 myotubes were exposed to 15 units of LLSA for increasing amounts of time (Fig. 7). The results indicate a time dependent process with maximal increases in 2-deoxy-D-glucose uptake occurring 12-17 hrs after exposure of the cells to the monokine preparation.

Fig. 5. **Characterization of Lipoprotein Lipase Suppressive Activity in 3T3-L1 Cells.**

Monolayers of fully differentiated 3T3-L1 cells were incubated in the presence of increasing concentrations of crude conditioned medium from RAW 264.7 macrophages for 17 hrs in a total volume of 2 ml. The activity of the heparin releasable fraction of lipoprotein lipase was measured as described in "Experimental Procedures". One unit of lipoprotein lipase suppressive activity is defined as the amount of crude conditioned medium required to achieve 50% suppression of lipoprotein lipase activity.

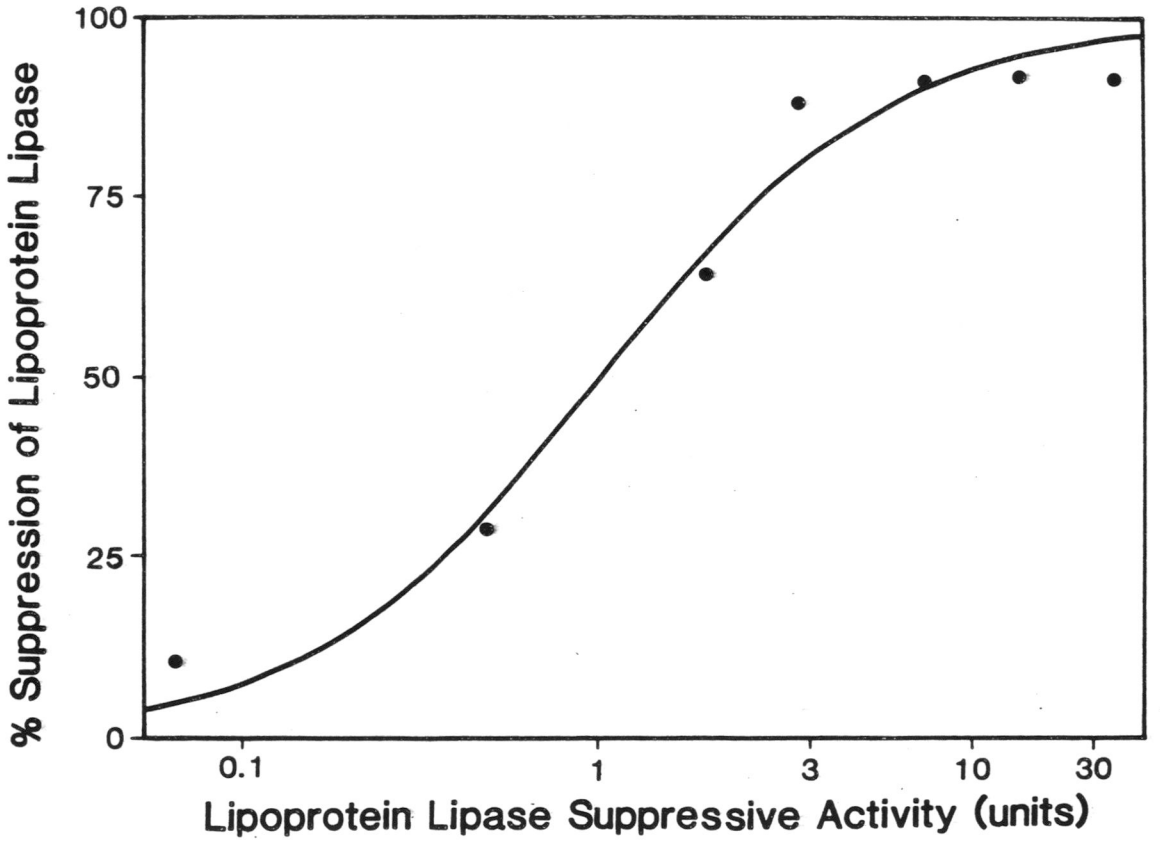

% Suppression of Lipoprotein Lipase
100
75
50
25
0
0.1
1
3
10
30
Lipoprotein Lipase Suppressive Activity (units)

Fig. 6. Monokine Dependent Increase in 2-Deoxy-D-Glucose in L6 Myotubes.

Fully differentiated myotubes were incubated for 17 hrs in the presence of increasing concentrations of lipoprotein lipase suppressive activity. Rates of 2-deoxy-D-glucose uptake were determined by incubating the cells for 5 min in 2 ml of Krebs-Ringer-Hepes buffer containing 1% BSA and [^{3}H]2-deoxy-D-glucose at a final concentration of 0.1 mM. The reaction was terminated by the addition of cold (4°C) Kreb's-Ringer-Hepes buffer containing 25 mM D-glucose. Cells were then air dried and lysed with 0.4 N NaOH, sonicated, and neutralized by the addition of 0.4 N HCl. Radiolabeled 2-deoxy-D-glucose uptake was then determined by liquid scintillation.

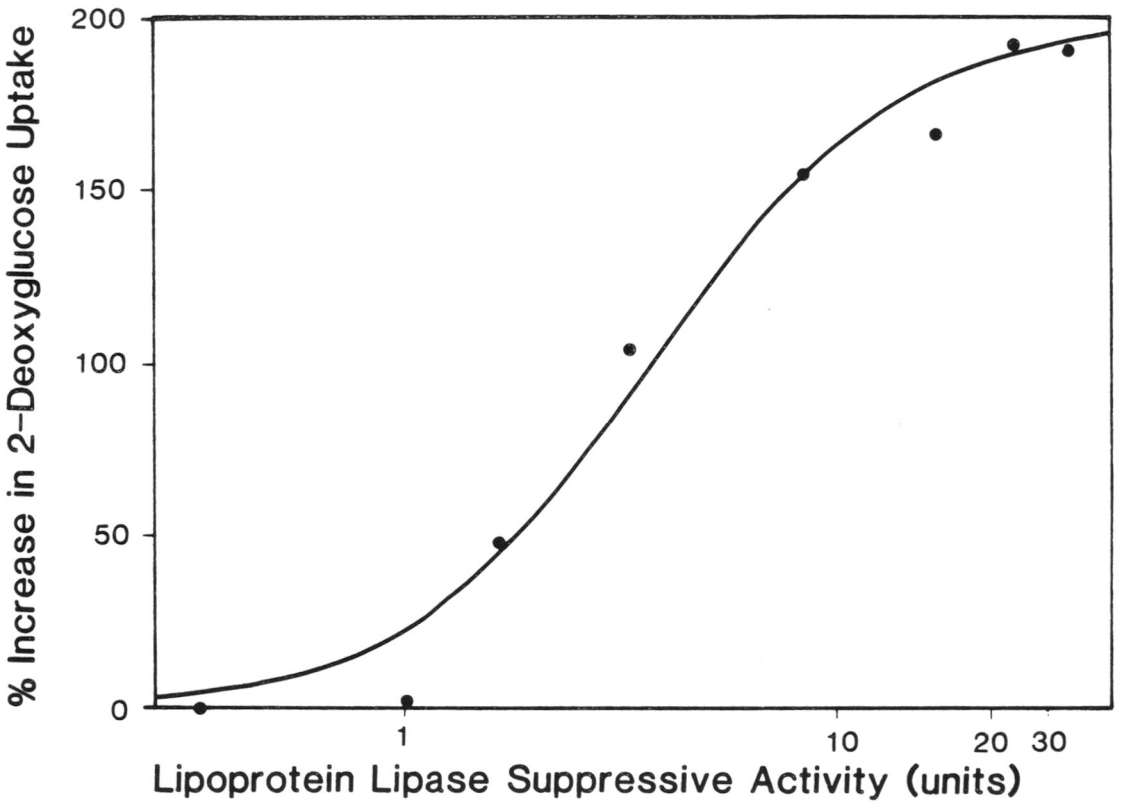
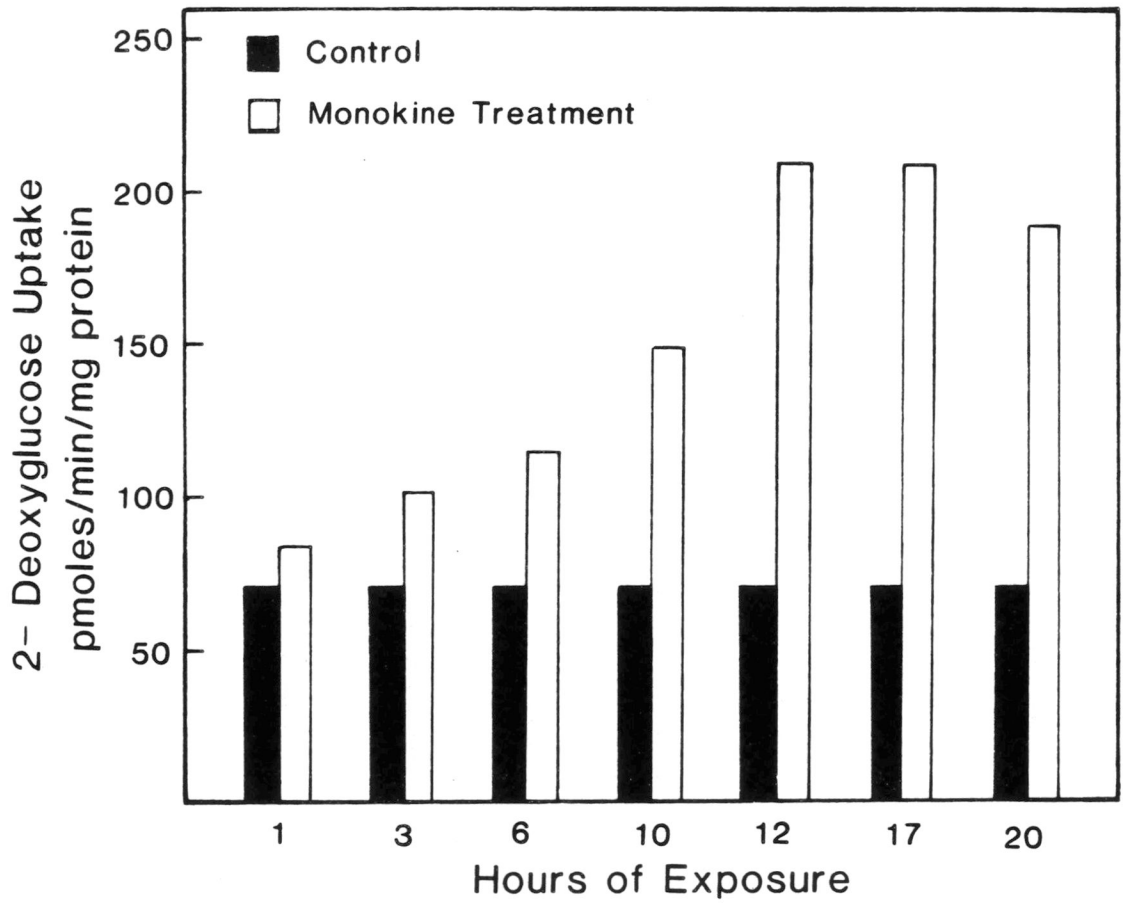

