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Monokine Regulation of Glucose Transporter mRNA in L6 Myotubes

Peter Cornelius, M. Douglas Lee, Melissa Marlowe, and Phillip H. Pekala*

Department of Biochemistry
School of Medicine
East Carolina University
Greenville, NC 27858

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Endotoxin-induced macrophage secretory proteins (monokines) have been shown to stimulate hexose uptake in L6 myotubes (1). In those studies a doubling of the Vmax for hexose uptake was observed which correlated with elevated numbers of glucose transporters (GT) in both plasma and microsomal membranes. To determine if these changes in transporter populations were due to increased GT mRNA, we performed Northern blot analysis using L6 cell RNA and a cDNA to the HepG2 glucose transporter. The L6 myotubes contained a single 2.8 kb species of GT mRNA that increased 2.5-fold after an 8h exposure to the monokine preparation. β-Actin mRNA levels were unaltered by the treatment, indicating specificity of monokine action. Glucose transporter mRNA content appeared to reach a maximum 8h after exposure to the monokine. Over the next 16h the levels of this mRNA gradually decreased, approaching control levels. Data obtained from nuclear transcription run-on assays suggest that increased levels of GT mRNA are due to an increased rate of gene transcription. A second transporter, the insulin-sensitive glucose transporter, was also observed to be expressed in the L6 cells. Monokine treatment resulted in a 60% suppression of the mRNA coding for this protein.

We have recently demonstrated that endotoxin can stimulate RAW 264.7 cells to synthesize and secrete monokines capable of modulating hexose utilization by L6 rat muscle myotubes in culture (1). Within minutes following exposure of the myotubes to the monokine preparation, a rapid depletion of glycogen is initiated that is complete within three hours. As this loss of intracellular energy reserves proceeds, a gradual stimulation of hexose transport is observed. Although the increase is initiated prior to depletion of glycogen, maximal increase in the rate of hexose entry into the cells does not occur until well

*To whom reprint requests should be addressed.

Abbreviations used: GT, homolog of the HepG2 glucose transporter; GT1, 3T3-L1 homolog of the HepG2 glucose transporter; GT2, the putative insulin-sensitive glucose transporter cloned from 3T3-L1 cells; poly(A)$^+$ RNA, polyadenylated RNA; TNF, tumor necrosis factor.

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after glycogen is depleted. Photoaffinity labeling as well as equilibrium binding studies with [^{3}H] cytochalasin B indicated that this response resulted from an increase in the number of D-glucose-specific transporters in both the microsomal and plasma membrane fractions. These results were consistent with an observed increase in the Vmax for hexose uptake. The monokine-induced increase in transport was blocked by exposure of the cells to cycloheximide, suggesting that synthesis of new transporters was the basis of the stimulation. This hypothesis is supported by the observation that increased glucose transport correlates directly with the previously measured increase in numbers of GT proteins. The magnitude of increased transport is apparently sufficient to meet cellular requirements since depleted glycogen stores begin to resupply as maximum levels of transport are obtained (1). In the present study, we examine this hypothesis by determining the effect of monokine treatment on rates of GT gene transcription as well as transporter mRNA accumulation. Our data support the hypothesis of transcriptional control of transporter mRNA synthesis.

EXPERIMENTAL PROCEDURES

Materials- The HepG2 glucose transporter cDNA and the actin cDNA were obtained from Dr. Mike Mueckler, Washington University, St. Louis, MO (2) and Dr. Don Cleveland, The Johns Hopkins University, Baltimore, MD (3) respectively. L6 cells and RAW 264.7 macrophages were from the American Type Culture Collection.

L6 Cell Culture - The L6 muscle cells were cultured as described by Lee *et al.* (1).

RAW 264.7 Macrophage Cell Culture and Monokine Preparation - RAW 264.7 macrophages were grown and incubated with endotoxin to stimulate monokine production as described by Mahoney (4). To measure the TNF content of our monokine preparation, cytotoxicity assays using LM cells were performed as described previously (5). The TNF concentration of the stock monokine preparation utilized in these experiments was 4800 units/ml. The L6 cells were routinely exposed to no more than 300 units/ml. There was no insulin present in the monokine preparation as judged by radioimmunoassay.

