CACHECTIN/TNF-MEDIATED LACTATE PRODUCTION IN CULTURED MYOCYTES IS LINKED TO ACTIVATION OF A FUTILE SUBSTRATE CYCLE

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The cytokine cachectin/TNF induces a rapid increase in lactate production and in glucose metabolism in L6 myocytes in culture; glucose uptake was maximal after 17 h, while elevated glucose utilization and lactate production persisted for up to 32 h. These increases are suggestive of increased glycolytic activity, and were associated with a 10% decrease in cellular oxygen consumption and a comparable decrease in the production of ^{14}C-labelled CO_2 from ^{14}C-labelled glucose. This decrease in aerobic metabolism, however, could account for only a small fraction of the energetic requirement for increased glycolytic activity. Furthermore, maximal stimulation of pyruvate dehydrogenase (PDH) by dichloroacetate (DCA) treatment in conjunction with cachectin/TNF abolished lactate production, but increased glucose uptake persisted. Taken together, this suggests that the primary effect of cachectin/TNF on myocyte carbohydrate metabolism is to increase glycolysis. Correspondingly, we postulated that cachectin/TNF must activate one or more ATP-depleting cellular processes to account for the lack of feed-back inhibition on glycolysis by the ATP produced. This led to the identification of a futile substrate cycle between fructose 6-phosphate and fructose 1,6-bisphosphate as a novel energy sink that is activated by cachectin/TNF. Cachectin/TNF treatment led to increased activity of both phosphofructokinase (PFK) and fructose bisphosphate phosphatase (FBP) in myocytes in culture, detectable after 1 h of incubation and persisting for up to 16 h. The possible role of cachectin/TNF-mediated futile substrate cycling in increased glycolytic activity, increased energy expenditure, heat production and tissue wasting during bacterial infections is discussed.

Increased lactate production by peripheral tissues is an early feature of the septic syndrome, and both the time of appearance and the magnitude of the rise in serum lactate correlate with infection severity. Elevation of serum lactate requires a compensatory response of the acid-base balance system, and clinical management of the septic patient is severely compromised when normal pH can no longer be maintained and blood pH drops. Along with lactic acidosis, increased carbohydrate utilization and elevated energy expenditure are also prominent metabolic changes during acute bacterial infections,[1,2] but links between these metabolic changes have remained obscure.

Lactic acidosis similar to that induced by the presence of bacteria in the bloodstream can be reproduced in experimental animals by injection of bacterial endotoxins or lipopolysaccharide (LPS). In response, monocytes and other cells of the immune system release cytokines and other endogenous mediators that are known to be directly responsible for a series of metabolic and physiological alterations that characterize the septic syndrome. Among these cytokines, cachectin/TNF has been shown to be a pivotal endogenous mediator of LPS toxicity,[3] increased glucose uptake by muscle and other tissues,[4,5] and the rise of serum lactate,[1,3] fever and increased energy expenditure[6–9] like that noted during sepsis. Moreover, we have previously reported that exogenous cachectin/TNF produces an increase in carbohydrate metabolism in an in vitro system of cultured L6 myo-

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cytes, where cytokine treatment induces glycogen degradation, increased glycolytic activity and greater glucose transport, culminating in increased production of lactic acid.[4]

Although increased energy expenditure and enhanced carbohydrate degradation are known to occur in vivo during actual bacterial infections and in experimental models of endotoxemia, little is known about the metabolic events and endogenous mediators that are responsible for these catabolic changes at the cellular level. Administration of LPS to experimental animals induces a rapid increase in the degradation of liver and muscle glycogen, and increased production of lactic acid.[10] A subsequent increase in glucose uptake occurs in muscle and other tissues once glycogen stores have been depleted.[1] These increases in carbohydrate utilization form part of the generalized catabolic state which prevails during bacterial infections. During experimental endotoxic shock, peripheral muscles release much of the lactate that appears in the serum.[11] Such observations support the hypothesis that during sepsis, muscle is a major contributor to the state of net catabolic activity, and suggest a direct effect of cytokines on muscle glycolytic activity.[4] We have focused on the biochemical mechanisms involved in enhanced carbohydrate catabolism in muscle cell cultures that culminate in increased lactate release, and the role that cachectin/TNF plays in these cellular metabolic changes.

RESULTS

Figure 1 shows the changes in glucose and lactate concentrations in control and cachectin/TNF-treated L6 myocytes cultures over 32 h of incubation. Throughout the period of cytokine treatment, cachetin/TNF increased the rate of glucose extraction by about two-fold, from an average of 271 ± 47 nmol/mg/h in control cultures to 660 ± 61 nmol/mg/h in cachectin/TNF-treated cell cultures (see Table 1). The rate of lactate production in cachectin/TNF-treated cells was also increased about two-fold, from 535 ± 66 nmol/mg/h in control cultures to 1286 ± 63 nmol/mg/h in cachectin/TNF-treated cells. These results indicate that >90% of the incorporated glucose was metabolized to lactate in cachectin/TNF-treated cultures. Glycogen was fully depleted during the first 3 h after TNF-treatment and then remained undetectable for the first 16 h of the treatment period (see Table 1). After this prolonged initial phase, glycogen levels eventually rose (evident at the 32 h timepoint), correlating in time with a period of excess glucose uptake relative lactate production. Fresh medium must be

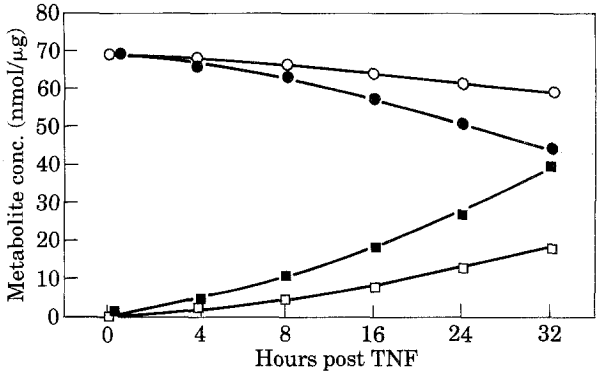

Figure 1. Glucose utilized from and lactate produced into the culture medium of non-treated control and cachectin/TNF-treated L6 cells.