% Increase in 2-Deoxyglucose Uptake
200
150
100
50
0
1
10
20
30
Lipoprotein Lipase Suppressive Activity (units)

Fig. 7. **Time Course for Stimulation of 2-Deoxy-D-Glucose Uptake in L6 Myotubes by the Monokine Preparation.**

Fully differentiated L6 myotubes were maintained as previously described and incubated for the indicated times in the presence (□) or absence (■) of 15 units of lipoprotein lipase suppressive activity. 2-Deoxy-D-Glucose was then measured as described under "Experimental Procedures".


Control
Monokine Treatment
2- Deoxyglucose Uptake
pmoles/min/mg protein
250
200
150
100
50
1
3
6
10
12
17
20
Hours of Exposure

Protein synthesis appeared to be required for monokine stimulation of hexose uptake, as cycloheximide effectively blocked the increase without affecting basal 2-deoxy-D-glucose uptake (Fig. 8).

Using 2-deoxy-D-glucose as the substrate for determining the kinetics of monokine stimulation of glucose uptake in the L6 cells, it was important to confirm that transport was rate-limiting. The data in Table II indicates that 80-90% of the total intracellular 2-deoxy-D-glucose was phosphorylated after 5 min at a final concentration of 0.1 mM. Furthermore, we determined that the monokine preparation had no significant effect on the degree of phosphorylation.

The effect of exposure of the myotubes to the monokine preparation on the rate of 2-deoxy-D-glucose uptake as a function of hexose concentration is shown in Figure 9. The data were fitted to the Michaelis-Menten equation and kinetic constants determined using a non-linear curve-fitting routine employing the simplex algorithm (Nelder and Mead, 1965; Deming and Morgan, 1973) as suggested by Dowd and Riggs (1965). The data indicate that after exposing the L6 myotubes to the monokine preparation, no change was observed in the Km for the system (1.1 mM) while the Vmax increased from 912 to 2400 pmoles/min/mg protein.

In order to compare the action of the monokine to that of insulin, cells were exposed to 10^{-7} M insulin for 30 min, resulting in only a 63% stimulation of 2-deoxy-D-glucose uptake (Table III). This is identical to that first observed by Klip et al. (1984). Moreover, this stimulation by insulin could not be blocked by cycloheximide (Table III). Longer exposure to insulin resulted in no further stimulation of 2-deoxy-D-glucose. In contrast, cells exposed to the 10^{-7} M insulin for

Fig. 8. **Effects of Cycloheximide on Monokine-Stimulated 2-Deoxy-D-Glucose Uptake in L6 Myotubes.**

Cells were cultured and maintained as previously described and then incubated under the following conditions. (☐) control cells, no addition; (▨) 15 U of lipoprotein lipase suppressive activity; (▩) 15 U of lipoprotein lipase suppressive activity and 11 μM cycloheximide; (▒) 11 μM cycloheximide only. After the indicated times, 2-deoxy-D-glucose uptake was determined as described under "Experimental Procedures".

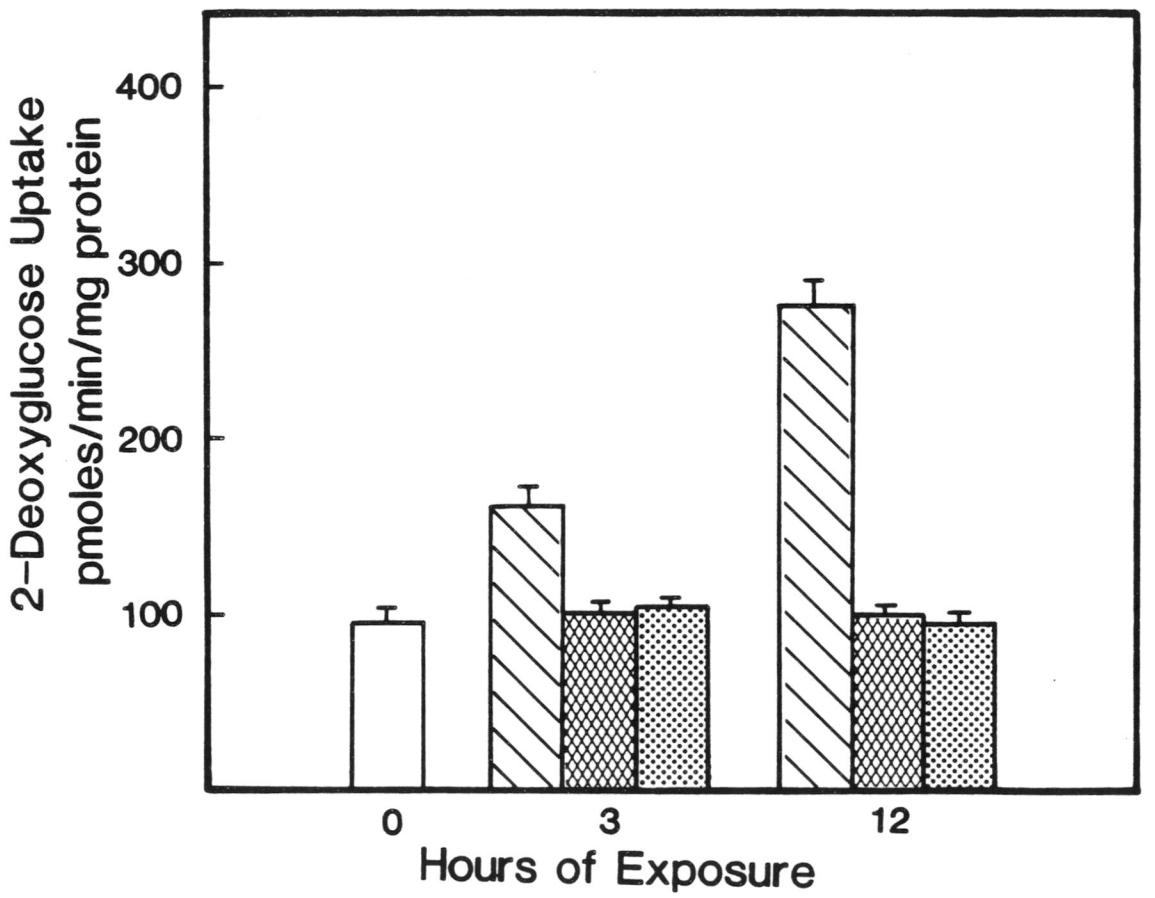

2-Deoxyglucose Uptake
pmoles/min/mg protein
400
300
200
100
0
3
12
Hours of Exposure

TABLE II

Distribution of Transported Hexose Between Phosphorylated and Nonphosphorylated Forms

Cell Treatment	Distribution of Radioactivity after Transport of 2-Deoxy-D-Glucose		
	CPM		
	2-deoxy-D-glucose	2-deoxy-D-glucose-6-phosphate	Total
Control	415 ± 153	2377 ± 127	2788 ± 134
Monokine-treated	1194 ± 500	5342 ± 586	6536 ± 967

Fully differentiated L6 myotubes were incubated in the presence or absence of 15 units of lipoprotein lipase suppressive activity for 17 hrs in a final volume of 2 ml. The cells were then incubated in the presence of 2-deoxy-D-[^{3}H]glucose and unlabeled 2-deoxy-D-glucose at a final concentration of 0.1 mM. After 5 min the reaction was terminated and 1 ml of ice cold 5% TCA was added. After an additional 5 min, cells were scraped and the amount of phosphorylation determined as described in "Experimental Procedures". Results are expressed in cpm ± standard deviation.

