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## 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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**Kraegen, Edward W., Asish K. Saha, Elaine Preston, Donna Wilks, Andrew J. Hoy, Gregory J. Cooney, and Neil B. Ruderman.** Increased malonyl-CoA and diacylglycerol content and reduced AMPK activity accompany insulin resistance induced by glucose infusion in muscle and liver of rats. *Am J Physiol Endocrinol Metab* 290: E471–E479, 2006. First published October 18, 2005; doi:10.1152/ajpendo.00316.2005.—Glucose infusion in rats for 1–4 days results in insulin resistance and increased triglyceride, whole tissue long-chain fatty acyl-CoA (LCA-CoA), and malonyl-CoA content in red skeletal muscle. Despite this, the relation between these alterations and the onset of insulin resistance has not been defined. We aimed to 1) identify whether the changes in these lipids and of diacylglycerol (DAG) precede or accompany the onset of insulin resistance in glucose-infused rats, 2) determine whether the insulin resistance is associated with alterations in AMP-activated protein kinase (AMPK), and 3) assess whether similar changes occur in liver and in muscle. Hyperglycemia (17–18 mM) was maintained by intravenous glucose infusion in rats for 3 or 5 h; then euglycemia was restored and a 2-h hyperinsulinemic clamp was performed. Significant ( $P < 0.01$ ) muscle and liver insulin resistance first appeared in red quadriceps and liver of the glucose-infused group at 5 h and was associated with a twofold increase in DAG and malonyl-CoA content and a 50% decrease in AMPK and acetyl-CoA carboxylase (ACC) phosphorylation and AMPK activity. White quadriceps showed qualitatively similar changes but without decreases in AMPK or ACC phosphorylation. Triglyceride mass was increased at 5 h only in liver, and whole tissue LCA-CoA content was not increased in liver or either muscle type. We conclude that the onset of insulin resistance induced by glucose oversupply correlates temporally with increases in malonyl-CoA and DAG content in all three tissues and with reduced AMPK phosphorylation and activity in red muscle and liver. In contrast, it was not associated with increased whole tissue LCA-CoA content in any tissue or triglyceride in muscle, although both are observed at later times.

adenosine monophosphate-activated protein kinase; hyperglycemia; glucose clamp; triglyceride; long-chain acyl-coenzyme A; glucotoxicity

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 PKC $\epsilon$  (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-

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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  $22 \pm 0.5^\circ\text{C}$  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. Cannulas were implanted into the right jugular vein and the left carotid artery under aseptic conditions. Catheters were exteriorized at the back of the neck. Rats were isolated in separate cages after surgery and handled daily to minimize stress. Only those rats that reached presurgery weight or above were used for experiments. Average body weight on the experimental/day was  $350 \pm 2$  g.

### Glucose Infusion

Six to seven days after cannulation surgery, rats were randomly divided into treatment groups. The experimental infusion protocol is shown in Fig. 1. Rats were infused either with 50% glucose or with 0.9% saline at a rate of 2 ml/h (control) for 3 or 5 h. Chronic infusion was performed via the carotid cannula by use of a peristaltic roller pump (101U/R; Watson-Marlow, Falmouth, UK). A blood sample (300  $\mu\text{l}$ ) was collected via the jugular cannula 10 min before the infusion started to determine basal levels of whole blood glucose and plasma glucose, insulin, nonesterified fatty acids (NEFA), and leptin.

A blood sample was then taken every 30 min, and the glucose level was adjusted, when necessary, to maintain a whole blood glucose concentration of  $\sim 12$  mM (corresponding to a plasma glucose level of 17–18 mM). Red blood cells from each sample were resuspended in normal saline and returned to the rat. After each infusion period of

either 3 or 5 h, the glucose infusion was reduced progressively over a 45-min period in three 15-min steps to minimize effects of a sudden decrease of plasma glucose and to prevent hypoglycemia from developing (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  $0.25 \text{ U} \cdot \text{kg body wt}^{-1} \cdot \text{h}^{-1}$  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. 2-deoxy- $^3\text{H}$ glucose ( $^3\text{H}$ JDG) and  $^{14}\text{C}$ glucose were administered as an intravenous bolus 75 min after commencement of the clamp. Blood samples (200  $\mu\text{l}$ ) 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  $-80^\circ\text{C}$  for subsequent determination of glucose uptake into specific tissues ( $\text{Rg}'$ ), triglyceride, glycogen content, and  $^{14}\text{C}$ 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- $\mu\text{l}$  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  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled tracers were measured as described previously, as were tissue glycogen concentration and  $^{14}\text{C}$ 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

