Increased malonyl-CoA and diacylglycerol content and reduced AMPK activity accompany insulin resistance induced by glucose infusion in muscle and liver of rats

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CHRONIC HYPERGLYCEMIA IS INCREASINGLY RECOGNIZED as a factor contributing to the insulin resistance and impaired insulin secretion associated with diabetes (48). It has been linked to insulin resistance in type 1 diabetic subjects in poor glycemic control (51) and in muscle (11, 16, 27, 28, 39) and liver (10) of rodents infused with glucose for 1–5 days. In addition, prolonged exposure of muscle (4 h) (38) and vascular endothelium (24 h) (17) to a high glucose concentration in vitro has been shown to impair insulin action.

The precise mechanisms by which a sustained oversupply of glucose to tissues generates insulin resistance are not clear. We have previously obtained evidence that one factor could be changes in intracellular lipid metabolism. Thus, when insulin resistance was produced in the rat by a glucose infusion at a rate threefold greater than normal basal glucose turnover for 1–4 days, increases in triglyceride, malonyl-CoA, and whole tissue long-chain fatty acyl-CoA (LCA-CoA) were observed in red muscle, as was an increase in membrane-associated PKCε (27, 28). Whether these events antedate or accompany the onset of insulin resistance has not been studied, nor has the possibility that these changes, as well as the insulin resistance, could be related to decreases in the activity of AMP-activated protein kinase (AMPK) activity and/or increases in the activity of acetyl-CoA carboxylase (ACC) (40). The latter possibilities are attractive, since a glucose excess increases the activity of ACC, leading to an increase in the concentration of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase I (CPT I), which controls the entrance of long-chain fatty acids into mitochondria. In this scenario, ACC may be increased directly by a glucose-induced increase in the concentration of its allosteric activator citrate (42) or by a glucose-induced decrease in the activity of AMPK (19), which, when activated, phosphorylates and inhibits ACC. The net result of one or both of these events would be a reduction in fatty acid oxidation and an increase in fatty acid esterification (40). Taken with earlier in vitro findings in adipocytes and fibroblasts (30, 35), our data suggested involvement of the diacylglycerol (DAG)-protein kinase C (PKC) pathway in muscle glucose-induced insulin resistance in vivo (28). DAG content was not measured in our original investigation, however, and it was not clear whether its accumulation is an early or late event in the onset of glucose-induced insulin resistance in muscle.

In the present study, we aimed to identify the time of onset of insulin resistance in both red and white skeletal muscle and liver during a glucose infusion, with a view to elucidating the changes that are coincident with or precede it. Toward this end, changes in muscle and liver triglyceride and whole tissue LCA-CoAs and DAGs were determined before and during the onset of insulin resistance. In addition, we examined whether the onset of insulin resistance in muscle and liver was associ-
ated with defined changes in key parameters of the AMPK-malonyl-CoA fuel-sensing mechanism, including the enzymes AMPK and ACC, and the concentration of malonyl-CoA (40).

**RESEARCH DESIGN AND METHODS**

**Animals**

All surgical and experimental procedures performed were approved by the Animal Experimentation Ethics Committee (Garvan Institute/St. Vincent’s Hospital) and were in accordance with the National Health and Medical Research Council of Australia’s guidelines on animal experimentation.