Fig. 9. Kinetics of 2-Deoxy-D-Glucose uptake in L6 myotubes.

Cells were cultured as described in Figure 6 and incubated for 17 hrs in the presence of 15 Units of lipoprotein lipase suppressive activity. 2-Deoxy-D-Glucose uptake was then initiated at the indicated concentrations. In order to insure that phosphorylation was not rate limiting at the higher concentrations, the reaction was terminated after 1 min. 10 μM cytochalasin B was added to duplicate plates in order to subtract the cytochalasin B insensitive component of uptake. 2-Deoxy-D-Glucose uptake was then measured as described in Figure 6.

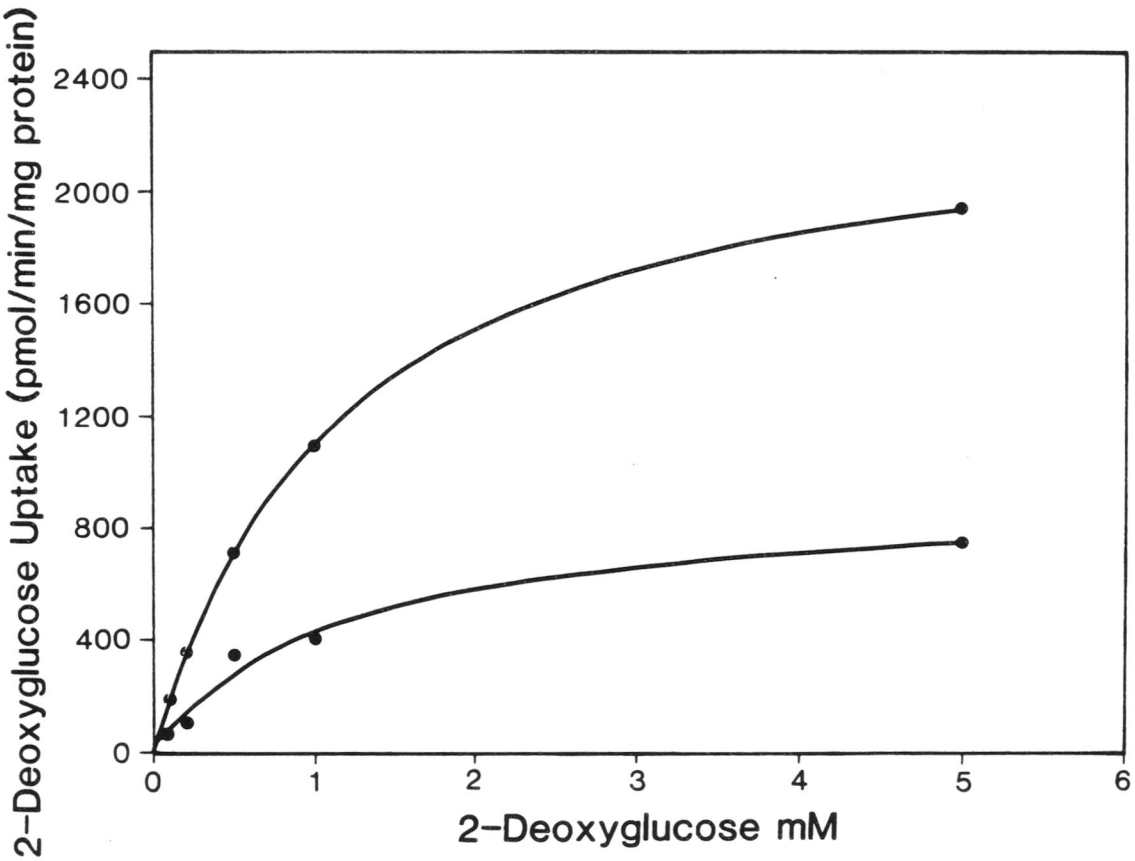

2-Deoxyglucose Uptake (pmol/min/mg protein)
2400
2000
1600
1200
800
400
0
0
1
2
3
4
5
6
2-Deoxyglucose mM

TABLE III

Insulin Stimulation of 2-Deoxy-D-Glucose Uptake in L6 Myotubes

Additions to Culture	2-Deoxy-D-Glucose Uptake pmol/min/mg protein	% Stimulation
No Additions, control	122 ± 5	--
11 μM cycloheximide	128 ± 8	--
0.1 μM Insulin	199 ± 11	63
0.1 μM Insulin and 11 μM cycloheximide	206 ± 12	61
Monokine treated	276 ± 17	126
Monokine treated, then 0.1 μM Insulin	286 ± 18	134

Fully differentiated L6 myotubes were incubated in serum-free medium for 12 hrs in the presence or absence of 15 μg/ml of the monokine preparation. The cells were then incubated in the presence or absence of 10^{-7} M insulin and 11 μM cycloheximide for 20 min at 37°C. Uptake of 2-deoxy-D-[^{3}H]glucose was then measured as described in "Experimental Procedures".

20 min after a 17 hr exposure to the monokine preparation failed to achieve any greater increases in 2-deoxy-D-glucose uptake than cells exposed to the monokine preparation alone (Table III).

In both control and monokine stimulated cells, hexose transport could be inhibited by greater than 90% by 10 μM cytochalasin B indicating transport via the specific D-glucose transporter.

Characterization of Plasma Membranes and Microsomes

In order to determine the purity of our membrane preparations, specific marker enzymes were assayed in the six 1.5 ml fractions from the sucrose gradient. The marker enzyme used to characterize the plasma membrane fractions was γ-glutamyl-transpeptidase, which catalyzes the transfer of the γ-glutamyl moiety of a γ-glutamyl donor to a variety of acceptors such as amino acids or peptides. The microsomal marker enzyme used was glucose-6-phosphatase. The results in Table IV indicate that the peak of enzyme recovery for γ-glutamyl-transpeptidase was found in fractions F1 and F2. Recovery of γ-glutamyl-transpeptidase in these fractions relative to the initial cell homogenate was 11.79%. The purification index is defined as the ratio of specific activity (μmol/h/mg protein) of a given fraction to the activity of the crude homogenate. The data in Table V indicate that fractions F1 and F2 had purification indexes of 159 and 129 respectively for γ-glutamyl-transpeptidase. This data, in conjunction with the total enzyme recovery data from Table IV, enabled us to conclude that fractions F1 and F2 could be combined and considered to be the plasma membrane fraction.

The peak of glucose-6-phosphatase activity was determined to reside

in fractions F4 and F5 of the gradient and revealed an 8.8% yield relative to the initial cell homogenate. Purification studies of fractions F4 and F5 yielded purification indexes of 42.2 and 46.13 respectively. Consequently, these two fractions were combined and designated as the microsomal membrane fraction. Because fractions F3 and F6 exhibited significant activity for both marker enzymes, they were not used in the subsequent experiments.

Effect of Monokine Exposure on the Distribution of D-Glucose Transporters.

Control and monokine treated monolayers of L-6 myotubes were exposed to [^{3}H]cytochalasin B in the presence or absence of D-glucose, as described in "Experimental Procedures". The monolayers were briefly irradiated with intense 260-400 nm light, after which the remaining free ligand was removed and the plasma and microsomal membrane fractions isolated by sucrose density gradient centrifugation (detailed in "Experimental Procedures"). Radioactivity specifically competable by D-glucose was considered to be associated with the hexose transporter. The results displayed in Table VI indicate that after exposure to the monokine, the [^{3}H]cytochalasin B associated with the plasma membrane increased 2.2 fold while that associated with the microsomal membranes increased nearly 4-fold.

These results were confirmed and extended using equilibrium binding techniques. Membrane preparations isolated from both control and monokine treated myotube cultures were exposed to 76 nM [^{3}H]cytochalasin B both in the presence and absence of D-glucose. Radioactivity specifi-

TABLE IV

Analysis of Marker Enzymes

Marker	Crude Homogenate	F1	F2	F3	F4	F5	F6
Total Protein (μg)	20,240	8.6	7.91	19.21	19.95	20.20	24.1
γ-glutamyl-Pase (Units)	3.34	0.226	0.168	0.104	0.0185	.0016	0.029
% Yield	--	6.77	5.02	3.12	0.55	0.35	0.87
Glucose-6-Pase (Units)	18.76	0.0081	0.014	0.183	0.78	0.864	0.76
% Yield	--	0.04	0.07	0.98	4.20	4.60	4.07

Fully differentiated L6 myotubes were homogenized and placed on a sucrose gradient as described under "Experimental Procedures". Isolated fractions were then assayed for γ-glutamyl-transpeptidase and glucose-6-phosphatase activity as described under "Experimental Procedures". The enzyme values represent units of enzyme activity.