Myocytes were exposed to a saturating concentration (10^4 cytotoxic Units/ml) of cachectin/TNF. At different time points, aliquots of the culture medium were removed and assayed for glucose and lactate content and these data were normalized to the cell number using total cellular protein as an index. The values are averages of triplicate determinations, standard deviations were less than 10% of the average values. ○, glucose − TNF; ●, glucose + TNF; □, lactate − TNF; ■, lactate + TNF.

added to myocyte cultures after 32 h to prevent a sharp decline in cell viability, most probably due to exhaustion of nutrients from the medium. If cachectin/TNF-treated cultures were re-fed at 32 h with fresh medium (lacking cachectin/TNF), glucose uptake and lactate production returned to normal, basal levels 24 h after the medium change (data not shown).

The increases in glucose uptake and lactate production induced by cachectin/TNF suggest an increase in glycolysis. To trace the fate of this glucose, we monitored its metabolism by adding glucose labelled with ^{13}C in position C1 to the medium and identifying the metabolites emitting a ^{13}C NMR signal. Glucose labelled in position C1 provides signals at 93 and 97 ppm, corresponding to the alpha and beta anomer forms, respectively. Figure 2 shows a reduction in the glucose signals over time, and reveals the progressive accumulation of a signal at 20 ppm that corresponds to lactate labeled in position C3. This signal appeared in the medium 1 h after initiation of cachectin/TNF treatment and increased steadily throughout the following 31 h. After 4 h, a much smaller signal appeared at 24 ppm and increased slightly throughout the incubation period. This signal most likely corresponds to a methyl group, possibly a C2 methyl group from acetate or C4 methyl group from acetoacetate. The magnitude of this signal compared to that of C3 of lactate remained very small at all time points. No signal for pyruvate or any other metabolite was detectable (i.e., more than three times greater than background). Based on standard curves prepared for ^{13}C-C3-labelled lactate and ^{13}C-C1-labelled glucose, lactate

TABLE 1. Metabolic parameters in cachectin/TNF-treated L6 myocite cultures.

Parameter	Control	Cachectin/TNF-treated	(N)
Rate of glucose extraction			
(nmol/mg/h) (enzymatic assay)	271 ± 47	660 ± 61	(8)
(nmol/mg/h) (^{13}C-label)	280 ± 26	670 ± 33	(4)
Rate of lactate production			
(nmol/mg/h) (enzymatic assay)	535 ± 66	1286 ± 63	(8)
(nmol/mg/h) (^{13}C-label)	540 ± 59	1260 ± 81	(4)
Glycogen content (μg/mg)	30 ± 3	2 ± 1	(3)
Rate of oxygen utilization (nmol O_2/mg/h)	81 ± 4	73 ± 3	(8)
Rate of $^{14}CO_2$ production (nmol $^{14}CO_2$/mg/h)	172 ± 23	156 ± 46	(8)
Rate of oxygen utilization in the presence of KCN (nmol O_2/mg/h)	1.6 ± 0.1	1.6 ± 0.1	(8)
Intracellular ATP (nmol/mg)	950 ± 25	940 ± 30	(4)
Ca^{2+} and Mg^{2+}-dependent ATPase activity (nmol/mg/h)	132 ± 18	129 ± 17	(3)
Ca^{2+}-dependent ATPase activity (nmol/mg/h)	96 ± 16	99 ± 14	(3)
Mg^{2+}-dependent ATPase activity (nmol/mg/h)	78 ± 15	76 ± 19	(3)
Incorporation of ^{3}H-thymidine (cpm/well/17 h)	7600 ± 80	7700 ± 70	(4)

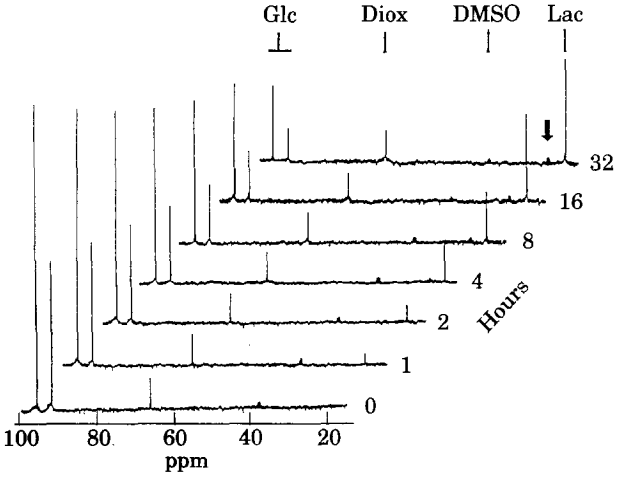

Figure 2. NMR signal of ^{13}C-labelled metabolites of ^{13}C-C1-glucose: conversion to only ^{13}C-C3-L-lactate by L6 myocytes treated with cachectin/TNF (10^4 cytotoxic Units/ml).

The signals correspond to: ^{13}C-C1-labelled glucose (Glc) at 97 and 93 ppm, ^{13}C-labelled dioxane (Diox) at 66 ppm added to all samples as an internal control, dimethyl sulphoxide (DMSO) at 38 ppm added to lock on the sample, and ^{13}C-C3 labelled lactate at 20 ppm. Aliquots of medium from cachectin/TNF-treated L6 myocyte cultures were removed and analysed, as described in Materials and Methods.

production accounted for at least 90% of the glucose consumed after 16 h incubation with cachectin/TNF (about 1.9 moles of lactate produced per mole of glucose taken up). Rates of glucose uptake and lactate production induced by cachectin/TNF treatment were estimated from the changes in ^{13}C signals, and were in close accordance with parallel estimates based on enzymatic measurements (see Table 1).

In parallel cultures, ^{3}H-2-deoxy-glucose uptake was monitored at several time points following cachectin/TNF addition. By this method, myocyte glucose uptake was also shown to be significantly increased by cachectin/TNF treatment, reaching a maximum 2.4-fold increase after about 17 h (from 210 ± 44 to 510 ± 96 nmol/mg/h; $P < 0.0001$). In subsequent experiments, we studied cellular responses after 17 h exposure to cachectin/TNF, at which time the rate of glucose uptake and lactate production had reached a significantly elevated plateau.