Determination of 2-Deoxyglucose Transport - Assay of 2-deoxyglucose uptake was performed as described previously (1).

RNA Isolation - Total RNA was isolated from the cells by extraction with guanidine isothiocyanate and centrifugation through 5.7 M CsCl as described by Chirgwin *et al.*(6). Poly(A)$^+$ RNA was isolated from total RNA by using oligo(dT)-cellulose as per the manufacturer's instructions.

RNA Analysis - For Northern analysis, 10 µg of poly(A)$^+$ RNA were separated by electrophoresis in 1.1% agarose/2.0 M formaldehyde gels and transferred to NitroPlus 2000, as described by Maniatis *et al.* (7). Hybridizations were carried out overnight in the presence of $1x10^7$ cpm/ml of a nick translated

glucose transporter cDNA probe and $0.5x10^6$ cpm/ml of a nick translated β-actin probe. After hybridization the filters were washed in 0.1X SSPE/0.1% SDS for 2h at 64°C with constant agitation and then subjected to autoradiography.

When cRNA probes were used for glucose transporter mRNA analysis, prehybridization and hybridization were carried out as described by Kahn et al. (8).

Nuclei Isolation - Nuclei were prepared as described by Marzluff and Huang (9).DNA concentrations were measured by lysing an aliquot of nuclei in 0.1% SDS, and determining the absorbance at 260 nm and 230 nm (10).Nuclei were frozen quickly in a dry ice-isopropanol bath and stored at -80°C until use.

Nuclear Run-on Transcription Assay - Elongation of nascent mRNA in isolated L6 nuclei was performed according to the method of Linial et al. (11). Filters contained 2 μg each of gel purified cDNA or linearized pGEM plasmid that had been denatured in NaOH, neutralized, and applied to NitroPlus 2000 with a Bethesda Research Laboratory Hybri-Slot manifold. Prehybridization and hybridization conditions were as described by Linial et al.(11).

RESULTS AND DISCUSSION

In order to characterize the effect of the monokine preparation on GT mRNA, L6 cells were exposed to saturating levels of RAW 264.7-cell monokines for 12h. This time course was selected based on previous studies demonstrating that maximal levels of GT activity were observed 12-17h after monokine treatment (1). From control and monokine-treated cells 10 μg of poly(A)$^+$ RNA were used for Northern analysis. Optimal detection of GT mRNA occurred when poly(A)$^+$ RNA was utilized in these analyses. In this experiment (Fig. 1), glucose transporter mRNA was determined to be of molecular size, 2.8 kb. This is identical to that described for other cells or tissues using the same cDNA probe (2,12-15). In this experiment a 150% increase in glucose transporter mRNA was observed 12h after the cells were exposed to the monokine preparation (Fig. 1, Lanes A and B). Glucose transport activity measurements taken at the same time indicated values of 70 pmol·min^{-1}·mg^{-1} protein for control cells while monokine-treated cells exhibited a 200% increase to 210 pmol·min^{-1}·mg^{-1} protein. Thus the magnitude of the transporter activity increase correlates well with the increase at the level of transporter mRNA. The specificity of this enhancement is suggested by the fact that no change in the amount of β-actin mRNA was observed in the same poly(A)$^+$ RNA preparation (Fig. 1).

To examine the time course of monokine-induced alterations in GT mRNA, poly(A)$^+$ mRNA was isolated at various times following exposure of L6 myotubes to