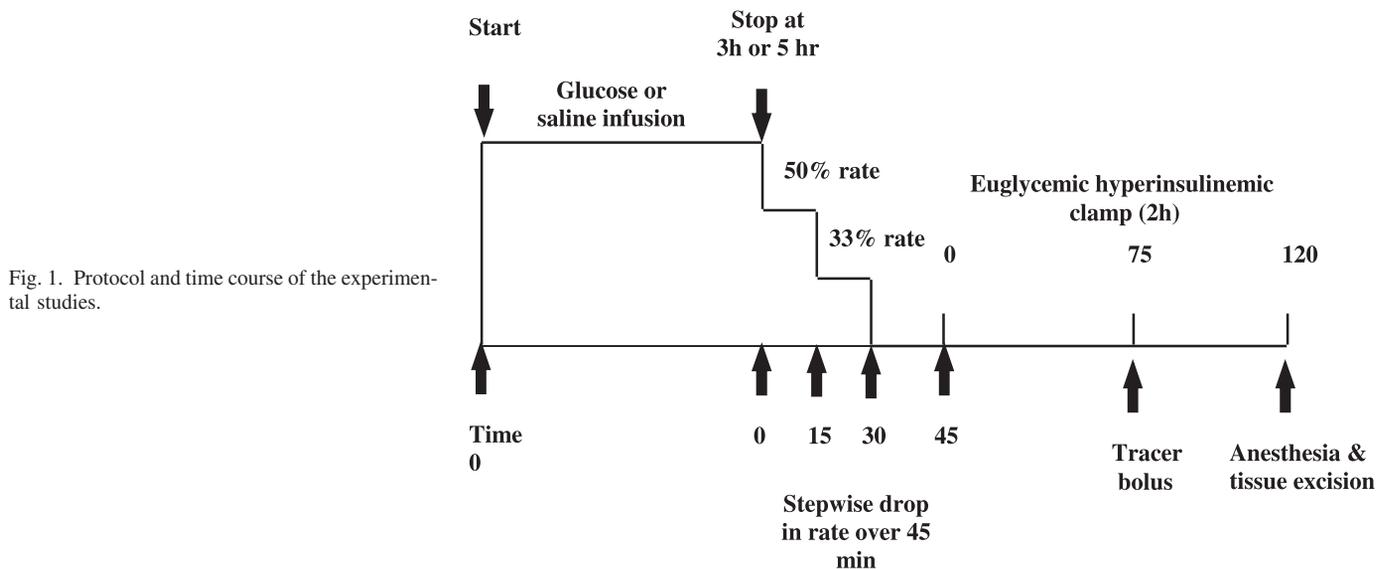


Fig. 1. Protocol and time course of the experimental studies.

Table 1. Basal, infusion, and preclamp plasma parameters of 3- and 5-h saline- and glucose-infused rats

Plasma Parameter	3-h Infusion Group			5-h Infusion Group		
	Basal	3-h	Preclamp	Basal	5-h	Preclamp
<b>Saline infusion</b>						
Glucose, mM	8.1±0.2	7.3±0.2	7.4±0.2	8.3±0.3	7.2±0.4	7.5±0.3
Insulin, mU/l	20±12	54±12	43±7	59±4	57±2	48±8
Leptin, ng/l	ND	2.6±0.1	ND	2.6±0.3	1.7±0.1	2.3±0.9
NEFA, mM	0.41±0.06	0.43±0.04	0.44±0.04	0.28±0.01	0.42±0.04	0.41±0.05
<b>Glucose infusion</b>						
Glucose, mM	7.8±0.2	18.1±0.8*	6.0±0.3*	8.3±0.3	17.3±0.9	6.3±0.1*
Insulin, mU/l	40±4	508±73*	53±8	64±6	531±88	85±9
Leptin, ng/l	ND	6.6±0.5*	5.7±0.7	3.0±0.3	5.9±0.7*	4.2±0.7
NEFA, mM	0.40±0.04	0.20±0.02*	0.19±0.02*	0.35±0.06	0.24±0.03*	0.20±0.02*

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  $\alpha 1$ - and  $\alpha 2$ -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 ( $R_d$ ) 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 ( $R_g'$ , derived from [ $^3$ H]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  $R_g'$  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  $R_g'$  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).

