Control of Glycogen Synthesis in Cultured Human Muscle Cells*

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The regulation of glycogen synthesis and associated enzymes was studied in human myoblasts and myotubes maintained in culture. Both epidermal growth factor (EGF) and insulin stimulated glycogen synthesis approximately 2-fold, this stimulation being accompanied by a rapid and stable activation of the controlling enzyme glycogen synthase (GS), EGF also caused inhibition of glycogen synthase kinase 3 (GSK-3) and activation of the α isoform of protein kinase B (PKB) with the time-course and magnitude of its effects being similar to those induced by insulin. An inhibitor of the mitogen-activated protein (MAP) kinase pathway did not prevent stimulation of GS by EGF, suggesting that this pathway is not essential for the effect. A partial decrease in the fold activation of GS was, however, observed when p70S6K activation was blocked with rapamycin, suggesting a contribution of this pathway to the control of GS by either hormone. Wortmannin, a selective inhibitor of phosphatidylinositol 3’-kinase (PI-3 kinase) completely blocked the effects of both EGF and insulin in these cells. These results demonstrate that EGF, like insulin, activates glycogen synthesis in muscle, acting principally via the PKB/GSK-3 pathway but with a contribution from a rapamycin-sensitive component that lies downstream of PI-3 kinase.

A key step in the lowering of blood glucose levels by insulin is the promotion of uptake of glucose into muscle and its subsequent storage as glycogen. This involves recruitment of additional glucose transporters to the plasma membrane and stimulation of the enzyme glycogen synthase (GS)1 (reviewed in Ref. 1). The activity of GS is regulated by reversible phosphorylation at a number of sites. The key sites involved in the regulation of GS are collectively referred to as sites 3, phosphorylation of which by glycogen synthase kinase 3 (GSK-3) leads to inactivation of GS (2). The dephosphorylation and re-activation of GS is catalyzed by a glycogen-bound form of protein phosphatase 1 (PP1G) (3).

Insulin activates GS via dephosphorylation of the protein. There is evidence for insulin exerting this stimulatory effect on GS by both activating PP1G and by inhibiting GSK-3. Recently, attention has focused on the mechanism by which insulin inhibits GSK-3, with a plausible scheme emerging to link events at the plasma membrane with regulation of GSK-3. GSK-3 is inactivated by phosphorylation at a single serine residue close to its amino terminus (4). At least three insulin-stimulated protein kinases, namely p70S6K, p90S6K, and protein kinase B (PKB) are capable of catalyzing this phosphorylation in vitro. Each of these kinases lies downstream of an insulin-stimulated cascade (5). However, current evidence indicates that PKB is responsible for this insulin-stimulated phosphorylation of GSK-3 in a variety of cell types (5–7). PKB is itself phosphorylated and activated in response to insulin, this being mediated, at least in part, by 3-phosphoinositide-dependent protein kinase 1 (PDK1)(8–9). The phosphorylation of PKB by PDK1 is dependent on the presence of phosphatidylinositol (3–5) triphosphate (10), the major lipid product of PI-3 kinase, an enzyme activated by insulin via binding to insulin receptor substrate 1 at the plasma membrane (reviewed in Ref. 11).

In addition to insulin, several other growth factors and hormones influence glycogen synthesis and the signaling pathways involved. Of particular interest is the action of epidermal growth factor (EGF) which apparently has different effects on glycogen metabolism in different cell types. In isolated rat adipocytes, EGF stimulates neither glycogen synthesis nor GS activity, despite stimulating p70S6K and p90S6K (12). Consistent with the failure to activate GS is the observation that, unlike insulin, EGF has only a small and transient effect on the activities of PKB and GSK-3 (13). In contrast, EGF inhibits GSK-3 in hepatocytes to the same extent as insulin (14) and yet it does not stimulate glycogen synthesis, indeed it antagonizes the stimulatory effect of insulin on this metabolic effect (15). This antagonistic effect is not however observed in adipocytes (12).

It is well established that insulin inactivates GSK-3 and activates GS in a variety of muscle systems (5, 6, 13, 16, 17) but little work has been carried out using EGF, with the exception of one study in rat diaphragm which reported that EGF does not stimulate GS (18). This laboratory has utilized cultured human muscle cells to study the control of glycogen synthesis by insulin (6, 17). Here we report that EGF stimulates GS and glycogen synthesis in both human myoblasts and myotubes and that this is apparently mediated via the PKB/GSK-3 pathway.