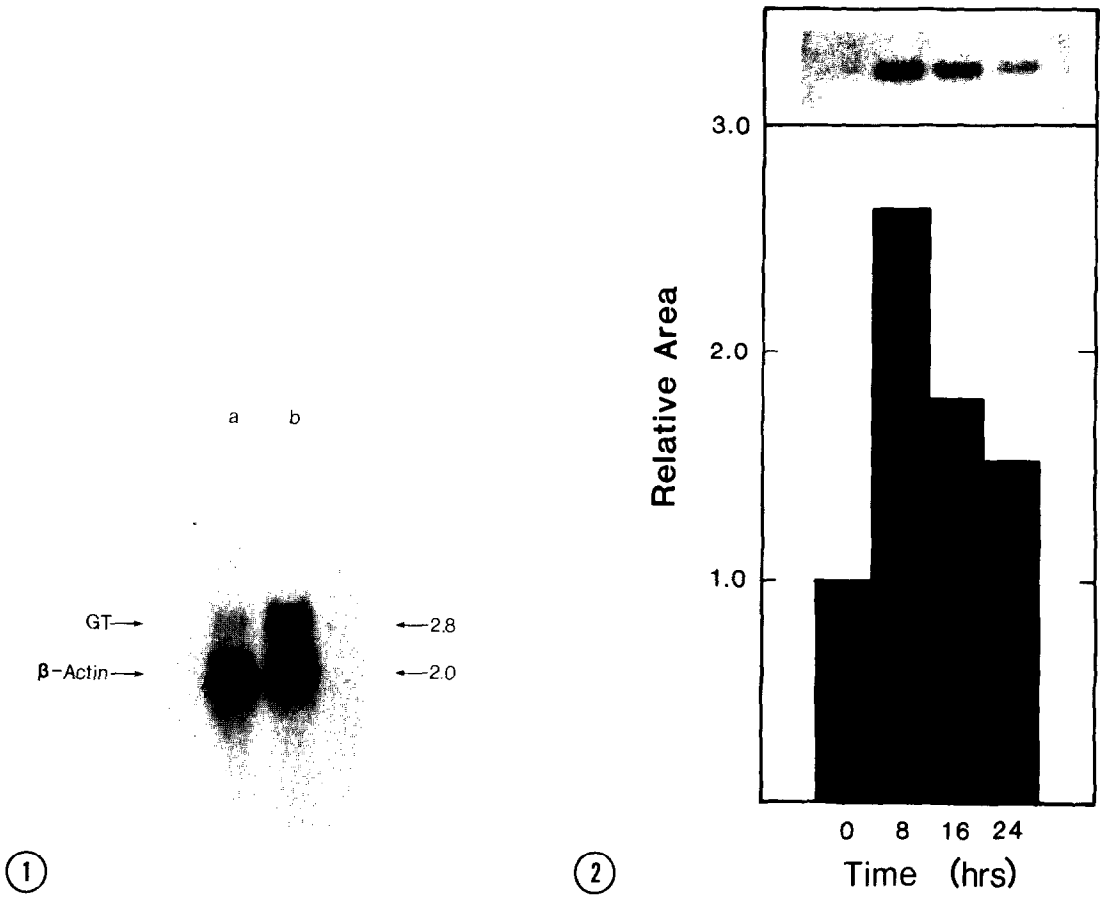

Fig. 1. Northern Analysis of Glucose Transporter and β-Actin mRNA in L6 Myotubes.
Analysis of poly(A)$^+$ RNA (10 µg/lane) was carried out as described in the Experimental section. **Lane a**, Control, untreated myotubes; **Lane b**, myotubes treated for 12 hours with 35 µg/ml of a monokine preparation.

Fig. 2. Time Course of Monokine-Induced Alteration in Glucose Transporter mRNA.
L6 myotubes were treated with 35 µg/ml of a monokine preparation, then at various times total RNA was isolated and subjected to oligo(dT)-cellulose chromatography. Analysis of poly(A)$^+$ RNA (10 µg/lane) was carried out using a GT antisense RNA probe as described in the Experimental section. The main Figure shows densitometric analysis of the autoradiogram in the top panel, with the results presented relative to control, untreated cells.

the monokine preparation. As demonstrated in Fig. 2, the time course for GT mRNA accumulation reached a maximum 8h after addition of the monokine preparation and by 16h had begun to gradually diminish. The accumulation of GT mRNA preceded the maximal rate of glucose transport activity observed in similar time course experiments (1).

Note that in this experiment to confirm the low abundance of GT mRNA in L6 cells, we prepared a ^{32}P-labeled antisense RNA and utilized this reagent to perform Northern analysis of poly(A)$^+$ RNA from the L6 myotubes. At high stringency of hybridization and washing, a single 2.8 kb transcript was detected in all samples (Fig. 2). The relative abundance was consistent with previous studies using a cDNA probe.