To account for the increased glycolytic activity induced by cachectin/TNF we pursued several alternative mechanisms: that the increase might have resulted from impaired aerobic metabolism; or might have developed in response to the activation of an energy utilizing process; or from a combination of such metabolic changes. Cachectin/TNF did induce a small but consistent decrease in total O_2 utilization: a 10% reduction in basal O_2 consumption that was equivalent to 8 nmol O_2/mg/h after 17 h of cytokine treatment (Table 1). Cachectin/TNF treatment, however, had no effect on the KCN-insensitive component of oxygen consumption (1.63 ± 0.13 vs 1.59 ± 0.16 nmol/mg/h for control and cachectin/TNF-treated cells, respectively; Table 1). In direct correspondence with the 10% reduction in oxygen consumption, cachectin/TNF also induced a 10% reduction in the rate of production of $^{14}CO_2$ from L6 myocytes, equivalent to 16 nmol CO_2/mg/h. This reduction was measured in cultures incubated in the presence ^{14}C-C6-labelled glucose for 1 h following 17 h of cachectin/TNF treatment. Comparing the rate of glucose extraction to oxygen consumption, we estimate that only about 10% of the incorporated glucose was oxidized, whether measured in control or cytokine-treated cultures.

We also considered whether the decrease in oxygen consumption and increased glycolysis might have resulted from impaired activity of pyruvate dehydrogenase (PDH). No difference in total PDH activity could be measured when the enzyme was assayed in cell extracts prepared from control versus cachectin/

TNF-treated cells, suggesting that increased lactate release in response to cachectin/TNF in vitro is not due to reduced PDH activity. As a further test, dichloroacetate (DCA) treatment was used to promote the activation of PDH by inducing its dephosphorylation; this promotes complete mitochondrial metabolism of pyruvate and consequent reduction in lactate production.[12] Figure 3A shows that treatment with 0.3 mM DCA abolished the cachectin/TNF-mediated increase in lactate production (1011 ± 86 nmol/mg/h with cachectin/TNF alone versus 394 ± 37 nmol/mg/h in the presence of both cachectin/TNF and DCA). Despite the presence of dichloroacetate, however, glucose uptake persisted at elevated rates: 547 ± 32 and 530 ± 42 nmol/mg/h in cachectin/TNF-treated and DCA + cachectin/TNF-treated cultures respectively (Fig. 3B). Control treatment with DCA alone induced only a negligible reduction in H^3-2-deoxy-glucose uptake, and a comparably small change in lactate production (Fig. 3B).

This small but measurable decrease in oxygen consumption, which was borne out by a corresponding decrease in CO_2 production, suggested that cachectin/TNF-treated cells exhibit comparatively reduced oxidative phosphorylation, and we wondered whether this reduction in cellular ATP generation might be sufficient to account the observed increase in glycolytic activity. Therefore, we estimated the expected ATP deficit by comparing the reduction in oxygen utilization to the increase in lactate production (see Table 1), where, according to equation E1,

$$E1\ (1\ C_6H_{12}O_6 + 2\ ADP + 2\ Pi \rightarrow 2\ C_3H_6O_3 + 2\ ATP)$$
$$E2\ (1\ C_6H_{12}O_6 + 36\ ADP + 36Pi + 6\ O_2 \rightarrow 6\ CO_2 + 36\ ATP + 42\ H_2O)$$

an increased rate of lactate production of 751 nmol lactate/mg/h (1286–535 nmol lactate/mg/h), implies a similar increase in the rate of ATP production (751 nmol ATP/mg/h). According to equation E2, the rate of oxygen utilization in control cells (81 nmol O_2/mg/h) requires 13.5 nmol glucose/mg/h (81 ÷ 6 = 13.5), and should yield 468 nmol ATP/mg/h (13.5 × 36 = 486) assuming that all the oxygen utilized was linked to glucose oxidation. Thus the observed 10% reduction in oxygen utilization in cachectin/TNF-treated cells would result in a deficit of about 50 nmol ATP/mg/h, which compares very unfavourably with the observed increase in ATP production of about 750 nmol ATP/mg/h in cytokine-treated cultures.

Therefore, although decreased oxygen utilization and correspondingly decreased ATP production might contribute slightly to the need for increased glycolysis, this mechanism would seem to account for only a small fraction (5–10% at most) of the enhancement in glucose metabolism. Of course, this line of reasoning assumes that glucose is the only aerobic substrate, when in fact fatty acids and amino acids, glutamine in particular, have been shown to serve as major aerobic substrates in cultured cells.[13] Accounting for the oxidation of these substrates would further

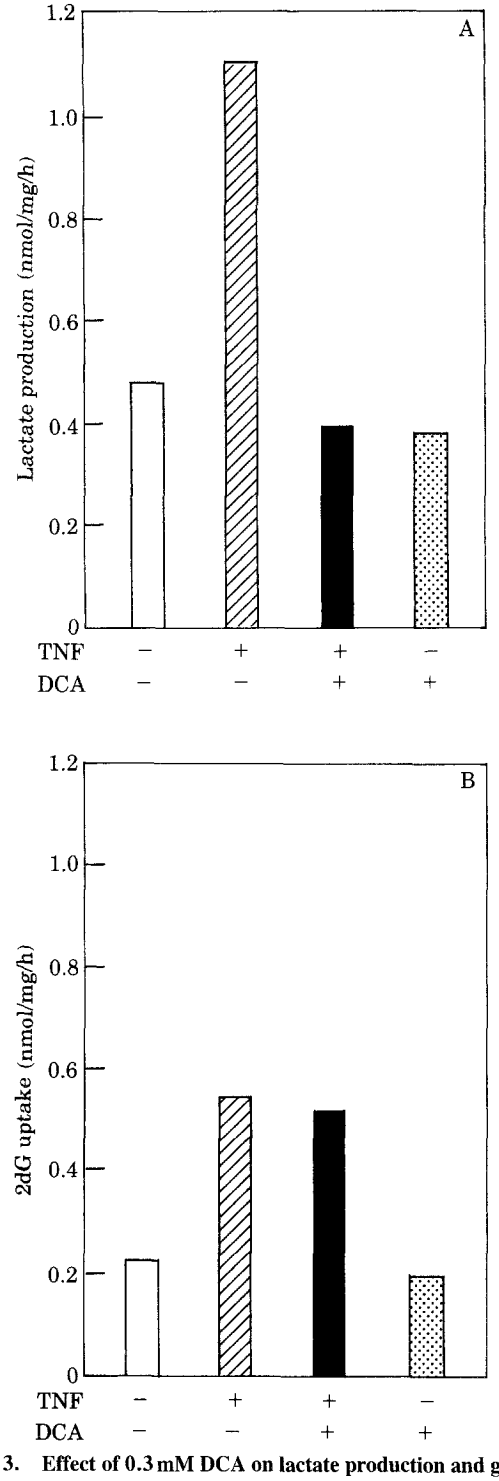

Figure 3. Effect of 0.3 mM DCA on lactate production and glucose uptake.