Adult male Wistar rats, supplied from the Animal Resources Centre (Perth, Australia), were communally housed at 22 ± 0.5°C with a controlled 12:12-h light-dark cycle (light from 0600 to 1800). They were fed ad libitum a standard chow diet (Rat Maintenance Diet; Gordons Specialty Feeds, Sydney, Australia) containing 5% fat, 69% carbohydrate, and 21% protein plus fiber, vitamins, and minerals. After 1 wk of acclimatization, rats were anesthetized with a combination of Ketalar (ketamine, 80 mg/kg) and Ilium Xylazil (xylazine, 20 mg/kg) injected intraperitoneally. Catheters were exteriorized at the back of the neck. Rats were divided into treatment groups. The experimental infusion protocol is illustrated in Fig. 1. At this point, subsets of rats were euthanized and tissues taken for basal analyses (see below). A euglycemic-hyperinsulinemic clamp was commenced on the remaining rats, as previously described (20). In brief, rats were infused with insulin (Actrapid HM; Novo Nordisk, Bagsvaerd, Denmark) via the carotid cannula at a rate of 0.25 U·kg body wt⁻¹·h⁻¹ for 120 min. During the insulin infusion, euglycemia was maintained by a variable-rate glucose infusion using 30% glucose. Blood glucose was measured at 5- to 10-min intervals and the glucose infusion rate (GIR) adjusted to maintain euglycemia. Blood samples (200 μl) were taken at 2, 5, 10, 15, 20, 30, and 45 min after administration of the bolus for estimation of plasma tracer and glucose concentration. After this, animals were euthanized with an overdose of pentobarbital sodium (Nembutal). Tissues were rapidly removed, freeze-clamped, and stored at −80°C for subsequent determination of glucose uptake into specific tissues (Rg), triglyceride, glycogen content, and [14C]glucose incorporation rates into lipid and glycogen. Analyses were performed in red and white quadriceps (RQ and WQ) muscle and liver.

**Analytic Methods**

Blood and plasma glucose levels were determined by an immobilized glucose oxidase method (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH) using 25-μl samples. Plasma insulin and leptin levels were measured by radioimmunoassay kit (Linco, St. Louis, MO). Plasma NEFA levels were determined spectrophotometrically with a commercial kit (NEFA-C; WAKO Pure Chemical Industries, Osaka, Japan).

Plasma and tissue levels of 3H- and 14C-labeled tracers were measured as described previously, as were tissue glycogen concentration and [14C]glucose incorporation rates into glycogen (20). Tissue LCA-CoAs and triglyceride were extracted and measured as described previously (7).

Malonyl-CoA was assayed radioenzymatically by a modification of the method of McGarry (see Ref. 41) and phosphorylated AMPK and ACC by Western blotting using commercially available antibodies, as described previously (46). Liver and muscle were homogenized as described previously (22), and immunoprecipitated AMPK was assayed in a 500-g supernatant fraction by a modification of the methods.
Table 1. Basal, infusion, and preclamp plasma parameters of 3- and 5-h saline- and glucose-infused rats

<table>
<thead>
<tr>
<th>Plasma Parameter</th>
<th>3-h Infusion Group</th>
<th>5-h Infusion Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>3-h</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>8.1±0.2</td>
<td>7.3±0.2</td>
</tr>
<tr>
<td>Insulin, mU/l</td>
<td>20±12</td>
<td>54±12</td>
</tr>
<tr>
<td>Leptin, ng/l</td>
<td>ND</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>NEFA, mM</td>
<td>0.41±0.06</td>
<td>0.43±0.04</td>
</tr>
<tr>
<td>Glucose infusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>7.8±0.2</td>
<td>18.1±0.8 *</td>
</tr>
<tr>
<td>Insulin, mU/l</td>
<td>40±4</td>
<td>508±73*</td>
</tr>
<tr>
<td>Leptin, ng/l</td>
<td>ND</td>
<td>6.6±0.5*</td>
</tr>
<tr>
<td>NEFA, mM</td>
<td>0.40±0.04</td>
<td>0.20±0.02*</td>
</tr>
</tbody>
</table>

Shown are means ± SE of results from 5–7 rats per experimental group. NEFA, nonesterified fatty acids; ND, not determined. *P < 0.01 vs. saline infusion.

of Vavvas et al. (49). The activities of the α1- and α2-isoforms of AMPK were determined as previously described (33).

Statistical Analysis

Statistical analyses were performed by use of a statistics package (StatView; Abacus Concepts, Berkeley, CA). Differences among relevant groups were assessed using ANOVA or unpaired Student’s t-test as appropriate. A P value of <0.05 is considered significant.