EXPERIMENTAL PROCEDURES

Materials—All tissue culture trays were from Costar (Cambridge, MA). Culture media, penicillin/streptomycin, and trypsin-EDTA were...
from Life Technologies, Inc. (Paisley, UK). Chick embryo extract was obtained from ICN (Costa Mesa, California).

Anti-GSK-3α and anti-GSK-3β antibodies and antibodies to the PH-domain of PKBα were as described previously (16, 5). (γ-32P)ATP (148 TBq/mmol) was obtained from ICN. [γ-32P]ATP (12.5 GBq/mmol) and [γ-32P]ATP (22.5 GBq/mmol) were from American Pharmaceutical Biotech (Buckinghamshire, UK). Wortmannin and rapamycin were from Sigma (Poole, UK), and PD98059 was from New England Biolabs (Massachusetts). Actrapid insulin was from Novo Nordisk (Copenhagen, Denmark). Mouse EGF was from Sigma.

**Cell Culture**—Human myoblasts were grown from needle biopsy samples taken from the gastrocnemius of healthy subjects with no family history of Type 2 diabetes and with normal glucose tolerance and normal insulin sensitivity as assessed using the short insulin tolerance test.

Myoblasts were maintained in growth medium consisting of Ham's F-10 nutrient mixture containing 20% fetal calf serum, 1% chick embryo extract, 10,000 units/ml penicillin, and 2 mg/ml streptomycin. Myoblasts were fused to form myotubes by incubation for up to 5 days in α-minimal essential medium containing 2% fetal calf serum, 2% l-glutamine, 10,000 units/ml penicillin, and 2 mg/ml streptomycin. All experiments were performed using cells between the fifth and sixteenth passage at greater than 90% confluence. Prior to hormone treatment, cells were incubated for at least 2 h in serum-free medium.

**Extraction of Protein Kinase Assays**—Preparation of Cell Extracts for Assay of Glycogen Synthase—Following the indicated treatments, cells were rapidly washed five times in ice-cold phosphate-buffered saline. Cells were lysed by the addition of 20% (v/v) KOH and excess liquid was removed. Cells were then scraped into kinase extraction buffer (100 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 2 mM MgCl2, 10 mM Na2MoO4, 1 μg/ml leupeptin, 1 μg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride). Cells were then disrupted by sonicating for 10 min and the pellets discarded. Glycogen synthase activity was assayed as incorporation of "H-glucose from uridine-5'-diphosphate [U-32P]ATP (approximately 4000 cpm/pmol), 10 mM MgCl2, 0.5 mM benzamidine, 0.5 mM dithiothreitol, 0.05 mM Na3VO4, and 2.5 mM inhibitory peptide of cyclic AMP-dependent protein kinase (PKA) (6). After incubation for 30 min at 30 °C, samples were centrifuged at 13,000 × g for 3 min, and 15 μl of the supernatant containing the radiolabeled peptide product was spotted onto 1-cm2 Whatman P81 phosphocellulose paper. After washing in 175 μl phosphoric acid with four changes, the papers were dried and phosphate incorporation was determined by liquid scintillation counting. PKB assay was assayed in an identical manner except that activity was measured against the PKB substrate Crotstide (100 μM) (5). One milliunit of enzyme activity was defined as that which catalyzes the incorporation of 1 nmol of phosphate into peptide substrate in 1 min.

**RESULTS**—In preliminary experiments, the effect of varying concentrations of EGF on glycogen synthesis in myoblasts was investigated. Incubation of cultured myoblasts with EGF (100 nm) caused a maximal increase in the incorporation of glucose into glycogen from 193 ± 18 to 360 ± 49 pmol/min/mg (n = 7, p < 0.05, compared with control). This increase was similar to that evoked by insulin which stimulated the rate of glycogen synthesis to 462 ± 55 pmol/min/mg (n = 7 independent experiments in cells from three subjects, p < 0.05, compared with control).

Incubation with EGF also caused a time-dependent increase in the activity ratio of GS, with stimulation being observed within 5 min and reaching a maximum of approximately 2-fold after 10–15 min (Fig. 1a). The magnitude and time courses are similar to those observed previously in response to insulin (6), although the basal values in the present work are significantly lower than reported previously. No synergistic effect was observed when the two agonists were added simultaneously (Fig. 1b). Similar effects were observed in myotubes, where EGF (100 nm) caused a significant increase in the activity ratio of GS from 0.014 ± 0.0012 to 0.030 ± 0.0024 (n = 6 independent experiments in cells from three subjects, p < 0.05, compared with control).