L6 myocytes were treated with 10^3 cytotoxic Units of cachectin/TNF/0.1 ml and/or 0.3 mM DCA. Production of lactate (A) and uptake of ^{3}H-2-deoxy-glucose (2dG) (B) were measured after 17 h incubation. The values are averages of triplicate determinations; standard deviations were less than 10% of the average values.

reduce any estimate of the maximum degree (5–10%) to which a decrease in total oxygen consumption might account for the observed increase in glucose uptake.

Taken together, the limited degree to which reduced oxygen consumption could account for the magnitude of cytokine-induced increases in glucose uptake and utilization suggested to us that other cellular mechanisms must play the major role. Perhaps cachectin/TNF might directly increase glycolytic activity or alternatively, activate an ATP-consuming energy sink that in turn could account for the increase in anaerobic glycolysis. A link between increased energy expenditure and increased glycolytic activity in cachectin/TNF-treated L6 myocytes was confirmed by inducing a partial block of Na^+/K^+-ATPase activity. This enzyme is essential for maintenance of the resting membrane potential and cellular volume, and is a major site for cellular ATP utilization.[14] Ouabain is a potent inhibitor of this enzyme and can thereby serve to reduce total cellular ATP utilization.[15] Figure 4

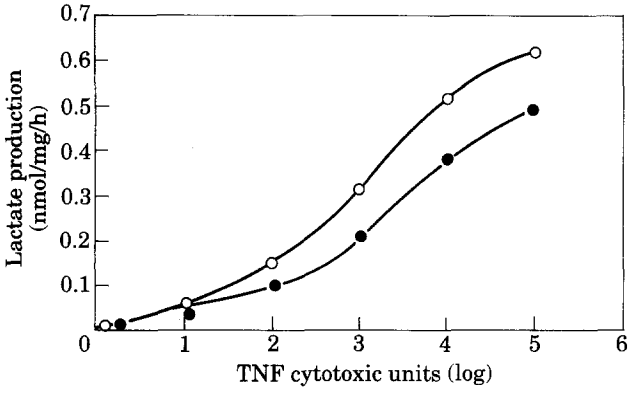

Figure 4. Concentration-dependent lactate production in L6 myocytes in the absence or in the presence of 0.5 mM ouabain.

Lactate concentration was assayed after 17 h continuous exposure to cachectin/TNF. The values are the averages of triplicate determinations; standard deviations were less than 10% of the average values. ○, absence of ouabain; ●, presence of ouabain.

shows that ouabain treatment induced a shift to the right in the dose-response curve of lactate release dependent on the concentration of applied cachectin/TNF. Thus, in a setting of reduced cellular demand for ATP, cachectin/TNF did not induce as large an increase in glycolysis as might otherwise be expected. In that ouabain-induced reduction of ATP expenditure results in reduced lactate production in response to a given dose of cachectin/TNF, we concluded that the cachectin/TNF-induced increases in glycolysis and lactate release require enhanced net ATP utilization. This suggested that the primary effect of TNF might be to activate an 'ATP-wasting energy sink' and that enhanced glycolysis is a reflection of the induced ATP debt. Given these metabolic links it should be possible to identify the increased activity of such an energy sink which consumes ATP in cachectin/TNF-treated cells.

Several alternatives that might serve as ATP-depleting energy sinks were examined and rejected (Table 1). Intracellular ATP levels remained unchanged in cachectin/TNF-treated L6 myocytes, as did the Ca^{2+}- or the Mg^{2+}-dependent ATPase activities in cell lysates prepared from cachectin/TNF-treated cells (data not shown). Exposure of primary rat myocyte cultures to cachectin/TNF induced an increase in glucose utilization and lactate production similar to that observed in L6 myocytes (Table 2).

TABLE 2. Metabolic parameters in cachectin/TNF-treated primary rat myocyte cultures.

Parameter	Control	Cachectin/TNF	(N)
Primary myocytes			
Rate of glucose extraction (nmol/mg/h)	312 ± 47	660 ± 61	(8)
Rate of lactate production (nmol/mg/h)	535 ± 66	1286 ± 63	(8)
Resting membrane potential (mV)	−73 ± 4	−68 ± 5	(22)
Peak action potential (mV)	+12 ± 5	+13 ± 5	(20)

Cachectin/TNF treatment induced a small (5 mV) but significant ($P < 0.001$) decrease in the resting membrane potential of primary myocytes, suggestive of increased ion flux across the membrane (Table 2). Using primary cultures we found that cachectin/TNF had no effect on the development of autonomous action potentials, nor on myocyte contraction, nor on the coupling between action potentials and fibre contraction, nor in the frequency or the magnitude of spontaneous action potentials (Table 2). Comparison of the current to voltage (I/V) plots obtained under voltage clamp revealed no difference between the normal and cachectin/TNF-treated cells (data not shown).

In the absence of a gross defect in membrane polarization, we turned instead to potential metabolic energy sinks as possible explanations for cytokine-enhanced glycolysis. This led to the identification of a set of metabolic changes in cachectin/TNF-treated cells that can effectively deplete cellular ATP and account for enhanced glycolysis and the lack of its feedback inhibition. In particular, this energy sink comprises a futile substrate cycle between fructose 6-