To determine if the accumulation of GT mRNA was due to increased synthesis we measured GT gene transcription in nuclei from L6 myotubes following exposure to the monokine preparation. In addition, β-actin gene transcription was measured, since β-actin mRNA levels were unaffected by monokine treatment (Fig.1).

Nuclei isolated from L6 myotubes over a 16h time course treatment demonstrate a substantial increase (150% over control) in GT gene transcription occurred between 2 and 4h of exposure to the monokines (Fig. 3) GT transcription was highest 16h following monokine treatment, despite declining steady-state levels of GT mRNA. This may reflect dissociation of transcriptional events and subsequent mRNA accumulation or decreased stability of the GT mRNA. The rate of β-actin gene transcription increased slightly (50% over control) two hours after monokine treatment, but remained unchanged over the next 14h. This increase, however, did not lead to an increased accumulation of actin mRNA, suggesting that the cellular content of this mRNA may be regulated at steps distal to transcription. These data suggest that the sequence of events leading to monokine stimulation of glucose transport are as follows: First, the monokines interact with the cell and initiate a catabolic cascade which results in a rapid depletion of glycogen stores (1). Second, as glycogen levels drop, homeostatic mechanisms are invoked to enable the cell to meet energy requirements. As an early event this would include an increase in GT gene transcription. Increased transcription leads to accumulation of GT mRNA which precedes the expressed activity of this protein. Thus, the cell has responded to decreased energy stores by increasing the number of plasma membrane glucose