TABLE V

Analysis of Membrane Purity

Marker	Crude Homogenate	F1	F2	F3	F4	F5	F6
Total Protein (μg)	20,240	8.6	7.91	19.21	19.95	20.2	24.1
γ-glutamyl-Pase (μmol/h/mg protein)	.165	26.29	21.23	5.42	.925	.575	1.21
Purification Index	—	159	129	33	5.6	3.48	7.33
Glucose-6-Pase (μmol/h/mg protein)	.927	.947	1.76	9.55	39.12	42.76	31.69
Purification Index	1.02	1.90	10.30	42.2	46.13	34.19	34.0

Fully differentiated L6 myotubes were homogenized and placed on a sucrose gradient as described under "Experimental Procedures". Isolated fractions were then assayed for γ-glutamyl-transpeptidase and glucose-6-phosphatase activity as described under "Experimental Procedures". The enzyme values represent specific activity (μmol/hr/mg protein).

TABLE VI

Effect of Monokine Treatment on the Distribution of D-Glucose Competable Cytochalasin B Binding Between Plasma and Microsomal Membranes

Preparation	[^{3}H]-Cytochalasin B Binding	
	Photoaffinity Labeling	Equilibrium Binding
	pmol/mg protein	
Control Membranes		
plasma	8.4 ± 1.5	15.4 ± 1.60
microsomal	3.5 ± 0.30	6.6 ± 0.63
Monokine-Treated Membranes		
plasma	18.7 ± 3.79	33.7 ± 4.9
microsomal	13.6 ± 1.72	20.1 ± 3.5

Fully differentiated L6 myotubes were incubated ± 20 units/ml of lipoprotein lipase suppressive activity for 17 hrs and the monolayers used either directly for the photoaffinity labeling procedure or for membrane preparation for the equilibrium binding studies as described in "Experimental Procedures". The results are the mean of duplicate experiments ± S.D.

cally associated with each membrane fraction was determined after a 20 hr incubation at 4°C. These data, displayed in Table VI, are consistent with the hypothesis that exposure of the cells to the mediator increases glucose transport by increasing available transporters. Moreover, these results point to the small number of transporters available for binding cytochalasin B present in microsomal membranes from control cells. This may represent the molecular basis for the minimal, acute stimulation (66%) of hexose transport by insulin (Table III).

The Effect of Recombinant Tumor Necrosis Factor on Lipoprotein Lipase Activity in 3T3-L1 Fatty Fibroblasts and on 2-Deoxy-D-Glucose Uptake in L-6 Myotubes.

A protein, present in the conditioned medium of endotoxin-treated RAW 264.7 cells and purified based on its ability to suppress the activity and synthesis of lipoprotein lipase in 3T3-L1 fatty fibroblasts, has been demonstrated to be identical to tumor necrosis factor (TNF) (Beutler et al., 1985). Purified recombinant TNF was first demonstrated to be active in its ability to suppress lipoprotein lipase in the 3T3-L1 fatty fibroblasts (Fig. 10). This monokine was very potent in this reaction, with less than 5 ng (76 pM) required for half maximal suppression of the lipase. However, it was a very poor agonist in its ability to stimulate 2-deoxy-D-glucose uptake in the L6 myotubes (Fig. 10). Approximately 2000 pM were required for half maximal stimulation with saturation occurring at over 3000 pM. At this extremely high concentration of TNF, the maximal stimulation obtained was only 85% above basal. This contrasts with the 200% stimulation observed with the crude mono-

kine preparation.

Attempts were also made to stimulate 2-deoxy-D-glucose uptake using purified recombinant interleukin-1 and gamma interferon. However, neither alone nor in combination with TNF did exposure of the myotubes to interleukin-1 or gamma interferon result in an enhancement of hexose uptake.

Effect of the Monokine Preparation on Hexose Metabolism in L6 Myotubes.

Exposure of the myotubes to the mediator preparation resulted in the rapid depletion of glycogen stores concomitant with an increased production of lactate (Fig. 11). The potent regulator of glycolysis, fructose-2,6-bisphosphate, increased rapidly in concentration to nearly two fold over basal. The time frame for this increase is coincident with that for glycogen depletion and lactate production.

Exposure of the cells to purified, recombinant tumor necrosis factor stimulated neither glycogen depletion nor lactate production.

Effect of Crude Monokine Preparation on General Protein Synthesis

In order to determine if the crude monokine preparation had any effect on overall protein synthesis, L6 myotubes were incubated in the presence or absence of the monokine preparation and [^{35}S]methionine incorporation into protein determined as described in "Experimental Procedures". Exposure of the cells to the crude monokine preparation for 1, 3, 6, and 17 hrs had minimal effect on acid-insoluble protein (Tables VII and VIII) in either the membrane fraction (Nonidet P-40 solubilized protein) or the cytosolic fraction (digitonin released protein). The

Fig. 10. Effect of Recombinant Tumor Necrosis Factor on Lipoprotein Lipase Activity in 3T3-L1 Cells and 2-Deoxy-D-Glucose Uptake in L6 Cells.

Fully differentiated 3T3-L1 cells were incubated in the presence of increasing concentrations of recombinant tumor necrosis factor for 17 hrs in a total volume of 2 ml. The lipase activity of the heparin-releasable fraction of lipoprotein lipase was measured as described in "Experimental Procedures". Fully differentiated L6 myotubes were incubated in the presence of increasing concentrations of recombinant tumor necrosis factor for 17 hrs. 2-Deoxy-D-Glucose uptake was then measured as described in Figure 3.

% SUPPRESSION OF
LIPOPROTEIN LIPASE ACTIVITY (●)
100
80
50
20
50
100
150
TUMOR NECROSIS FACTOR (ng)
100
80
50
20
% INCREASE IN 2-DEOXYGLUCOSE UPTAKE (○)

Fig. 11. Effect of Monokine Preparation on Metabolite Levels in L6 Myotubes.

Fully differentiated L6 myotubes were incubated in the presence of 15 units of lipoprotein lipase suppressive activity for the indicated times. The medium was then collected and lactate determined as described under "Experimental Procedures". The cell monolayer was digested in 1 N NaOH and fructose-2,6-bisphosphate levels determined as described under "Experimental Procedures". Duplicate monolayers were scraped in 10% TCA and glycogen levels determined as described under "Experimental Procedures". Results are the average of 3 experiments performed in duplicate.

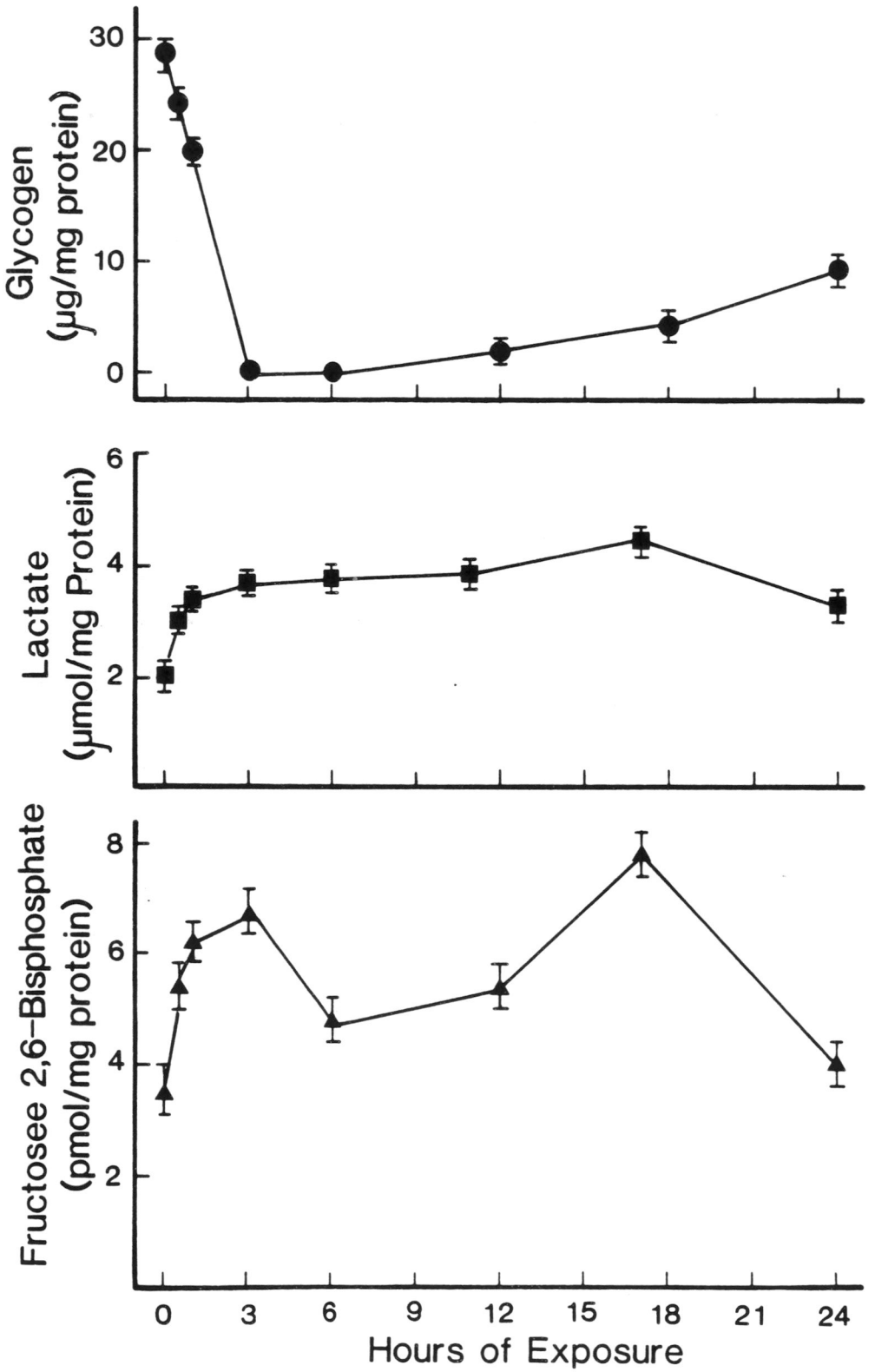

Glycogen
(µg/mg protein)
30
20
10
0
Lactate
(µmol/mg Protein)
6
4
2
Fructosee 2,6-Bisphosphate
(pmol/mg protein)
8
6
4
2
0
3
6
9
12
15
18
21
24
Hours of Exposure