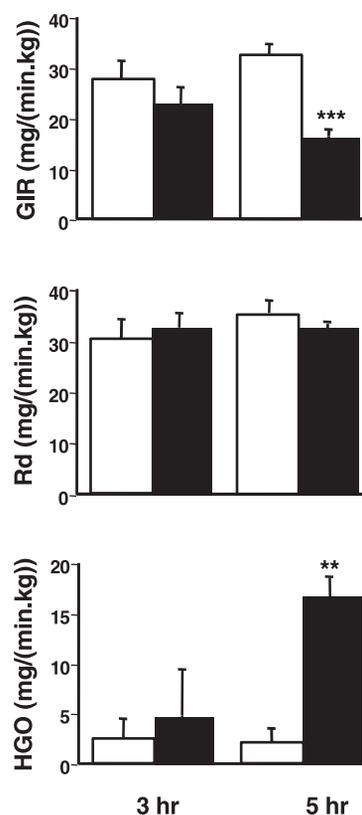


Fig. 2. Clamp parameters in rats previously administered an infusion for 3 or 5 h of saline (open bars) or glucose (filled bars). Means ± SE from 5–7 rats per experimental condition. GIR, clamp glucose infusion rate;  $R_d$ , rate of glucose disposal; HGO, hepatic glucose output. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. saline vehicle infusion.

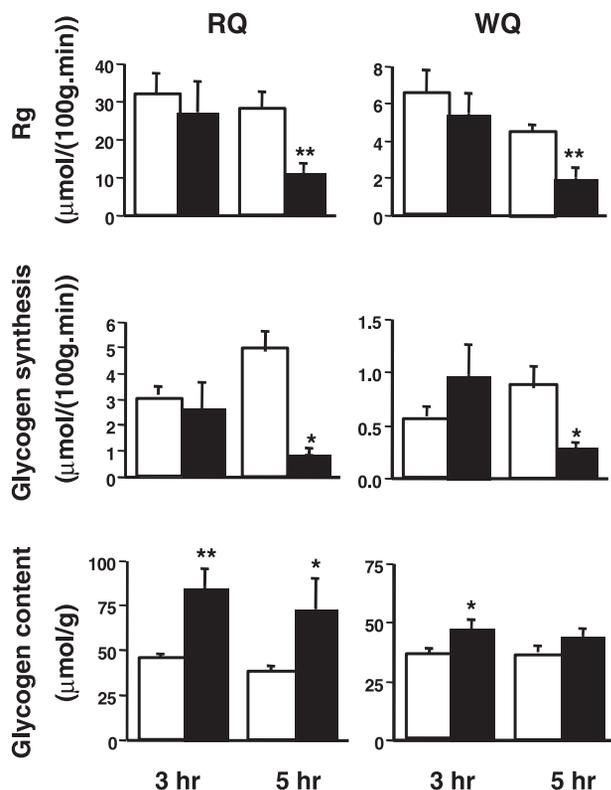


Fig. 3. Insulin-stimulated glucose uptake ( $Rg'$ ), glycogen synthesis rate, and glycogen content in red (RQ) and white quadriceps (WQ) muscle after 3- or 5-h infusion of saline (open bars) or glucose (filled bars). Means  $\pm$  SE of 5–7 rats per group. \* $P < 0.05$ , \*\* $P < 0.01$  vs. saline vehicle infusion.

**Tissue lipids.** 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 at 3 h or in 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.