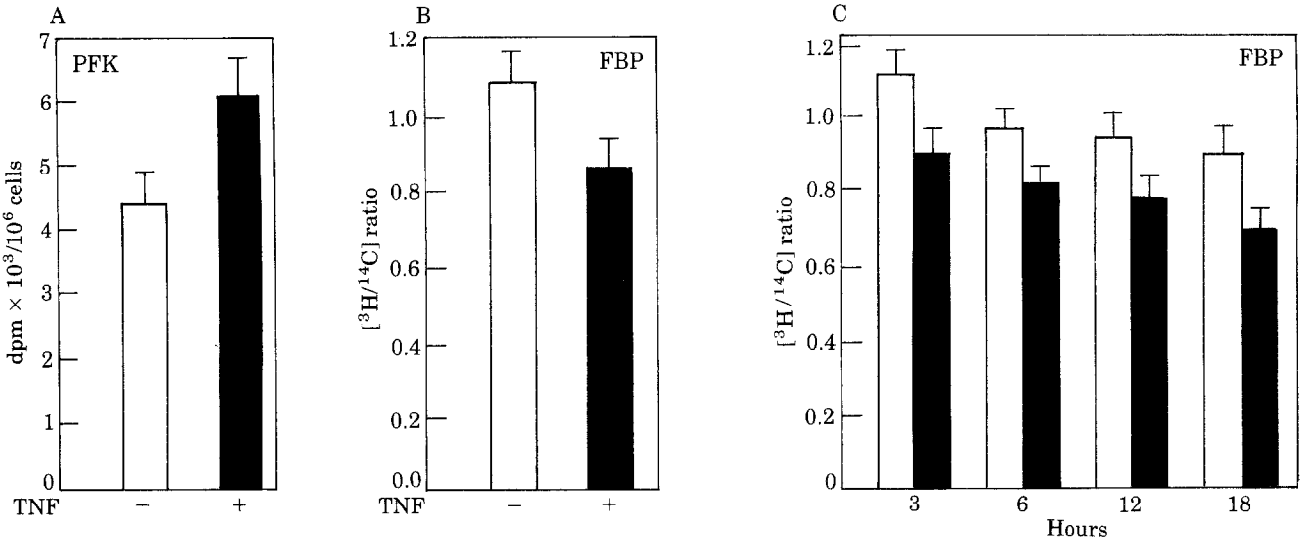

Figure 5. Activation of the enzymes involved in futile substrate cycling in control (empty bars) and cachectin/TNF-treated myocytes (solid bars).

A: Measurement of phosphofructokinase activity (PFK) as the release of ^{3}H into the medium after 1 h incubation with saline or cachectin/TNF-treated cells. B: Measurement of fructose 1,6-bisphosphatase activity (FBP) as the *decrease* in $^{3}H/^{14}C$ ratio in the pool of mono-phosphorylated sugars after 1 h incubation with saline or cachectin/TNF. C: Measurement of fructose 1,6-bisphosphatase activity (FBP) at different time points in control and cachectin/TNF-treated myocytes.

phosphate and fructose 1,6-bisphosphate that is activated by cachectin/TNF treatment in L6 myocytes. Futile substrate cycling (PFK/FBP cycle) was recognized by measuring simultaneous increases in the activity of both phosphofructokinase (PFK) and fructose 1,6-bisphosphatase (FBP) in intact cells treated with cachectin/TNF. Figure 5, A and B, shows that flux through both PFK (reflected by an increase in dpm) and FBP (reflected by a decrease in the $^{3}H/^{14}C$ ratio) was co-ordinately increased after 1 h exposure to cachectin/TNF (increased release of ^{3}H-H_2O into the medium, an index of flux through the forward reaction, increased from 215 ± 41 to 609 ± 51 nmol/mg/h, $P < 0.005$; and the $^{3}H/^{14}C$ ratio was reduced from 1.089 ± 0.056 to 0.851 ± 0.064, $P < 0.005$, indicating enhanced backward substrate flux through FBP). FBP activity remained elevated above control values for up to 16 h during continuous exposure to cachectin/TNF (Fig. 5C). Although FBP activity of control cells also increased throughout the 17 h of incubation ($^{3}H/^{14}C$ ratio shows a slight progressive decrease in Fig. 5C), FBP activity in cachectin/TNF-treated cells was always higher than in non-treated controls at the same timepoints (i.e., a lower $^{3}H/^{14}C$ ratio).

DISCUSSION

We report that cachectin/TNF induces increased utilization of glucose and its ultimate conversion to lactate in the muscle cell line L6 as well as in primary rat myocyte cultures. The principal driving force behind these changes appears to be the activation of a futile substrate cycle between PFK and FBP, resulting in the net hydrolysis of ATP and a consequent increase in glycolysis to compensate for the imposed energy loss. In addition, a small decrease in aerobic metabolism and a small depolarization of the resulting membrane potential may also be involved in triggering increased anaerobic glycolysis in cachectin/TNF-treated cells.

When cachectin/TNF was added to myocyte cultures, glucose uptake and lactate production were increased, reflected by the rapid conversion of ^{13}C-C1-labelled glucose into ^{13}C-C3-labelled lactate and suggesting that increased glycolytic activity accounts for enhanced glucose uptake (Figs 1 and 2). In a previous report we had shown that cachectin/TNF induces an increase in the intracellular levels of fructose 2,6-bisphosphate,[4] a potent allosteric regulator of phosphofructokinase. Similar results have been reported in synovial cells in culture, where treatment with IL-1 or IFN-γ increases fructose 2,6-bisphosphate levels, glucose utilization and lactate production.[16] In conjunction with this increase in glucose metabolism, we have observed an increase in GLUT1 glucose transporter mRNA levels and glucose transporter protein levels in cachectin/TNF-treated L6 myocytes (data not shown). A similar response in 3T3L1 pre-adipocytes results from stabilization of GLUT1 glucose transporter mRNA.[17]

These cellular metabolic changes in vitro are reminiscent of the increased lactate production that occurs during sepsis or experimental endotoxemia in vivo. Such in vivo changes have traditionally been explained as consequences of marked peripheral vasodilation leading to reduced tissue perfusion and finally resulting in lower oxygen availability in the tissues.[2,11,18] However, increased glucose utilization and enhanced lactic acid production occur even with milder endotoxin challenges where no haemodynamic alterations are observed,[19] suggesting that other mechanisms are involved in the development of lactic acidosis. The in vitro system used here eliminates possible variations in oxygen availability as a cause for lactate production, and suggests a possible role of cachectin/TNF as a direct mediator of septic acidosis.

The accumulation of small amounts of ^{13}C-labelled C4 acetoacetate or C2 acetate in the medium (Fig. 2) suggested a possible impairment of PDH activity in cachectin/TNF-treated cells, but in our hands cachectin/TNF had no detectable effect on PDH activity (data not shown). Under normal physiological conditions, only a fraction of cellular PDH is in the active form (dephosphorylated).[12,20] In animal models of chronic and acute sepsis, it has been reported that skeletal muscle PDH activity is eventually reduced (but only after several days of sepsis) and that this decrease in activity is due to a conversion of the PDH enzyme complex to its inactive, phosphorylated form.[21] Thus, although impaired PDH activity does not seem to account for the short term effects of cachectin/TNF on carbohydrate metabolism L6 myocyte cultures, it would be interesting to document whether cachectin/TNF is also responsible for altering the fraction of PDH which is in the active form in vivo over the longer term.