RESULTS

Plasma Parameters Before and After Chronic Infusions of Glucose or Saline

Plasma glucose, insulin, leptin, and NEFA levels before commencement of the infusions (basal), at the end of 3- or 5-h infusions of saline or glucose, and immediately before the start of the final 2-h clamp (preclamp) were determined (Table 1). These parameters did not differ among the groups in the basal state. During the glucose infusion, plasma glucose was increased approximately twofold in the 3-h and 5-h groups, and plasma insulin levels responded accordingly. Plasma NEFA levels were suppressed in the glucose-infused rats, and plasma leptin was elevated twofold compared with saline-infused controls (P < 0.01). All of these parameters were similar between the 3-h and 5-h glucose-infused groups in the preclamp period.

Clamp Responses

Whole body. Euglycemic hyperinsulinemic clamps were performed over a final 2 h in the rats previously infused for 3 or 5 h with either saline or glucose. Levels of euglycemia and hyperinsulinemia over the last hour of the clamp were not significantly different among groups (means in range 7.1–7.5 mM and 126–139 mU/l, respectively). Other whole body clamp responses are shown in Fig. 2. The GIR required to maintain euglycemia was significantly lower in the 5-h glucose-infused rats (P < 0.001) compared with controls but was not diminished after 3 h. Whole body glucose uptake (Rd) was not significantly different from that observed in the control rats at either time point. Insulin suppressibility of hepatic glucose output (HGO) in the glucose-infused rats was diminished after 5 h of glucose infusion (P < 0.01), but not after 3 h, compared with saline-infused controls.

Muscle parameters. Glucose uptake into individual tissues (Rq), derived from [3H]DG uptake was measured in RQ and WQ muscle during the final 45 min of the clamp. In the 5-h glucose-infused group, a significant reduction in Rq was observed in both muscle types compared with controls (RQ P < 0.01, WQ P < 0.05; Fig. 3), indicating muscle insulin resistance. In contrast, no significant difference in Rq was observed for RQ or WQ after 3 h of glucose infusion (Fig. 3).

At the conclusion of the euglycemic-hyperinsulinemic 2-h clamp, glycogen content was increased in the RQ of the 3-h and 5-h glucose infusion groups and to a lesser extent in the WQ of the 5-h-infused rats (P < 0.05; Fig. 3). Rates of glycogen synthesis in the muscles during the clamp were significantly reduced compared with controls only in the 5-h glucose-infused group (Fig. 3).
At the conclusion of the clamp, tissue triglycerides were increased threefold in the liver of the 5-h glucose-infused rats, but they were not increased in the liver or the RQ or WQ after either infusion period.

Whole tissue LCA-CoAs were significantly elevated in the WQ of the 3-h glucose infusion group (\(P < 0.05\) vs. controls). On the other hand, they were not increased at 5 h in the WQ or in the RQ or the liver at either 3 or 5 h (Fig. 4). In addition to these measurements made at the conclusion of the clamp study, the concentration of LCA-CoA was determined in the RQ at several other times. As shown in Table 2, at the end of the 5-h glucose infusion (no clamp), whole tissue LCA-CoAs were significantly suppressed. They returned to control values after the stepwise return to euglycemia, and during the clamp they were decreased to the same extent as in the saline-infused rats.

In contrast to triglyceride and whole tissue LCA-CoA, DAG content was consistently elevated in the three tissues (RQ, WQ, and liver) of the 5-h glucose-infused rats (Fig. 4; \(P < 0.05\) vs. controls). DAG content was not significantly different from that of saline controls after 3-h of glucose infusion.