#### Tissue AMPK, ACC, and Malonyl-CoA.

In parallel with the pattern of change of DAG content, Thr<sup>172</sup>-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

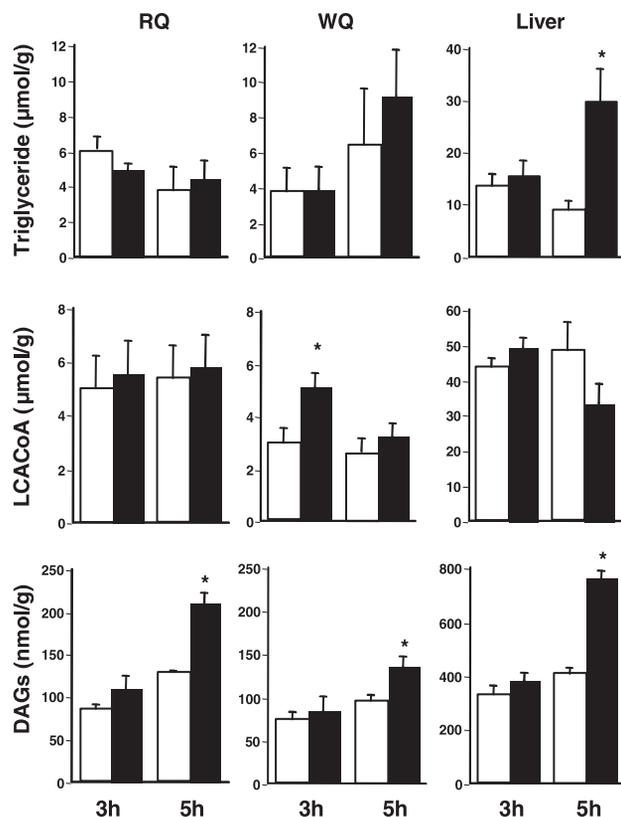


Fig. 4. Triglyceride, long-chain fatty acyl (LCA)-CoA, and diacylglycerol (DAG) in RQ, WQ, and liver after 3- or 5-h infusion period followed by hyperinsulinemic euglycemic clamp. Means  $\pm$  SE of 5–7 rats per group. \* $P < 0.05$  vs. saline vehicle infusion.

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

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

Study time point	Long-Chain Acyl-CoAs, $\mu\text{mol}/\text{g}$	
	Saline Infusion	Glucose Infusion
At end of 5-h infusion of glucose or saline	15 $\pm$ 2	4.5 $\pm$ 1*
After return to euglycemia	14 $\pm$ 1	12.5 $\pm$ 2.5
At end of clamp	6.0 $\pm$ 1	6.5 $\pm$ 1.5

Shown are means  $\pm$  SE. \* $P < 0.01$  vs. saline infusion.

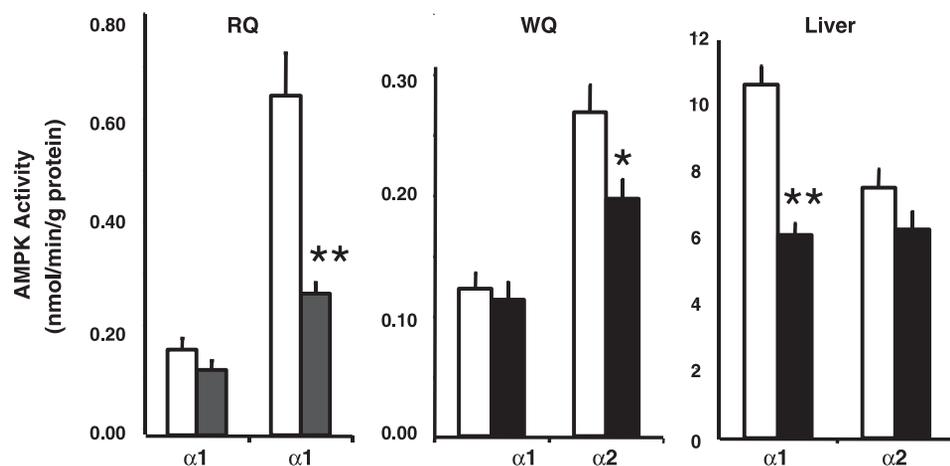


Fig. 5. AMPK $\alpha$ 1 and  $\alpha$ 2 subunit activities in RQ and WQ and liver after 5-h infusion of saline (open bars) or glucose (filled bars). Means  $\pm$  SE of 5–7 rats per group. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. saline vehicle infusion.

glucose compared with saline controls (RQ  $98.8 \pm 2.1$  vs.  $99.5 \pm 6.1$ ; WQ  $98.0 \pm 3.2$  vs.  $102.8 \pm 3.0$ ; liver  $173 \pm 4.8$  vs.  $163 \pm 14$  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 PKC $\epsilon$  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.