TABLE VII

Effect of Crude Monokine Preparation on [^{35}S]Methionine Incorporation into Cytosolic Protein

Time of Treatment	cpm [^{35}S]Methionine Incorporated/μl	% Change
0 hour control	77,029 ± 1,219	
1 hours	70,017 ± 91	9
3 hours	70,710 ± 2,558	8
6 hours	66,469 ± 2,366	14
17 hours	69,469 ± 1,980	10

Fully differentiated L6 myotubes were pulse labeled for 30 min with [^{35}S]methionine as described in "Experimental Procedures". The cytosolic protein fraction was obtained by digitonin treatment and [^{35}S]methionine incorporation was determined as described under "Experimental Procedures". Duplicate determinations were performed on duplicate plates and the results expressed are the mean ± standard deviation.

TABLE VIII

Effect of Crude Monokine Preparation on [^{35}S]Methionine Incorporation into Membrane Protein

Time of Treatment	cpm [^{35}S]Methionine Incorporated/µl	% Change
0 hour control	112,545 ± 14,343	
1 hours	134,884 ± 6,458	20
3 hours	115,698 ± 106	2.8
6 hours	115,116 ± 403	2.3
17 hours	120,566 ± 2,327	7.1

Fully differentiated L6 myotubes were pulse labeled for 30 min with [^{35}S]methionine as described in "Experimental Procedures". The membrane protein fraction was extracted with Nonidet P-40. [^{35}S]Methionine incorporation into this fraction was then determined as described in "Experimental Procedures". Duplicate determinations were performed on duplicate plates and the results expressed are the mean ± standard deviation.

Fig. 12. Effect of Monokine Preparation on Protein Synthesis in the Cytosolic Fraction of the Cells.

Autoradiogram of a 7.5% acrylamide/0.1% SDS gel analysis of [^{35}S]methionine-labeled cytosolic protein after exposure to 15 units of the monokine preparation. L6 cells were pulse-labeled and the soluble protein was obtained by digitonin treatment. Aliquots (2 x 10^6 cpm) of the cytosolic fraction from each time point were applied to the gel and electrophoresed. Lane A, control cells; lane B, exposure to the monokine preparation for 1 hr; lane C, exposure for 3 hrs; lane D, exposure for 6 hrs; lane E, exposure for 17 hrs. Protein standards, molecular weights x 10^{-3}, are indicated on the left margin.

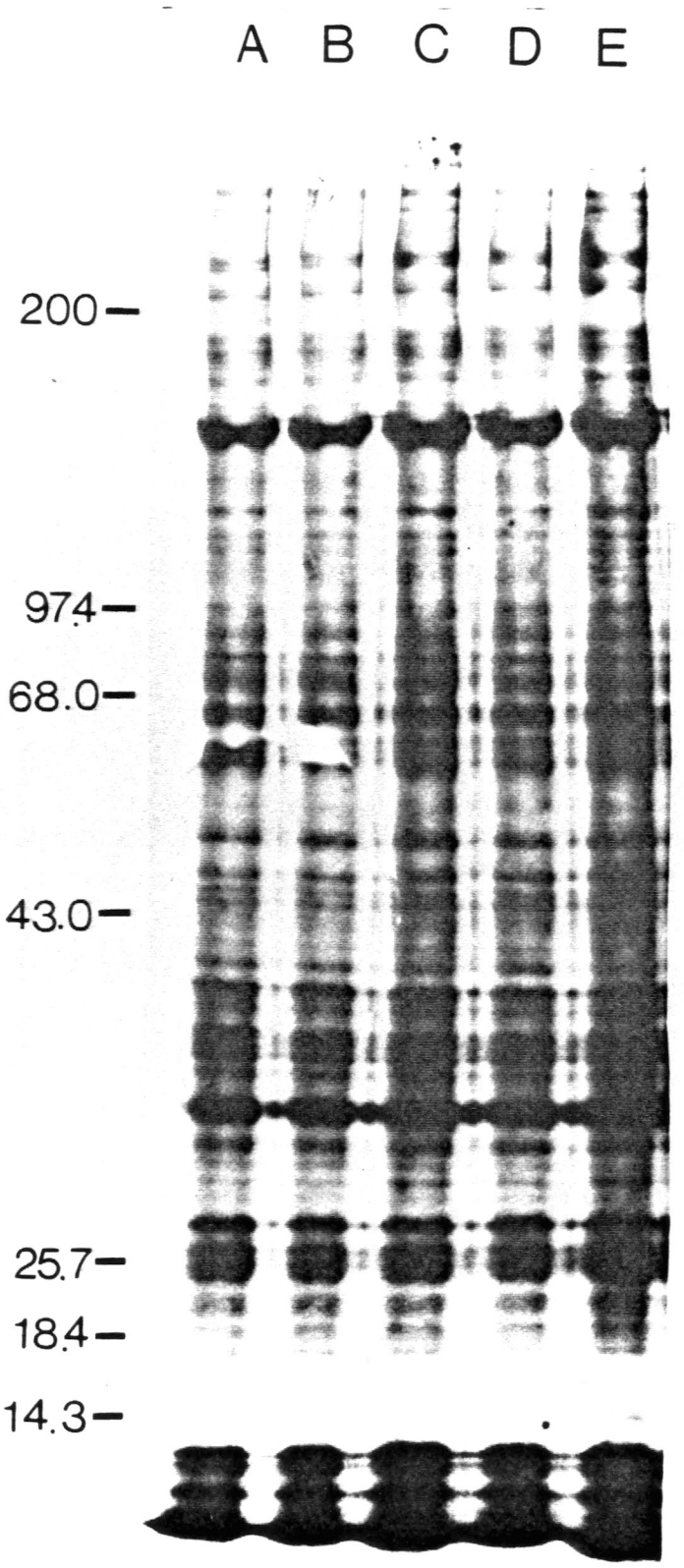
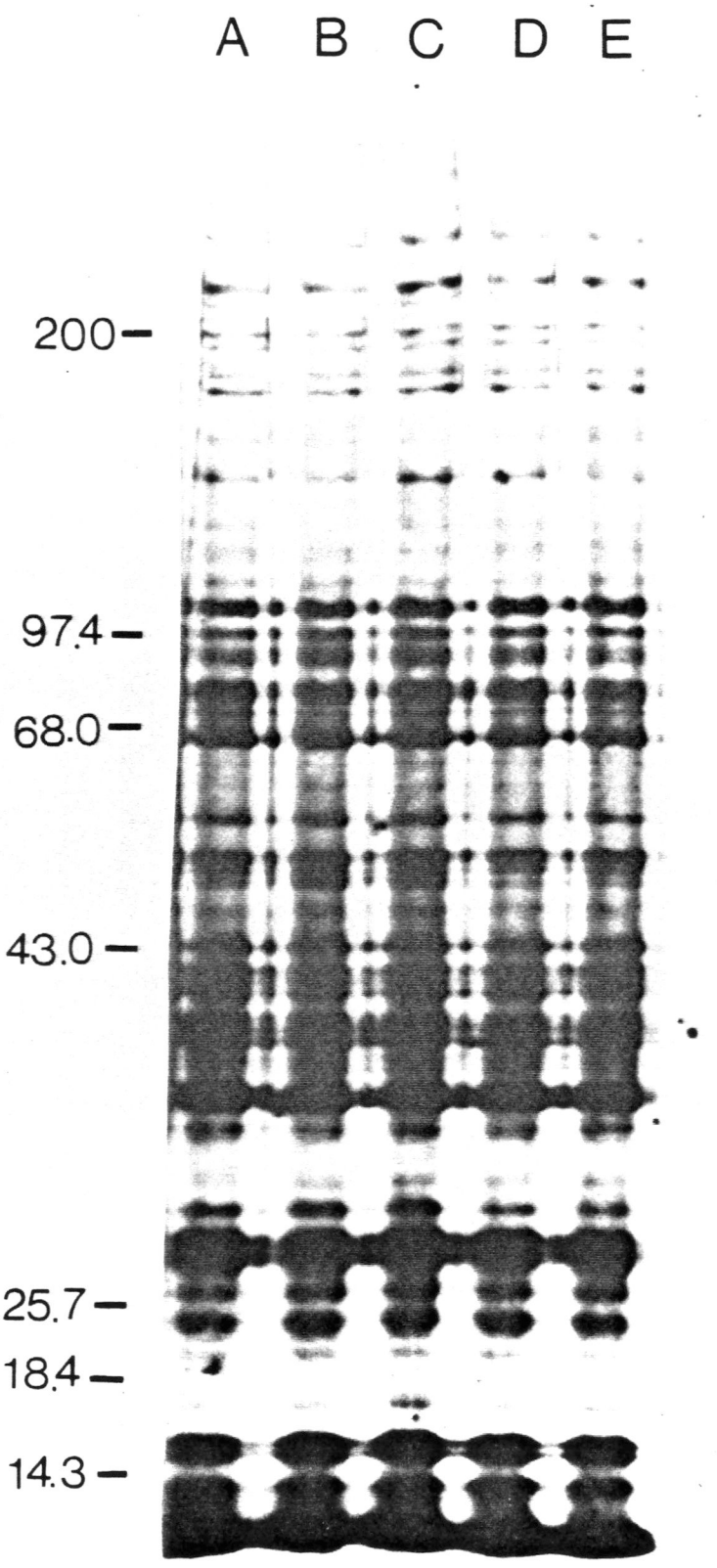