In parallel with the pattern of change of DAG content, Thr\(^{172}\)-phosphorylated AMPK (p-AMPK), a measure of activation, was diminished in RQ and liver after 5 h but not after 3 h of glucose infusion (\(P < 0.01\)). Consistent with the decrease in AMPK phosphorylation, the phosphorylation of ACC (p-ACC) was diminished in the tissues after 5-h but not after 3-h glucose infusion, in keeping with the decrease in p-ACC in these tissues. In contrast, neither of these parameters was significantly altered in the WQ at either time. The concentration of malonyl-CoA was significantly elevated in both the RQ and liver of the 5-h glucose-infused group. In contrast to p-AMPK and p-ACC, the content of malonyl-CoA was also significantly, but more modestly, increased in these tissues after 3 h of glucose infusion and in the WQ after 5 h of glucose infusion.

In view of the different p-AMPK responses of the RQ and WQ, measurements of isoform-specific AMPK activity were performed in the 5-h groups infused with glucose and saline (Fig. 5). In agreement with the p-AMPK changes in these tissues (Fig. 6), we found reduced AMPK\(\alpha_1\) activity in liver and RQ. In contrast, in the WQ, in which p-AMPK was not diminished, AMPK\(\alpha_2\) activity was significantly reduced, but to a much lesser degree than in the RQ muscle. No differences were found in AMPK abundance in the 5-h group infused with glucose.

In Table 2, the long-chain acyl-CoA content in red quadriceps muscle at various times during study is shown. The data show that at the end of 5-h infusion of glucose, the long-chain acyl-CoA was significantly increased compared to saline. After return to euglycemia, the levels remained elevated, and at the end of the clamp, they were significantly decreased compared to the saline group. The values are shown as means ± SE.

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**Table 2. Long-chain acyl-CoA content in red quadriceps muscle at various times during study**

<table>
<thead>
<tr>
<th>Study time point</th>
<th>Long-Chain Acyl-CoAs, (\mu\text{mol/g})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Infusion</td>
<td>Glucose Infusion</td>
</tr>
<tr>
<td>At end of 5-h infusion of glucose or saline</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>After return to euglycemia</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>At end of clamp</td>
<td>6.0 ± 1</td>
</tr>
</tbody>
</table>

Shown are means ± SE. *\(P < 0.01\) vs. saline infusion.
glucose compared with saline controls (RQ 98.8 ± 2.1 vs. 99.5 ± 6.1; WQ 98.0 ± 3.2 vs. 102.8 ± 3.0; liver 173 ± 4.8 vs. 163 ± 14 arbitrary units).

DISCUSSION

This study was performed to determine whether changes in cellular lipids, AMPK, and malonyl-CoA accompany the onset of insulin resistance in tissues of rats chronically infused with glucose. The principal findings are that insulin resistance was not present after 3 h of prior glucose infusion but appeared after 5 h, as evidenced by decreases in clamp GIR, insulin-mediated Rg, and glycogen synthesis in red and white muscle and impaired insulin suppressibility of HGO. In red and white muscle, the onset of insulin resistance at 5 h occurred without significant triglyceride and whole tissue LCA-CoA elevation but was accompanied by substantial increases in the content of DAG, an activator of conventional and novel PKCs and a possible mediator of insulin resistance (15, 24, 43). An increase in the concentration of malonyl-CoA occurred at 3 h in red muscle and at 5 h in both muscles; however, only in the red muscle was it associated with decreased AMPK and ACC phosphorylation. Changes similar to those in red muscle occurred in liver, except that triglyceride content was also increased at 5 h. As will be discussed below, the nature of the change in AMPK in white muscle is less clear.

Several studies, including our own, have demonstrated that chronic glucose infusion to rats for one to several days generates muscle and/or liver insulin resistance (11, 16, 27, 28, 47). These studies made use of chronic models, and the factors operative near the time of onset of the insulin resistance were not determined. From the results presented here, it appears that normal rats can cope with an oversupply of glucose and high insulin levels for a reasonable period of time without becoming insulin resistant but that ultimately changes in malonyl-CoA, DAG, and, in some tissues AMPK, occur and insulin action is impaired. At the whole body level, these changes were associated with an elevation in plasma leptin, indicative of a response of adipose tissue to an abundance of energy supply (29).