It has been reported that macrophages activated by LPS in vitro impair mitochondrial respiratory activity in L-1210 murine lymphocytic leukaemia cells,[22] and in cell lines derived from sarcomas, mammary tumours, and hepatic tumours.[23] This impaired cellular respiration, due to inhibition of the electron transport respiratory chain,[21] is accompanied by increased sugar extraction and lactate production. In L6 myocytes, however, the limited effect of cachectin/TNF treatment on oxygen utilization and CO_2 production (5–10% reduction in each) indicated that the dominant effect of cachectin/TNF on carbohydrate metabolism in L6 myocytes is not due to inhibition of cellular respiration, and instead must lie elsewhere. Our demonstration that glucose uptake can be uncoupled from lactate production by DCA treatment (Fig. 3) suggests the increased metabolism of glucose through the glycolytic pathway as the proximal site for cachectin/TNF action.

In order to account for the continuous cellular utilization of the 'excess' ATP produced by cachectin/TNF-enhanced glycolysis, we postulated that cytokine treatment must activate one or more cellular energy-consuming processes. Such a link between lactate production and energy metabolism was supported by experiments to inhibit the Na^+/K^+-ATPase with ouabain: after reducing total cellular ATP utilization by inhibiting the Na^+/K^+-ATPase, lactate production by cachectin/TNF-treated cells was reduced for a given dose of cachectin/TNF. Once increased activity of intracellular ATPases and altered membrane permeability had been eliminated as dominant sites of increased ATP utilization (Table 1), we considered an alternative explanation: that increased energy utilization resulted from cellular activation of a futile substrate cycle in cachectin/TNF-treated myocyte cultures.

Changes in futile substrate cycling have been proposed to participate in body weight regulation, by increasing energy expenditure to levels similar to those seen during sepsis.[24] In skeletal muscle the futile substrate cycle between fructose 6-phosphate and fructose 1,6-bisphosphate that results from the simultaneous activation of PFK and FBP is of particular interest in energy dissipation.[25] Activation of this PFK/FBP cycle results in the net hydrolysis of one

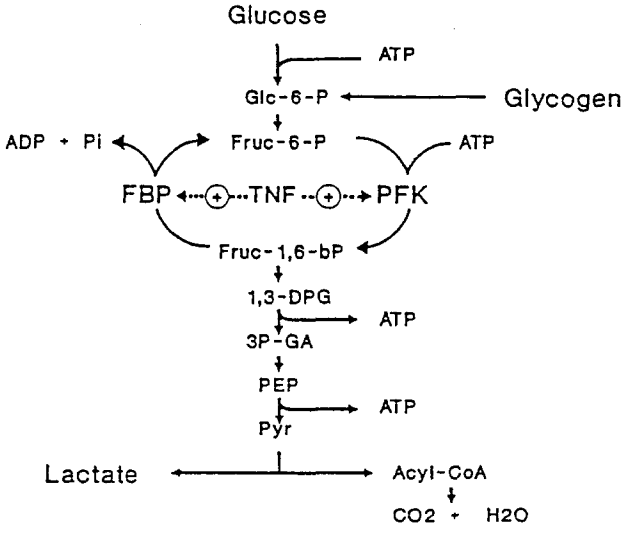

Figure 6. Futile substrate cycling between fructose 6-phosphate (Fruc-6-P) and fructose 1,6-bisphosphate (Fruc-1,6-bP).

Abbreviations correspond to glucose 6-phosphate (Glc-6-P), phosphofructokinase (PFK), fructose 1,6-bisphosphatase (FBP), glyceraldehyde 3-phosphate (GAD-3P), dihydroxyacetone phosphate (DHAP), 1,3-diphospho-glycerate (1,3-DPG), 3-phosphoglycerate (3PG), 2-phosphoglycerate (2PG), phosphoenolypyruvate (PEP), pyruvate (Pyr).

ATP for every turn of the cycle, as shown in Fig. 6. The increased substrate flux through both PFK and FBP which we measured in cachectin/TNF-treated cells (Fig. 5) implies the activation of such a futile substrate cycle between fructose 1,6-phosphate and fructose 6-phosphate, resulting in the imposition of an ATP-wasting metabolic energy sink. Cachectin/TNF evokes a rapid increase in fructose 2,6-bisphosphate,[4] a potent positive allosteric regulator of PFK, enhancing the forward reaction of the cycle (see Fig. 6).[26] The reverse reaction of the cycle is catalysed by FBP, which is allosterically regulated by fructose 2,6-bisphosphate and by AMP.[27] While fructose 2,6-bisphosphate acts as an inhibitor of the hepatic FBP isoenzyme, the muscle isoenzyme has been shown to be 10 times less sensitive to such inhibition.[28] In addition, the activity of the muscle FBP isoenzyme shows a biphasic regulatory response to fructose 2,6-bisphosphate: while low concentrations of the allosteric regulator have an inhibitory effect, higher concentrations actually stimulate enzymatic activity.[28] This effect may explain our observations, where activation of FBP occurs in parallel with an increase in fructose 2,6-bisphosphate.

We propose that once cachectin/TNF treatment triggers the PFK/FBP cycle, an energy deficit is created, and glycolysis is activated to compensate for the increased energy utilization. This increased glycolytic activity in turn would lead to increased utilization of glycogen and glucose with a consequent increase in lactate production. The net result of this catabolic activity induced by cachectin/TNF in vitro is the dissipation of energy in the form of heat and the utilization of ATP and glucose. It is possible that activation of futile substrate cycling in general, and of the PFK/FBP cycle in particular, may contribute to the increased energy expenditure induced by cachectin/TNF in vivo.[7]

An inevitable consequence of futile cycle activity is the release of heat,[24] which together with uncoupling of mitochondrial oxidative phosphorylation,[29] represent known metabolic thermogenic mechanisms. Both in vitro and in vivo increases in temperature correlate with responses that indicate a possible beneficial role for fever and locally increased temperature at sites of inflammation. Hyperthermia has been shown to reduce growth of some infectious agents,[30] and to increase viability of different vertebrates with bacterial infections.[31] In vitro temperatures similar to those in mild fever enhance cellular components of the host defence response, such as leukocyte mobility, lymphocyte activation and response to interferons.[30] Activation of futile substrate cycling leading to heat production may represent a portion of the metabolic contribution to increased local and systemic temperature during the host defence response to infection. An increase in glucose utilization, lactate production and heat generation has been reported in thioglycolate-elicited macrophages,[32] however, direct micro-calorimetric measurements revealed that glycolysis alone accounts for only 60% of the heat produced during increased glycolytic activity. It is therefore possible that futile substrate cycling may contribute to the remaining 40% of metabolic heat produced by such hypermetabolic cells.