Previous work from several laboratories has indicated that the activation of GS by insulin is mediated via inactivation by GSK-3. The effect of EGF and insulin on the activity of GSK-3 is shown in Fig. 2. EGF causes a rapid decrease in the activity of GSK-3 (Fig. 2a) and both the magnitude (Fig. 2b) and time course (Fig. 6) of this effect are similar to those observed with insulin.

To probe the events upstream of GSK-3, selective inhibitors of p70S6k activation (rapamycin), p90S6k activation (PD98059), and PI-3 kinase (wortmannin) were utilized. Fig. 3 demonstrates that inactivation of GSK-3 by EGF is not significantly affected by rapamycin or PD98059 but is blocked completely by wortmannin. These observations are consistent with those for insulin (5, 6), implicating PKB as the kinase responsible for phosphorylation and inactivation of GSK-3 in response to EGF. This is supported by the data in Fig. 4, which shows that PKB is rapidly activated by EGF, the activation being sufficiently rapid to account for the time course of GSK-3 inactivation (Fig. 4a). Once again the magnitude of activation in response to EGF was similar to that evoked by insulin (Fig. 4b).

The effect of these inhibitors on the activity of GS was also examined (Table 1). Whereas rapamycin had little effect on the basal activity of GS, both PD98059 and wortmannin caused a decrease in the fractional activity in the absence of agonists. Upon addition of EGF, full activation of GS was observed (the presence of PD98059), implying that the MAP kinase p90S6k pathway is not essential for the activation of GS by EGF, despite EGF being a potent stimulator of that pathway (12). Wortmannin completely blocked activation of GS by EGF. The EGF-induced stimulation of GS activity was, however, reduced in the presence of rapamycin (EGF alone at 1.8-fold versus EGF/rapamycin at 1.3-fold). We have previously reported a
similar effect of rapamycin on the activation of GS following insulin treatment of these cells (6).

**DISCUSSION**

Human muscle cells in culture represent an appropriate model system for the study of cell signaling. Myoblasts and myotubes in culture show many properties of mature muscle in terms of their responsiveness to insulin and other agonists, but they are immature cells and show several important differences from mature muscle (6, 17). One prime example is that myoblasts do not express significant levels of the insulin-responsive glucose transporter Glut-4 and show little increase in glucose uptake in response to insulin (21). Although this is a limitation of the cells, it does have the advantage that observed effects on glycogen synthesis are effectively independent of effects on glucose uptake and therefore presumably because of changes in the activity ratio of GS. Hence, although the cells represent a good model for study of events in human muscle in vivo, some of the data must be interpreted with caution when extrapolating to the situation in vivo, although these concerns apply even more when using immortalized cell lines taken from a variety of animal species.

The data reported here indicate that, in myoblasts obtained from healthy human volunteers, EGF causes an approximate 2-fold stimulation of the rate of incorporation of extracellular glucose into intracellular glycogen. This is associated with a relatively rapid and sustained activation of GS. In both cases, the extent of stimulation and the time course of the effects were similar to those observed in response to insulin (6).

Several observations suggest that common pathways are involved in the stimulation of GS by both EGF and insulin. First, both the time course and extent of the effects of the two agonists were remarkably similar for all the parameters examined. Second, the stimulation of GS activity by EGF and insulin was not additive if both agonists were present simultaneously. Finally, rapamycin was able to partially prevent the stimulation of GS by both EGF and insulin. Overall, the data suggest that the stimulation of glycogen synthesis in response to both EGF and insulin is mediated principally via the PKB/GSK-3 pathway, leading to the dephosphorylation of GS and stimulation of its activity, but with an additional contribution via a rapamycin-sensitive pathway that does not involve inhibition of GSK-3. Previous work on 3T3-L1 adipocytes has demonstrated involvement of a rapamycin-sensitive component in the stimulation of glycogen synthesis and GS by insulin (22). It has been reported previously, using A431 cells, that the phosphorylation and inactivation of GSK-3 in response to EGF is not...
Cultured human myoblasts were incubated in the absence by EGF. Alternatively, cells were treated with 100 nM rapamycin for 15 min (R EGF), 50 nM PD98059 for 1 h (PD EGF), or 100 nM wortmannin (W EGF) for 15 min prior to the addition of EGF. Addition of each inhibitor had no effect on the basal values of GSK-3. Extracts were prepared and assayed for GSK-3 activity following immunoprecipitation of the enzyme. Results are expressed as means ± S.E. (n = 6 independent experiments in three different patients). Activities are presented as a percentage of the basal activity. Statistical significance (p < 0.05) compared with the value obtained in cells treated with EGF alone is indicated by *.