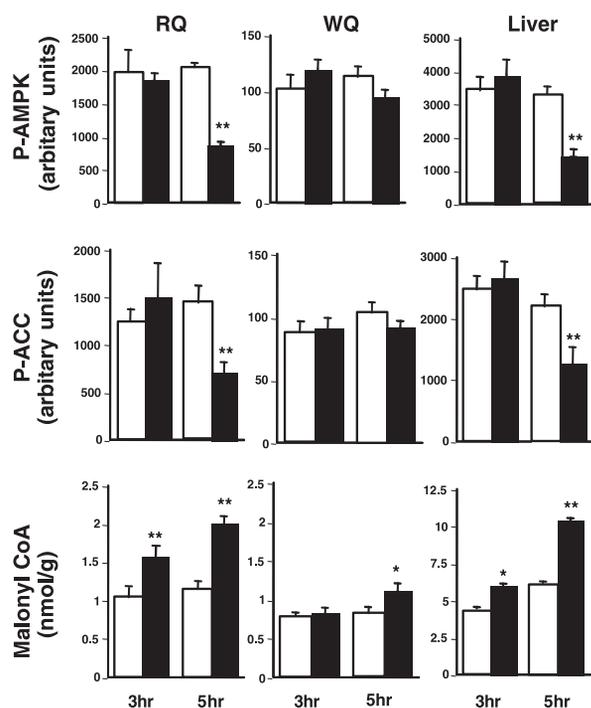


Fig. 6. Levels of phosphorylated AMPK (p-AMPK), phosphorylated acyl-CoA carboxylase (p-ACC), and malonyl-CoA in RQ, WQ, and liver after 3- or 5-h infusion period followed by a hyperinsulinemic euglycemic clamp. Means  $\pm$  SE of 5–7 rats per group. \* $P$  < 0.05, \*\* $P$  < 0.01 vs. saline vehicle infusion.

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, we (28) have previously reported that the DAG-sensitive isoform PKC $\epsilon$  and, to a lesser extent, PKC $\theta$  are activated in response to chronic glucose infusion (24 and 96 h) in both red and white muscle of the rat. Studies at earlier time points have not yet been conducted.

Although a case can be made for phosphorylation of IRS-1 by PKC as a cause of insulin resistance (52), other mechanisms could be involved. Oxidative stress, possibly caused by changes in PKC $\beta$  or PKC $\delta$  and/or NF- $\kappa$ B, has been linked to insulin resistance in muscle, adipose tissue, and possibly vascular cells (8, 18, 45). It has been suggested that activation of JNK and possibly IKK $\beta$  by oxidative stress might produce insulin resistance by phosphorylating serine/threonine residues on IRS (8), and recent studies have suggested that the mammalian target of rapamycin (mTOR) and p70 S6 kinase have a similar action (see Ref. 34). In addition to these findings, antioxidant infusion has been reported to oppose the development of hyperglycemia-induced insulin resistance in an in vivo model similar to that used in our study (10).

The role of a decrease in AMPK activity in mediating the development of insulin resistance is somewhat unclear. AMPK phosphorylation, a measure of its activity, was significantly decreased in both red muscle and liver after 5 h of glucose infusion in parallel with the increase in DAG content and the appearance of insulin resistance. Likewise, a decrease in the activity of the  $\alpha$ 2-isoform of AMPK in red muscle and the  $\alpha$ 1-isoform in liver paralleled these changes. AMPK activation has been reported to inhibit malonyl-CoA formation (40), DAG synthesis (17), NF- $\kappa$ B activation, and oxidative stress (4), all of which have been linked to insulin resistance. In addition, decreases in AMPK activity have been observed in muscle and liver of insulin-resistant rodents, including the ZDF and *fa/fa* rat and *ob/ob* mouse and IL-6 knockout mice (23, 40), and, where studied, activation of AMPK by pharmacological agents has diminished or prevented insulin resistance in these and other experimental models (3, 17, 40). Thus it is highly likely that the decrease in AMPK activity in liver and red

muscle contributed to the development of insulin resistance in these tissues.