A B C D E
200
97.4
68.0
43.0
25.7
18.4
14.3

Fig. 13. Effect of Monokine Preparation on Protein Synthesis in the Membrane Fraction of the Cells.

Autoradiogram of a 7.5% acrylamide/0.1% SDS gel analysis of [^{35}S]methionine-labeled membrane protein after exposure of L6 cells to the monokine preparation. Experimental design was identical to that described in Figure 12. Membrane proteins were obtained by Nonidet P-40 extraction. Lane A, control cells; lane B, exposure to the monokine preparation for 1 hr; lane C, exposure for 3 hrs; lane D, exposure for 6 hrs; lane E, exposure for 17 hrs. Protein standards, molecular weights x 10^{-3}, are indicated in the left margin.


A B C D E
200
97.4
68.0
43.0
25.7
18.4
14.3

autoradiograms of the soluble proteins obtained on digitonin treatment and those solubilized by Nonidet P-40 treatment of the L6 cells are shown in Figures 12 and 13. No change in any of the bands was observed.

Partial Characterization of the Monokine that Increases Hexose Metabolism in L6 Myotubes.

In order to characterize the protein from the conditioned medium of endotoxin stimulated RAW 264.7 cells which increases glucose metabolism in L6 myotubes, the medium was first dialyzed against 0.9% NaCl (1:2000, changed 3 times) for 24 hrs to see if activity still remained. Extensive dialysis had no effect on the ability of the crude conditioned medium to stimulate 2-deoxy-D-glucose uptake in L6 myotubes. The dialyzed material was then subjected to concentrations of trypsin (0.05 mg/ml, 0.5 mg/ml, 1 mg/ml) for 6 hrs, after which soybean trypsin inhibitor was added at a ratio of 2:1. The ability of this material to stimulate 2-deoxy-D-glucose uptake in L6 myotubes after a 17 hr incubation was tested and found to exhibit no activity. We then concluded that the mediator responsible for the increase in 2-deoxy-D-glucose uptake was indeed a protein.

In order to further characterize the monokine responsible for enhancement of 2-deoxy-D-glucose uptake in the L6 myotubes, we attempted to separate this monokine from the monokine responsible for the suppression of lipoprotein lipase in 3T3-L1 cells. We took 5 liters of crude conditioned medium from endotoxin stimulated RAW 264.7 cells and concentrated this preparation to a final volume of 11 ml. We then split the solution into two 5.5 ml fractions for successive separations on a TSK-3000 SW

HPLC gel filtration column. Assay for 2-deoxy-D-glucose from even-numbered fractions yielded a molecular weight of 75,000 daltons for the major peak of monokine activity (Fig. 14). Furthermore, assays for lipoprotein lipase suppressive activity off the gel filtration column yielded a major peak of activity at 46,000 daltons, indicating the distinct differences in the two monokines.

In order to determine the isoelectric point (pI) of the monokine in question, fractions 15-25 from the HPLC column were combined and the material electrofocused as described under "Experimental Procedures". After isoelectric focusing, fully differentiated L6 myotubes grown in miniwells were incubated in the presence of 15 µl of this material for 17 hrs. Uptake of 2-deoxy-D-glucose was then measured and the peak of monokine activity determined to reside in fraction 29 (pI 6.3). Although there was a smaller peak at fraction 24 (pI 5.1), further studies indicated that the majority of monokine activity had a pI = 6.3 (Fig. 15).

Fig. 14. Separation of Monokine Responsible for 2-Deoxy-D-glucose Uptake in L6 Cells from the Monokine Responsible for Lipoprotein Lipase Suppression in 3T3-L1 Cells by HPLC Gel Filtration.

5 liters of crude conditioned medium from endotoxin-stimulated RAW 264.7 cells were concentrated to a final volume of 11 ml and separated on a TSK-3000 SW HPLC gel filtration column. Even-numbered (0.5 ml) fractions were then tested for the ability to stimulate 2-deoxy-D-glucose uptake in L6 myotubes. Duplicate samples were tested for the ability to suppress lipoprotein lipase in 3T3-L1 cells. The arrow at A indicates the major peak of activity for 2-deoxy-D-glucose enhancement in L6 myotubes and corresponds to a molecular weight of 75,000 daltons. The arrow at B indicates the major peak of monokine activity responsible for the suppression of lipoprotein lipase in 3T3-L1 cells and corresponds to a molecular weight of 46,000 daltons.

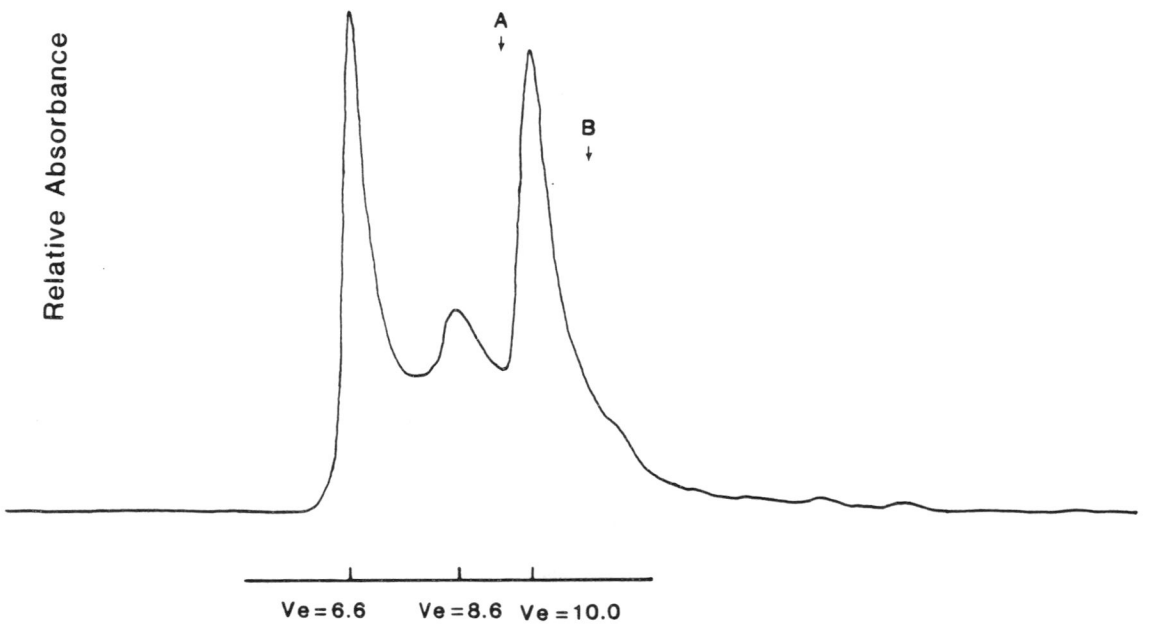
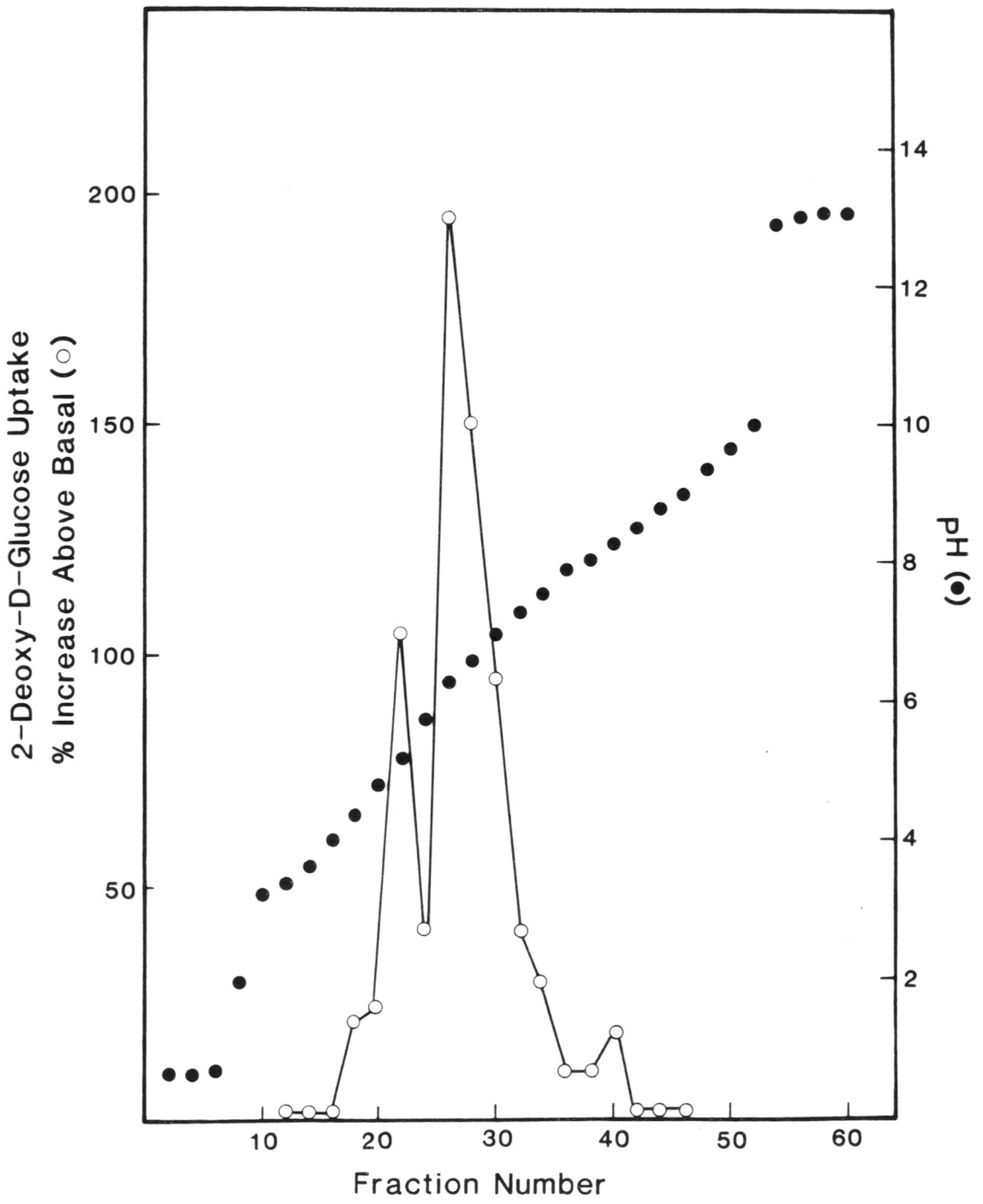