As a caveat, the metabolic response of muscle cells to cachectin/TNF in vitro and in vivo may differ in view of the fact that in vivo, muscle metabolism will also be influenced by stress hormones and other mediators, and by the presence of other energy substrates such as lipids and amino acids. Also, the concentration of cachectin/TNF in tissues is of particular importance and, although the highest concentrations used in this study (10^5 cytotoxic Units/ml) are above circulating physiological levels, we have observed a significant increase in glucose uptake with as little as 10^2 cytotoxic Units/ml,[4] a level compatible with cachectin/TNF concentrations in serum during sepsis. Is it possible, nonetheless, that energy dissipation by cachectin/TNF-mediated activation of futile substrate cycling could contribute appreciably to the overall increased energy expenditure in vivo during sepsis, and we can make rough calculations to determine whether this cellular metabolic change is large enough to account for the magnitude of increased energy expenditure that has been noted in sepsis. Assuming that ATP production due to increased glycolytic activity serves to compensate the energy debt created by the activation of the PFK/FBP cycle, then the rate of lactate production and the rate of ATP hydrolysis by the PFK/FBP cycle should be equal. Based on the increase in the rate of production of ^{13}C-lactate in cachectin/TNF-treated L6 myocytes that we measured (about 0.6 mmol/g/h; Table 1), we can estimate a rate of ATP hydrolysis equal to 0.6 mmol/g/h, which can be restated as 4.4 kcal/kg/h (equal to 0.6 mmol/g/h × 7.3 cal/mmol ATP). If a similar cellular process were to be activated in all of the muscle mass of a 70 kg septic patient, the increase in energy expenditure could be as high as 1900 kcal/day (equal to 18 kg muscle × 4.4 kcal/kg/h × 24 h). This is a significant increase over the normal basal metabolic expenditure of about 1500 kcal/day, and is within the range of other estimates of the increase in caloric expenditure in septic patients (as high as 3000 kcal/day).[6] Considering the amount of FBP present in human skeletal muscle, the maximal rate of excess energy expenditure due to activation of the PFK/FBP cycle has been calculated to be 1600 kcal/day.[24] Thus, although futile substrate cycling might not account for all of the metabolic heat produced during local infections or in sepsis, this cytokine-induced cellular metabolic change

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may contribute significantly to the increase in local and systemic heat production.

Increased energy metabolism and energy expenditure are observed clinically during Gram-negative sepsis and other infectious diseases. This wasteful process leads to heat production and depletion of energy reserves, and may, in the chronic state, contribute to catabolism of tissue mass and cachexia. Our in vitro results suggest a possible role for cachectin/TNF-mediated futile substrate cycling in muscle cells as a principle component of the excess energy dissipation typical of systemic sepsis in vivo.

MATERIALS AND METHODS

Reagents

Chemicals and enzymes were purchased from Sigma Chemical Company, St Louis, MO. [^{13}C]-labelled glucose was purchased from Aldrich Chemical Company, Milwaukee, WI. [^{3}H]-labelled 2-deoxy-glucose and [^{35}S]-labelled methionine were purchased from New England Nuclear, Boston, MA. Enzymes were purchased from Sigma and Boehringer Mannheim, Indianapolis, IN.

Cytokines

Recombinant murine cachectin/TNF was kindly provided by Dr Michael Palladino (Genetech, South San Francisco, CA), native murine cachectin/TNF was prepared as previously described.[33,34]

Cell Culture

Disposable tissue culture grade culture ware was purchased from Corning, Falcon and GIBCO Laboratories. Cells were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere. L6 rat myoblasts (ATCC cat# CRL 1458)[35,36] were grown as previously described.[4] Primary myocyte cultures were derived from dissociated fetal rat hind limbs.[35] The differentiation of L6 myocytes and rat primary myocytes was monitored visually by the formation of myotubes and confirmed by measuring the activity of phosphocreatine kinase (PCK). Increased lactate production and glucose utilization in response to cachectin/TNF occurred in both undifferentiated and differentiated cultures, where the magnitude of the effect in differentiated cultures represented only 60% of that of undifferentiated cultures (data not shown). All experiments shown here with primary myotubes and the L6 cell line were performed on cells expressing high levels of PCK.

2-Deoxy-glucose Uptake

Glucose transport was assayed by pulsing the cell monolayers for 5 min with [^{3}H]-labelled 2-deoxy-glucose (2dG) and counting the incorporated radioactivity as previously described.[4]

Enzymatic Assays

In all the samples used for enzymatic analysis of lactate and glucose, serum protein was removed by precipitation with trichloroacetic acid (TCA). Denatured protein was first removed by centrifugation, acid extracts were neutralized and metabolites were assayed in these supernatants. All spectrophotometric measurements were performed in a double beam spectrophotometer (Hewlett Packard, New York, NY). Lactate was assayed by monitoring NAD reduction in the presence of lactate dehydrogenase.[37] Glucose was assayed by measuring the reduction of NADP in the presence of hexokinase (HK), ATP and glucose 6-phosphate dehydrogenase (GDH).[38] ATP was assayed enzymatically in neutralized perchloric acid cell extracts using HK and GDH.[39] PCK activity was assayed in total cell lysates in 10 mM glycylglycine buffer, pH 6.5, using a diagnostic kit (Sigma CAT#47-20). Glycogen was assayed as previously described.[21]

Rates of glucose utilization were estimated from the data presented in Fig. 1, by subtracting the amount of glucose present in the medium at different time points from the amount present at time 0. The rate of lactate production was estimated by subtracting the amount of lactate present at time 0 from the amount at different time points. Basal rates of glucose extraction and lactate production appear in Table 1.

Ca^{2+}- and Mg^{2+}-dependent ATPases were assayed in the clear 800 × **g** supernatants derived from cell homogenates prepared in 250 mM sucrose, 10 mM HEPES, pH 7.4. ATPase activity was assayed in homogenization buffer in the presence or absence of 1 mM of $CaCl_2$, or 1 mM $MgCl_2$ or both. The reaction was started with 10 mM ATP, after 30 min at 37°C 100 μl of ice cold TCA were added. ATPase activity was reflected by the difference between the initial and final ATP concentrations.