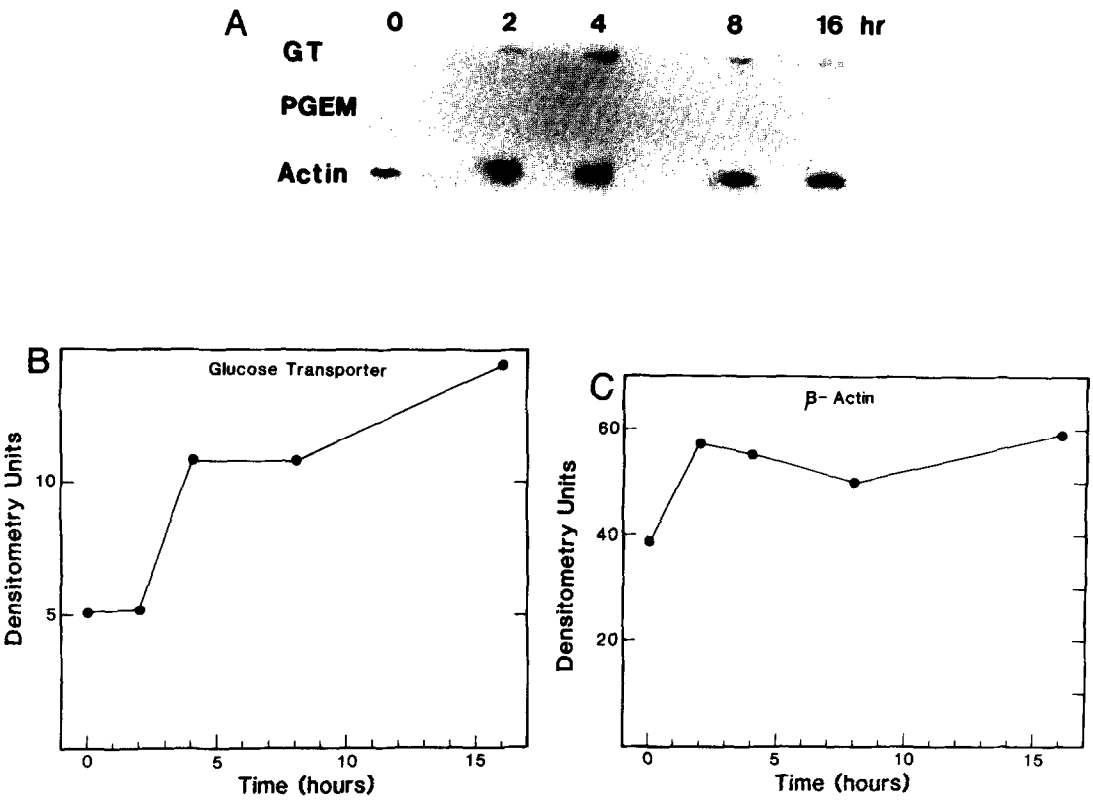

Fig. 3. **Effect of Monokine Treatment on Transcription in L6 Myotubes.** Following exposure of L6 myotubes to 35 µg/ml of a monokine preparation, nuclei were isolated at the indicated times and transcriptional analysis was carried out as described in the Experimental section. Following autoradiography, the results were quantified by laser densitometry of the x-ray film. Panel (A) is an autoradiogram showing hybridization of *in vitro* transcribed RNA to the indicated cDNAs. The filter was exposed to film for 3 days at -80°C using a Quanta III intensifying screen. Densitometric analysis of the autoradiogram in Panel (A) is presented in Panels (B) and (C) as the hybridization intensity of GT and β-actin transcripts, respectively, versus time after monokine treatment.

transport proteins, enabling the uptake of more glucose from the extracellular milieu.

In a separate experiment, the ability of the L6 cells to express mRNA for the putative insulin-sensitive glucose transporter (GT2) was examined. As shown in Fig. 4 (lane a), in addition to GT1, insulin-sensitive glucose transporter, GT2, was also expressed. In this experiment the reciprocal regulation of these two transporter mRNAs was observed after the L6 myotubes were exposed to the monokine preparation (Fig.4, lane b). When normalized to the mRNA levels for actin, previously shown not to change under these conditions, mRNA for GT1 increased by nearly 160% while GT2 mRNA decreased by 60%.

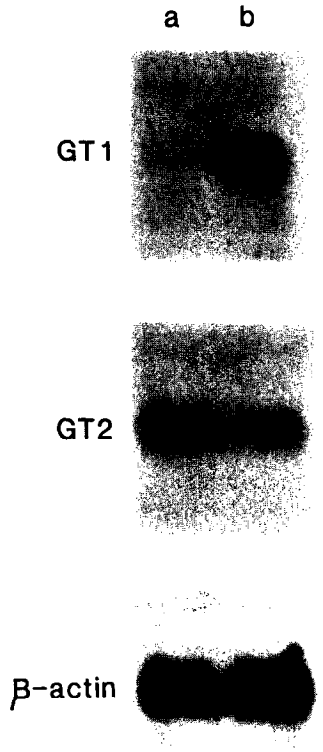

Fig. 4. **Monokine-Induced Reciprocal Regulation of GT1 and GT2.** Analysis of poly(A)$^+$ RNA (10 µg/lane) was carried out as described in the Experimental section. **Lane a**, Control, untreated myotubes: **Lane b**, myotubes treated for 12 hours with 35 ug/ml of a monokine preparation. The blot was sequentially probed with the three cDNAs. Two of these, GT1 (the murine homolog of the HepG2 transporter) and GT2 (a putative insulin-responsive glucose transporter of murine origin) were as we have previously described (16). Exposure time for autoradiography to obtain the GT1 panel was 8 h, while a 48 h exposure was required to obtain the GT2 panel. The figure displayed is representative of an experiment performed three times with different preparations of RNA, in all cases the results were identical.

Thus, although a second transporter is expressed in these cells, the stimulation of transporter activity observed after monokine treatment appears due to the accumulation of the GT1 mRNA followed by subsequent increased synthesis and placement in the plasma membrane of this transporter protein. The down-regulation of GT2 mRNA on exposure of the cells to monokines suggests an explanation for the observed insulin resistance of these cells after such treatment (Lee, M.D., and Pekala. P.H., unpublished results).

Earlier studies suggested the involvement of tumor necrosis factor as an essential mediator of these metabolic changes in L6 myotubes (1). Use of the

monokine preparation in the studies reported herein resulted in exposure of the L6 cells to a maximum TNF concentration of 300 units/ml. Use of 5nM (800 units/ml) recombinant TNF (Biogen, Cambridge, MA) resulted in at best an 80% increase in transport rate. Under these conditions neither an increase in GT mRNA, nor a decrease in intracellular glycogen levels was observed. Our data suggest that TNF alone is not responsible for the observed effects on L6 cell metabolism. Further work on the isolation of this monokine(s) is under way.

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