Effects of selective inhibitors on the inhibition of GSK-3 by EGF. Cultured human myoblasts were incubated in the absence (Bas) or presence of 100 nM EGF (EGF) for 10 min. Alternatively, cells were treated with 100 nM rapamycin for 15 min (R EGF), 50 nM PD98059 for 1 h (PD EGF), or 100 nM wortmannin (W EGF) for 15 min prior to the addition of EGF. Addition of each inhibitor had no effect on the basal values of GSK-3. Extracts were prepared and assayed for GSK-3 activity following immunoprecipitation of the enzyme. Results are expressed as means ± S.E. (n = 6 independent experiments in three different patients). Activities are presented as a percentage of the basal activity. Statistical significance (p < 0.05) compared with the value obtained in cells treated with EGF alone is indicated by *.

The finding of different effects of EGF on glycogen synthesis in different cell systems remains enigmatic (12–15). One possible explanation is that some cells may express a counter-regulatory pathway, triggered by EGF which acts to antagonize the stimulatory effects on GS and glycogen synthesis. Of particular interest is the comparison of the present data in muscle with observations with isolated rat hepatocytes where EGF inactivates GS by EGF–3 to the same extent as insulin and also causes a significant transient activation of PKB (14) and yet does not stimulate glycogen synthesis and indeed antagonizes the stimulatory effect of insulin on that metabolic parameter (15). It is clear, however, that EGF has a range of effects in hepatocytes, which possess both high and low affinity forms of the EGF receptor (24). The effects of EGF on glycogen synthesis in hepatocytes are complex in that they are dependent on conditions of cell culture (e.g., cell density) and the morphology of the cells. Furthermore, there appears to be a pertussis toxin-sensitive component to the action of EGF (15). The ability of EGF to activate phospholipase C in hepatocytes (25) leading to increases in the cytosolic levels of Ca2+ may also trigger a counter-regulatory response, which overrides the stimulatory effects of EGF (and insulin) on glycogen synthesis. In contrast, the major if not sole effect of EGF in muscle is to stimulate glycogen synthesis, via activation of GS. The stimulatory effects of EGF are observed in the absence of insulin and are not augmented by insulin, implying a common pathway and providing no evidence for an additional EGF-specific pathway.

It has been previously reported that EGF fails to stimulate the activity of GS in isolated rat diaphragm (18). The finding that EGF also fails to stimulate p70S6k in that system is consistent with the possibility that a rapamycin-sensitive pathway is the major pathway controlling GS by EGF, under the conditions studied in that system. The relative importance of the respective signaling pathways in human muscle in vivo remains to be determined.

The observation (Table I) that the MAP kinase/ERK kinase inhibitor PD98059 causes a decrease in the activity state of GS under basal conditions implies that the MAP kinase pathway is involved in the maintenance of GS activity under basal conditions in these cells. This is consistent with our previous observation that considerable p90S6k activity is present in human myoblasts in the absence of any known agonist (6). However, upon addition of EGF, the activity of GS rises significantly, and indeed, the increase in the presence of PD98059 is equivalent.
to the increase induced by EGF in the absence of the inhibitor. It is possible that the effect of PD98059 on the basal values of GS is because of the involvement of the MAP kinase pathway in the regulation of a GS phosphatase, possibly PP1G (3). Thus, appreciable levels of MAP kinase activity in the basal state may partially activate the GS phosphatase, whereas inhibition of MAP kinase activity with PD98059 would inactivate the phosphatase, suppressing GS activity. This scenario would allow for subsequent stimulation of GS via inhibition of GSK-3 alone, a process which is unaffected by PD98059.

Similarly, lowering of the basal activity ratio of GS by wortmannin implies a role for PI-3 kinase and the MAP kinase pathway (16) in maintaining the basal state of activity.

In summary, the data reported here demonstrate that EGF stimulates both glycogen synthesis and GS in human muscle cells in culture. This is mediated primarily via the GSK-3/PKB pathway, but there is also a contribution to the activation of GS from a component which is rapamycin-sensitive and lies downstream of PI-3 kinase.

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