Relative Absorbance
A
B
Ve=6.6
Ve=8.6
Ve=10.0

Fig. 15. Isoelectric Focusing of Monokine Responsible for Stimulation of Hexose Metabolism in L6 Myotubes.

Fractions from the FPLC column exhibiting monokine activity were subjected to isoelectric focusing as described in "Experimental Procedures". L6 myotubes grown in miniwells were then incubated for 17 hrs in the presence of 15 µl from 0.5 fractions collected from theisoelectric focusing column. After this time 2-deoxy-D-glucose was then performed as described in "Experimental Procedures". The results indicate % increase over basal in 2-deoxy-D-glucose (O) as a function of pH (●).


2-Deoxy-D-Glucose Uptake
% Increase Above Basal (○)
200
150
100
50
14
12
10
8
6
4
2
pH (●)
10
20
30
40
50
60
Fraction Number

DISCUSSION

The studies demonstrate the presence of an endotoxin-inducible macrophage secretory protein capable of modulating the critical first step of hexose oxidation by peripheral tissue, that of hexose transport. Preliminary characterization indicates that this monokine is a protein of molecular weight 75,000 daltons and pI = 6.3. Data demonstrating that it is non-dialyzable and readily degradable by trypsin support the contention that the monokine is indeed a protein.

After standardization of the monokine preparation by monitoring its ability to suppress lipoprotein lipase activity in 3T3-L1 fatty fibroblasts, fully differentiated L6 myotubes were demonstrated to exhibit a dose and time dependent activation of 2-deoxy-D-glucose uptake upon exposure to the monokine preparation. Maximal stimulation of uptake was 200% above basal and occurred 12-17 hrs after the cells were exposed to the monokine preparation. The observed increase was due to an increase in the Vmax of the system with no change in the Km. Protein synthesis was also demonstrated to be required for the monokine stimulation of hexose uptake in the L6 cells. The increased hexose uptake was readily blocked by cytochalasin B, indicating the use of the D-glucose specific transporter.

Using 2-deoxy-D-glucose as the substrate for determining the kinetics of monokine stimulation of glucose uptake in the L6 cells, studies were performed in order to confirm that transport was rate limiting. Studies have indicated (Graffe et al., 1984) that uptake assays utilizing this substrate may underestimate actual initial velocities of transmembrane

transport if ATP levels are sufficiently depleted and phosphorylation becomes rate limiting. Direct assay revealed that 80-90% of the total intracellular 2-deoxy-D-glucose was phosphorylated at a final concentration of 0.1 mM (Table II). Furthermore, the monokine preparation had no significant effect on the degree of phosphorylation. We therefore concluded that under the usual assay conditions of the experiment, the capacity of the phosphorylating system was not exceeded. These results are in agreement with Standaert et al. (1984) using the differentiating nonfusing muscle cell line BC3H-1. Hence, any errors associated with phosphorylation of intracellular 2-deoxy-D-glucose are sufficiently small to conclude that monokine induced changes in 2-deoxy-D-glucose uptake primarily reflect changes in transmembrane transport. Therefore, the data presented here clearly indicates that monokine stimulation of 2-deoxy-glucose transport in L6 myotubes is due to an increase in Vmax of the system. This is in agreement with previous studies in other cell systems (Czech, 1976; Graff et al., 1978; Standaert et al., 1984).

Photoaffinity labeling as well as steady state binding studies using [^{3}H]cytochalasin B indicate that the cells respond to exposure to the monokine by increasing the number of D-glucose specific transporters in both the microsomal and plasma membrane fractions. The increase in plasma membrane glucose transporters is consistent with the increase observed in the Vmax for 2-deoxy-D-glucose uptake.

Purity of membrane preparations from the sucrose gradient were determined by assaying for marker enzymes specific for either the plasma membrane or microsomal fraction. Recovery of the plasma membrane marker enzyme γ-glutamyl-transpeptidase in fractions F1 and F2 relative to the

initial homogenate was 11.79% (Table IV). Furthermore, Table V indicated an average purification index of 144 in these fractions. All other fractions had markedly lower purification yields for γ-glutamyltranspeptidase relative to the initial homogenate. This data, in conjunction with the fact that there was virtually no glucose-6-phosphatase activity in fractions F1 and F2 (Tables IV and V), led us to conclude that fractions F1 and F2 consisted primarily of plasma membrane protein. Further studies indicated that the microsomal marker enzyme glucose-6-phosphatase resided primarily in fractions F4 and F5 (Tables IV and V). Since fractions F3 and F6 exhibited significant yields for both enzymes studied, it was concluded that there was enough cross contamination in these fractions to warrant excluding them from the binding studies. Consequently, the data clearly indicate that the fractions used for [^{3}H]cytochalasin B binding and photoaffinity labeling studies were relatively pure membrane preparations.

The increased number of plasma membrane glucose transporters support a model for stimulation of glucose utilization by peripheral tissue during endotoxicosis based on regulation of transport into the cell. Therefore, increased utilization is dependent on monokine stimulation of the synthesis of hexose transporters and their subsequent placement in the plasma membrane. This model for increased glucose utilization contrasts with the current theory of insulin action. Insulin, on binding to its receptor, is thought to activate mobilization of pre-existing glucose transporters from an intracellular compartment associated with the light microsomes (Cushman and Wardzala, 1980; Kono *et al.*, 1981; Oka

and Czech, 1984). This mobilization is rapid and not dependent on protein synthesis (Kono et al., 1981).

Klip et al. (1984) were the first to describe an acute effect of insulin on glucose transport in L6 myotubes. After exposure to 10^{-7} M insulin for 60 min, an approximate 57% increase in glucose transport in serum-deprived L6 myotubes was observed. At higher concentrations (10^{-5} M) a 120% increase was observed. Our results support this description of a rapid response by the L6 myotubes to insulin (Table III). Furthermore, we were able to document that the increase is not dependent on protein synthesis. These data suggest that the myotubes in culture respond to physiological concentrations of insulin in a manner compatible with the current model of insulin action. The inability to achieve higher than a 65% stimulation of hexose transport in L6 myotubes after prior exposure to physiological (10^{-7} M) concentrations of insulin may be due to the scarcity of a large intracellular pool of transporters available for mobilization. Both the photoaffinity labeling and [^{3}H]cytochalasin B binding studies indicate a small pool of transporters in microsomal fractions of control cells relative to the plasma membrane. Another interesting aspect involving the monokine stimulation of hexose transport is the inability of insulin (10^{-7} M) to further stimulate 2-deoxy-D-glucose uptake in fully differentiated L6 myotubes after a 17 hr exposure to the monokine preparation. This could be due to the fact that the L6 cells have at this time reached their maximum transport capacity, which would preclude further stimulation of 2-deoxy-D-glucose uptake by insulin. We further noted that radioimmunoassays performed to examine the presence of insulin in the monokine preparation were nega-

tive, which ruled out any long term effect on hexose uptake due to insulin exposure.