The current studies provide “snapshots” of stages just before and just after the onset of insulin resistance in muscle and liver of glucose-infused rats. We were particularly concerned with the possible involvement of accumulation of cytosolic lipid metabolites, such as triglyceride, LCA-CoAs, and DAG, all of which have been considered factors leading to insulin resistance (32, 44). Our previous studies involving 1–4 days’ glucose infusion reported significant muscle triglyceride and LCA-CoA accumulation accompanying the insulin resistance. Although DAG mass was not measured in these long-term studies, its elevation by a glucose infusion has been documented, as has an increase in membrane-associated PKCe in muscle of rats infused with glucose for 1 and 4 days (28).

Similarly, in studies in which insulin resistance was produced by fat feeding (32), by overexpressing lipoprotein lipase in muscle (24) or by raising plasma FFA levels during a clamp study (5, 52), elevation of multiple cellular lipid pools was observed.
There has been uncertainty as to which lipid pool may be most important in linking a sustained surfeit of glucose to impaired insulin action. Our current data stress the importance of DAGs, which are significantly elevated in both types of muscle and in liver when these tissues become insulin resistant. In contrast, it is apparent that insulin resistance in muscle can develop before significant elevations of triglyceride and whole tissue LCA-CoAs. The time course of changes in LCA-CoAs determined during glucose infusion (Table 2) stresses the labile nature of these metabolites; indeed, until euglycemia was reestablished, these whole tissue LCA-CoA levels were suppressed in muscle, probably as a result of reduced fatty acid supply. The fact that LCA-CoAs were not elevated does not rule out the possibility of increased flux through LCA-CoAs to DAGs. In addition, because of compartmentation in the cell, it cannot be stated with certainty that whole tissue levels of LCA-CoA reflect its concentration in the cytosol.

DAG was the only lipid measured that was consistently elevated in both muscle and liver at the time of onset of insulin resistance. Among possible mechanisms whereby increased DAG mass could generate insulin resistance is the activation of DAG-sensitive PKCs (9, 28, 43), leading to impaired insulin signaling, possibly via serine phosphorylation of insulin receptor substrate-1 (IRS-1) (52). In keeping with this possibility, signaling, possibly via serine phosphorylation of insulin receptor substrate-1 (IRS-1) (52), leading to impaired insulin resistance, and that decreases in AMPK occur later. As reviewed elsewhere (21, 40), decreases in AMPK activity could sustain insulin resistance both by effects on malonyl-CoA and by independent effects on ceramide and DAG synthesis, oxidative stress, the activation of mTOR and UFBK, and mitochondrial function. Evidence that an increase in the concentration of malonyl-CoA leads to changes in lipid metabolism and insulin resistance independently of AMPK comes from two recent reports: one showing an increase in insulin sensitivity in mice genetically deficient in ACC2 (1) and the other showing an increase in insulin sensitivity in fat-fed rats treated with a pharmacological ACC inhibitor (14).

Events in the WQ were not as clear cut as in RQ in that p-AMPK and p-ACC were not diminished after 5 h of glucose infusion. However, a small but significant decrease in AMPK activity was observed, suggesting that AMPK was altered. The reason for the apparent discrepancy between the p-AMPK and activity measurements remains to be determined. Possibly, the activity measurement is a more sensitive indicator since it distinguishes the α1- and α2-AMPK isoforms, whereas p-AMPK abundance does not. Alternatively, the activity measurement detects AMPK activation earlier. If so, studies in which glucose is infused for periods in excess of 5 h should enable us to determine whether more substantial effects on p-AMPK and AMPK occur later in WQ. Such a finding would be consistent with a previous study, in which incubation of the extensor digitorum longus, another white muscle, with an elevated glucose concentration (25 vs. 5 or 0 mM glucose) for 4 h caused significant decreases in both AMPK phosphorylation and activity (19).