Pyruvate dehydrogenase (PDH) was assayed by coupling the synthesis of acetyl-CoA to the acetylation of 4-methyl-aniline, followed at 405 nm.[40]

Nuclear Magnetic Resonance (NMR) Spectroscopic Analysis

The NMR signal of ^{13}C-labelled compounds was identified in spectra generated with a 300 MHz NMR spectrometer (General Electric NMR Instruments, Foremont, CA). All measurements were performed on solutions containing 10% deuterated dimethyl-d_6-sulphoxide and ^{13}C-labelled dioxane (1% v/v). Spectra were obtained using 'Charm' software (General Electric), with a 6 μs pulse width at 30°, a 819.20 ms acquisition time, and recycling time of 1 s. The decoupler was used with a standard-64 modulation, and a frequency of 4 ppm to saturate the water signal. The free induction decay signal was recorded over 960 accumulated acquisitions.

The experiment was started by adding fresh DMEM containing 15 mM ^{13}C-C1-glucose supplemented with dialysed FBS with or without 10^4 cytotoxic units of cachectin/

TNF. Medium samples were recovered at time points ranging from 1 to 32 h, treated with TCA, neutralized and supplemented with DMSO and dioxane. Glucose and lactate concentrations were estimated by comparing the intensities of the ^{13}C-C1-glucose and ^{13}C-C1-lactate signals to a standard curve of ^{13}C-labelled compounds. The rates of glucose utilization and lactate production (Table 2) were estimated from data like that presented in Fig. 2, by calculating the difference in metabolite concentration at different time points.

Oxygen Consumption from L6 Cells in Suspension

The oxygen measurements were performed using a Clark electrode at 37°C in a jacketed glass chamber. Oxygen content in the chamber containing 1 ml of 0.150 M sucrose, 20 mM HEPES was titrated with $Na_2O_4S_2$ and estimated to be 250 nM.

After 17 h with or without cytokine treatment (5×10^3 cytotoxic Units/ml), myocytes were detached from the plates using trypsin/EDTA and washed in DMEM without serum, L6 myocytes in suspension were placed in medium containing DMEM supplemented with 10 mM glucose. Cells (2×10^6) were injected into the chamber and oxygen consumption was monitored for 10 min; after addition of KCN (final concentration of 0.1 mM) oxygen consumption was monitored for another 10 min.

Production of ^{14}C-labelled CO_2

Cell suspensions supplemented with 10% dialysed FBS were prepared as described for oxygen consumption. Myocytes were transferred to reaction vessels to capture ^{14}C-labelled CO_2,[41] wherein 2.5×10^6 cells were incubated in 2.5 ml of the same saline solution supplemented with 5 mM glucose and 15 μCi/ml ^{14}C-C6- or ^{14}C-C1-labelled glucose (specific activity 3 μCi/μmole). Reaction vessels were sealed and incubated at 37°C for 60 min in a shaker at 100 cycles/min. Cell viability was 90% or greater, as estimated by Trypan blue exclusion at the end of the incubation period. In the experimental reaction vessels, cells were lysed by injecting 250 μl 50% perchloric acid (PCA), followed by injection of 200 μl of hyamine into Eppendorf tubes suspended within the vessels. Addition of 250 μl PCA (10% v/v) was enough to obtain a quantitative recovery of ^{14}C-labelled CO_2. After further incubation for 90 min, the rubber seal was removed and the hyamine in the Eppendorf tubes was transferred to scintillation vials and mixed with 5 ml of scintillation cocktail and counted in a scintillation spectrophotometer. All assays were performed in triplicate.

Phosphofructokinase

The flow of substrate through ATP:D-fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.11) (PFK) was assayed by measuring the amount of ^{3}H released as ^{3}H-H_2O into the medium when cells were incubated with ^{3}H-5-glucose.[42,43]

Differentiated L6 myocytes were incubated in DMEM supplemented with 1 mM glucose and 200 μCi/ml ^{3}H-5-glucose. After 3 h of incubation, cachectin/TNF or saline was added and the samples were incubated for periods of time ranging from 1 to 17 h. Samples of culture medium from control and treated cells were collected at specified time points and frozen for later analysis. Labelled water was recovered by lyophilization and 200 μl of each water sample were counted with 5 ml scintillation cocktail.

Fructose-bisphosphatase

D-fructo-1,6-bisphosphate 1-phosphohydrolase, (EC 3.1.3.11) (FBP) activity was assayed by measuring the ratio of ^{3}H/^{14}C in the pool of mono-phosphorylated sugars (glucose- and fructose 6-phosphate).[42,43]

Myocytes were pre-incubated in the presence of 1 mM glucose, including 200 μCi/Ml of ^{3}H-5-glucose and 200 μCi/ml of ^{14}C-1-glucose for 3 h. Cachectin/TNF was added and mono-phosphorylated sugars were isolated after incubation for various time intervals ranging from 1 to 17 h. Perchloric acid extracts were neutralized and de-ionized before separation in an anion-exchange cellulose thin layer chromatography system.[44] Radioactivity was quantified using a thin layer chromatography radioactivity linear analyser (Berthold, Pittsburgh, PA). The position of the different sugars was confirmed by comparing the migration of cold standards. Regions of the TLC plate containing the different sugars were scraped and counted to obtain the ratio of ^{3}H/^{14}C. The results are the average of triplicates.

Electrophysiology

Primary myocytes were cultured in 35 mm petri dishes. Resting and action membrane potentials were recorded at room temperature (25°C) in viable cells using glass microelectrodes with a tip resistance of 30 MΩ.[45] Data were digitized, and stored in memory for later analysis using a personal computer. After exposure to medium supplemented with saline or cachectin/TNF, cells were impaled and the initial voltage deflection was recorded as the resting membrane potential. The same experimental conditions used to measure the resting membrane potential were used for whole-cell voltage clamping.[46] Each voltage step was sustained for 400 ms while the deflections in membrane current were stored in memory for later analysis. A total of 15 voltage steps of 10 mV were used, ranging from −100 to +50 mV. The same software program was used to generate the transient and steady state current/voltage (I/V) curves.[45]

Proliferation Assays

Cell proliferation was measured by [^{3}H]-thymidine incorporation assays.[47] Briefly, cells were plated (5×10^4/cm^2), and 1×10^4 cytotoxic units of cachectin/TNF were added 2 h after plating. ^{3}H-thymidine was added after 24 h to a final concentration of 10 μCi/ml. After 6 h, cell monolayers were washed with ice-cold PBS and fixed with 95% methanol. Incorporated ^{3}H-thymidine was extracted with 0.1 M NaOH and counted in scintillation cocktail. All assays were done in triplicate.

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