The use of purified recombinant interleukin-1 and γ-interferon indicated an inability of these monokines to significantly enhance 2-deoxyglucose uptake in fully differentiated L6 myotubes. However, tumor necrosis factor at very high concentrations (3000 pM) was able to stimulate 2-deoxy-D-glucose uptake by as much as 85% over basal. When compared to the 100 pM concentration required to suppress activity of lipoprotein lipase in 3T3-L1 fatty fibroblasts and the 10 nM concentration needed to obtain a maximal cytotoxic response in sensitive tumor or transformed cell lines (Sugarman et al., 1985), the high concentration of TNF required for the maximal response in the L6 cells suggests two possibilities: 1) the high concentration of TNF required to achieve only an 86% stimulation of 2-deoxy-D-glucose uptake in the L6 cells is merely the result of cross-reactivity of TNF with a receptor similar to the TNF receptor, and 2) the inability of TNF to achieve a 200% increase in 2-deoxy-D-glucose uptake alone may be the result of a second monokine acting synergistically with TNF. The former hypothesis is supported by the recent work of Satomi et al. (1985) who demonstrated that in some mouse strains, hypoglycemia occurred following administration of large doses of endotoxin but that TNF production was still low. They further demonstrated that hypoglycemia was not induced by administration of highly purified TNF and only the endotoxin-induced tumor necrosis was accompanied by hypoglycemia.

Changes in carbohydrate metabolism are also observed in L6 myotubes

after exposure to the monokine preparation. Rapid depletion of glycogen reserves with a concomitant increase in lactate levels occurred within the first 3 hrs of monokine exposure (Fig. 11). In order to determine if the increased lactate levels were the result of increases in the glycolytic flux, fructose-2,6-bisphosphate was measured during the 24 hr time period after exposure of the L6 cells to the monokine preparation. Fructose-2,6-bisphosphate, by regulation of phosphofructokinase I, has been reported to control the rate of glycolysis in various tissues (Pilkis et al., 1981; Hers and Hue, 1983). Figure 16 depicts the relationship between fructose-2,6-bisphosphate and the activity of phosphofructokinase I. The rapid increase observed in fructose-2,6-bisphosphate (Fig. 11) is consistent with that observed by other investigators in muscle tissue (Van Schaftingen and Hers, 1981; Bosca et al., 1985; Narabayashi et al., 1985; Tornheim, 1985), and supports the hypothesis that fructose-2,6-bisphosphate controls the rate of glycolysis. The rapid depletion of glycogen levels after exposure to the monokine preparation indicates that the energy from glycogen is shunted through glycolysis. Since these cells produce most of their energy needs through anaerobic metabolism, increases in lactate levels would be expected with little change in the rate of the tricarboxycylic acid cycle (aerobic respiration). The fact that fructose-2,6-bisphosphate levels remain high at 17 hrs even though lactate production has leveled off indicates that fructose-2,6-bisphosphate may not control the rate of glycolysis over extended periods of time and may only regulate initial bursts in glycolysis. The rapid glycogen depletion and increased lactate levels observed in these L6 cells are consistent with results observed in whole

animal models during endotoxicosis (Filkins, 1984). The gradual increase seen in glycogen levels observed after 12 hrs (Fig. 11) is coincident with maximal glucose uptake into the cells (Fig. 7). Since lactate production has leveled off at this time, it appears that the increase in glucose uptake is the result of the myotube attempting to regain homeostasis within the cell by restoring depleted glycogen levels, possibly via an increase in glycogen synthase I levels.

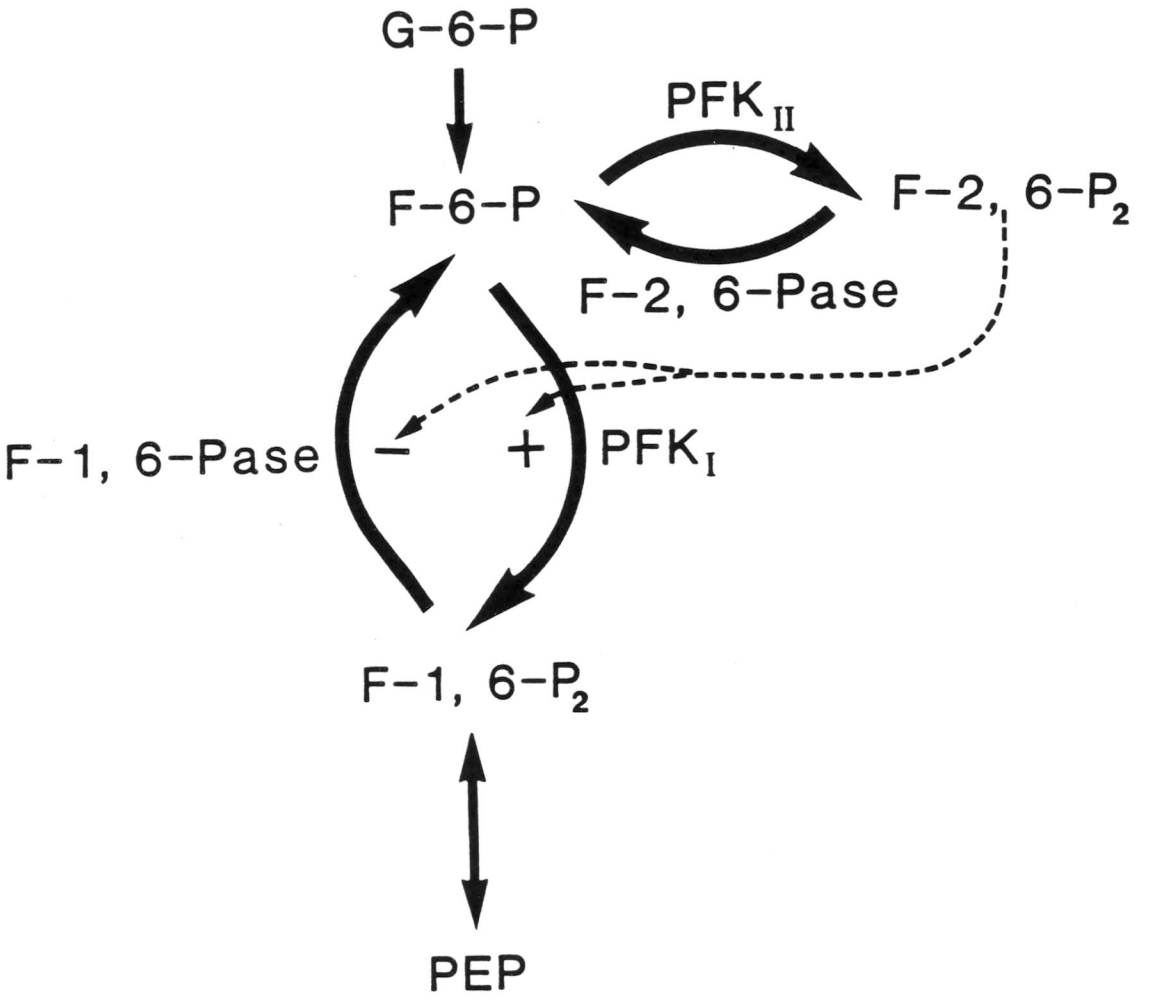
In order to examine the possibility that the observed effect on glucose uptake could be explained by general increases in protein synthesis, the effect of the monokine preparation on [^{35}S]methionine incorporation into protein was investigated. The results displayed in Tables VII and VIII indicate no significant change in the total amount of [^{35}S]methionine incorporation into protein after exposure of the L6 myotubes to the monokine preparation. There are also no detectable changes in the protein patterns or the autoradiograms (Figs. 12 and 13) indicating that the change observed in glucose uptake in the L6 myotubes is not due to a general increase in protein synthesis.

Preliminary characterization of the monokine in question further supports the hypothesis that the monokine responsible for the increased glucose uptake and subsequent metabolism is different from tumor necrosis factor. Gel filtration studies (Fig. 14) yielded a protein of molecular weight 75,000 daltons, which is significantly larger than that of TNF (MW=48,000 daltons). In addition, isoelectric focusing (Fig. 15) yielded a pI = 6.3 whereas TNF has a pI = 4.9.

In summary, the current work presents an attractive model system

for the study of the monokine regulation of carbohydrate metabolism in muscle tissue. The use of RAW 264.7 cells to provide the endotoxin-induced monokine preparation coupled with the fully differentiated L6 myotubes should provide an adequate system to further investigate the molecular mechanism of monokine action on muscle metabolism. Moreover, the results presented here indicate the presence of one or more monokines responsible for the changes in carbohydrate metabolism observed in muscle tissue during sepsis or endotoxicosis.

Fig. 16. Regulation of Fructose-1,6-Diphosphate by Fructose-2,6-Bisphosphate.


G-6-P
PFK II
F-6-P
F-2, 6-P2
F-2, 6-Pase
F-1, 6-Pase
–
+
PFK I
F-1, 6-P2
PEP

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