Independently of whether a decrease in AMPK activity is a cause of insulin resistance, the current study widens the perspective of the role of AMPK in the regulation of energy metabolism. The classic view is that AMPK is activated in response to a decrease in energy state, caused by such stresses as ischemia, fuel deprivation, and, in muscle, contraction. In these situations, the role of AMPK activation has been assumed to be the restoration of cellular ATP levels to normal values. It is now apparent that AMPK may additionally play a
bidirectional role in cellular energy regulation since, as shown in the present study, its activity is diminished in vivo in states of high glucose supply. Although the precise mechanism(s) responsible for the decrease in AMPK activity in this setting is not known, on the basis of the findings here and in studies with incubated muscle (19) and cultured Hep G2 hepatoma cells (53), we suggest that it is a part of a feedback mechanism that limits glucose uptake into muscle and prevents the inhibition of glucose release from liver. In keeping with this notion, a decrease in AMPK activity has been observed in the liver of 24- to 48-h-starved rats when refed a high-carbohydrate mea.

Furthermore, in the latter study, the decrease in AMPK activity was associated with a shift in hepatic enzymes that favored the net synthesis of malonyl-CoA, DAG, and triglyceride.

The reason AMPK activity is diminished by the glucose infusion in red muscle and liver is not known. Studies in rat muscles incubated with a high concentration of glucose suggest that it is not associated with a decrease in the AMP/ATP ratio. Alterations in an upstream AMPK kinase (13), phosphorylation and inhibition of AMPK by a serine kinase such as Akt (25), the action of specific phosphatases on phosphorylated AMPK, changes in redox state (37), and alterations in tissue (muscle) glycogen (36, 50) have all been suggested as possible AMPK regulators. However, their roles in the regulation of muscle or liver AMPK by glucose availability have not been tested. It is possible also that the high insulin levels during the glucose infusion may have a suppressive effect on AMPK activity, possibly via Akt activation (12). However, it is unclear whether this occurs in vivo; indeed, the fact that 3 h of substantially elevated insulin does not by itself lead to decreased AMPK activity argues against this. Additionally, we have new in vivo data (6), where Akt1 was substantially overexpressed locally in rat tibialis cranialis muscle by DNA electrophoresis (and glycogen content increased) without a concomitant reduction in AMPK phosphorylation.

Finally, it is somewhat puzzling that a significant decrease in Rd was not observed during the clamp in the 5-h glucose-infused rats, although there was a small tendency toward a reduction [amplified to 15–20% vs. controls when expressed as glucose clearance (data not shown)]. There may possibly have been a compensatory increase in glucose clearance by other lipogenic tissues (e.g., liver). In any event, it should be stated that our bolus tracer methodology Rd estimation is not as precise as the Rg estimation in individual muscles. Indirect evidence that the clamp Kg may be slightly overestimated in the 5-h glucose-infused group is provided by the estimated clamp HGO, which, at 15 mg·kg\(^{-1}\)·min\(^{-1}\), is 3–4 mg·kg\(^{-1}\)·min\(^{-1}\) above historical values for basal glucose turnover in our rats. There was, however, excellent agreement between the independent clamp Kg and glycogen synthesis estimates indicating muscle insulin resistance in the 5-h glucose-infused group.

In conclusion, our studies reveal that a coordinated set of metabolic responses come into play in response to an energy abundance produced by a systemic oversupply of glucose. An association in time was defined among 1) onset of muscle and liver insulin resistance, 2) accumulation of cellular lipids, particularly diacylglycerol, and 3) a decrease in AMPK activity in red muscle and liver and possibly white muscle. We suggest that these changes are part of an adaptation to regulate the tissue fate of excess glucose supply. How these alterations relate to changes in other pathways implicated in causing glucose-induced insulin resistance [e.g., hexosamine biosynthetic pathway, O-glycosylation of proteins (40)] remains to be determined. The experimental model and time sequences defined here should prove useful in defining the role of these pathways and other biochemical and genetic events that regulate insulin sensitivity in high energy availability states in vivo.

**GRANTS**

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