Three in vitro studies have suggested that cell glucose oversupply, independently of insulin and FFA, can cause insulin resistance. Incubation of rat muscle (19, 26), cultured Hep G2 hepatocytes (53), and human umbilical vein endothelial cells (17) with a high glucose concentration (20–30 mM) causes insulin resistance and diminished AMPK activity and, where studied, increased cytosolic lipid as observed in the 5-h glucose-infused group in the present study. For this reason, although plasma insulin and FFA levels might have an influence, we consider it likely that the responses observed here do not absolutely depend on changes in these parameters. We also believe that the demonstration here that similar changes to those in the in vitro studies can occur in vivo is new and that they raise the possibility that AMPK can be downregulated in a normal rodent with physiological consequences.

A possible dual mechanism whereby increased glucose availability leads to increased malonyl-CoA concentration and subsequent insulin resistance is summarized in Fig. 7. Our studies suggest that increases in malonyl-CoA and DAG are early events, which by themselves can produce 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 $\alpha$ 2 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  $\alpha$ 1- and  $\alpha$ 2-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

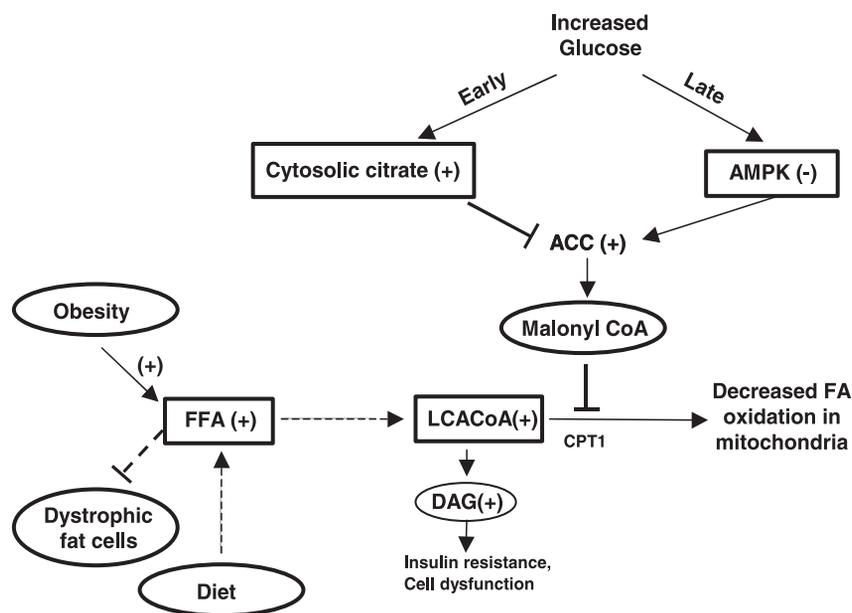


Fig. 7. Schema depicting dual mechanism by which increased glucose availability leads to an increase in concentration of malonyl-CoA. As shown by studies in incubated skeletal muscle, the increase in the cytosolic concentration of citrate can take place rapidly (min) (42), whereas decreases in AMPK, which phosphorylates and inhibits ACC, occur later (h) (19). Insulin resistance may occur as a consequence of the increase in concentration of DAG that takes place when malonyl-CoA diverts LCA-CoA from its oxidation in mitochondria to observed glycerolipid synthesis. A decrease in AMPK activity could both enhance these events and cause insulin resistance by its effects on oxidative stress (reactive oxygen species), ceramide synthesis, and the IKK $\beta$ /NF- $\kappa$ B system (40) (see text). As shown here, increases in plasma free fatty acids (FFA) could cause a picture similar to that produced by sustained glucose oversupply. CPT1, carnitine palmitoyltransferase I.

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 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 meal (2, 31). 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 electrotransfer (and glycogen content increased) without a concomitant reduction in AMPK phosphorylation.

Finally, it is somewhat puzzling that a significant decrease in  $R_d$  was not observed during the clamp in the 5-h glucose-

infused rats, although there was a small tendency toward a reduction [amplified to  $\sim 15\text{--}20\%$  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  $R_d$  estimation is not as precise as the  $R_g'$  estimation in individual muscles. Indirect evidence that the clamp  $R_d$  may be slightly overestimated in the 5-h glucose-infused group is provided by the estimated clamp HGO, which, at  $15 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ , is  $3\text{--}4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  above historical values for basal glucose turnover in our rats. There was, however, excellent agreement between the independent clamp  $R_g'$  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*.

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