Metformin Prevents the Development of Acute Lipid-Induced Insulin Resistance in the Rat Through Altered Hepatic Signaling Mechanisms

Mark E. Cleasby, Nicolas Dzamko, Bronwyn D. Hegarty, Gregory J. Cooney, Edward W. Kraegen, and Ji-Ming Ye

Metformin reduces the incidence of progression to type 2 diabetes in humans with obesity or impaired glucose tolerance. We used an animal model to investigate whether metformin could prevent acute lipid-induced insulin resistance and the mechanisms involved. Metformin or vehicle was administered to rats daily for 1 week. Rats were studied basally, after 3.75 h of intralipid-heparin or glycerol infusion, or after 5 h of infusion with a hyperinsulinemic-euglycemic clamp between 3 and 5 h. Metformin had no effect on plasma triacylglycerol or nonesterified fatty acid concentrations and did not alter glucose turnover or gluconeogenic enzyme mRNA after lipid infusion. However, metformin normalized hepatic glucose output and increased liver glycogen during lipid infusion and clamp. Basal liver (but not muscle or fat) AMP-activated protein kinase activity was increased by metformin (by 310%; $P < 0.01$), associated with increased phosphorylation of acetyl CoA carboxylase. Postclamp liver but not muscle phosphorylated/total Akt protein was increased, whereas basal c-Jun NH_2-terminal kinase-1 and -2 protein expression were reduced (by 39 and 53%, respectively; $P < 0.05$). Metformin also increased hepatic basal IκBα levels (by 260%; $P < 0.001$) but had no effect on tyrosine phosphorylation or expression of insulin receptor substrate-1 (IRS-1). In summary, metformin opposes the development of acute lipid-induced insulin resistance in the liver through alterations in multiple signaling pathways. *Diabetes* 53:3258–3266, 2004

The association among obesity, insulin resistance, type 2 diabetes, and the metabolic syndrome is now well recognized (1). Hyperlipidemia is an important component of this syndrome; in particular, elevation of circulating free fatty acid (FFA) concentrations has been associated with insulin resistance in many clinical studies (2,3). Furthermore, artificial elevation of systemic FFA in normal humans or animals by means of triacylglycerol-heparin infusion (4–10) or increasing dietary fat content (11,12) results in insulin resistance, usually involving a substantial impairment in the ability of insulin to suppress hepatic glucose output (HGO) (4,7,9). Several groups have identified changes in expression or activation of molecules linked to the insulin-signaling cascade as a result of increased supply of FFAs to the liver. Reductions in insulin receptor substrate (IRS) expression or IRS-associated phosphatidyl inositol 3 kinase activity were observed in three rodent models of hepatic FFA oversupply (13–15), whereas Ser473 and Thr308 phosphorylation of protein kinase B (PKB)/Akt (denoting activation) was reduced in the liver of acutely lipid-infused rats (8). Furthermore, c-Jun NH_2-terminal kinase (JNK) and IκB kinase-β (IKK-β), two kinases known to serine phosphorylate and thus inhibit IRS-1 activation in vitro (16), may be implicated, as deletion of IKK (17) or JNK-1 (18) prevented whole-body high-fat diet–induced insulin resistance in mice.

The biguanide compound metformin has been in wide clinical use for the alleviation of the hyperglycemia associated with type 2 diabetes for decades (19). Despite this, a comprehensive molecular basis for the antihyperglycemic mechanism of action of metformin has yet to be established. Whereas some studies have implicated increased peripheral glucose utilization, the majority have shown that reduction of basal hepatic glucose output and/or alleviation of the impaired insulin suppressibility of HGO observed in insulin resistance and type 2 diabetes is quantitatively the most important components of its activity in vivo (11,20–22). Recently, metformin was shown to ameliorate defects in insulin signaling in vitro, involving activation of IRSs and associated phosphatidyl inositol 3 kinase activity (23,24), although these findings were not replicated in muscle from diabetic human subjects (25). In addition, metformin activates AMP-activated protein kinase (AMPK) in hepatocytes (26,27), whereas enzyme activity was elevated in skeletal muscle of diabetic patients after 4 weeks of metformin treatment (28).

There is increasing interest in strategies to prevent the progression of impaired glucose tolerance and obesity to type 2 diabetes (1). Large-scale prospective studies have demonstrated the value of metformin administration in preventing the progression of impaired glucose tolerance

From the Diabetes and Obesity Program, Garvan Institute of Medical Research, Sydney, Australia.

Address correspondence and reprint requests to Edward W. Kraegen, PhD, Diabetes and Obesity Program, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, NSW 2010, Australia. E-mail: e.kraegen@garvan.org.au.

Received for publication 11 December 2003 and accepted in revised form 19 August 2004.

ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; FBP, fructose 1,6-bisphosphatase; FFA, free fatty acid; GIR, glucose infusion rate; HEC, hyperinsulinemic-euglycemic clamping; HGO, hepatic glucose output; IKK, IκB kinase; IRS, insulin receptor substrate; JNK, c-Jun NH_2-terminal kinase; PKB, protein kinase B; R_d, rate of glucose disposal; TAG, triacylglycerol.

to type 2 diabetes (29) or the incidence of type 2 diabetes in an overweight population (30), although the mechanisms for these effects are not known. We and others recently demonstrated the efficacy of short-term preadministration of thiazolidinediones in substantially preventing the insulin resistance normally observed in rats acutely infused with lipid (8,31). In this study, we sought to determine whether metformin was also efficacious in preventing lipid-induced insulin resistance in rats, with particular reference to its possible intrahepatic modes of action and its effects on signaling through AMPK and the insulin signaling cascade to suggest a mechanism for its protective effects.

RESEARCH DESIGN AND METHODS

Animal maintenance, treatment, and surgery. Male Wistar rats were obtained from the Animal Resources Centre (Perth, Australia) and habituated to their new surroundings for 1 week. Groups of two to three per cage were maintained at 22 ± 0.5°C under a 12-h day/12-h night cycle and were fed a standard chow diet (Norco, Kempsey, Australia; 18% fat, 33% protein, and 48% carbohydrate by energy content) ad libitum. Thereafter, they were randomly assigned to receive vehicle (0.5% methylcellulose; Con group) or metformin (120 mg · kg^{-1} · day^{-1}; Met group) daily by gavage for 1 week between 1500 and 1700, with an additional dose given on the morning of the end point experiment. Also, ~1 week before the study, the left carotid artery and right jugular vein were cannulated in rats that were being used for infusion studies (32). Anesthesia was induced and maintained with halothane in oxygen, the surgical site was irrigated with bupivacaine (0.5 mg/100 g) before closure, and 5 mg/kg ketoprofen was administered to provide postoperative analgesia. Rats were single housed and handled daily for the following week to minimize stress. At the end of the experiment, rats were killed by injection of pentobarbitone and appropriate tissues were rapidly dissected, cleaned of blood, and freeze clamped using liquid nitrogen-cooled tongs, commencing with the liver. All experimental procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

Lipid and tracer infusion. Conscious rats from vehicle- or Met-treated groups were studied after 5–7 h of fasting. Between 0900 and 1000, jugular cannulae were connected to a sampling line and carotid cannulae to an infusion line. Rats were allowed to acclimatize for 30–40 min, after which infusion of either 0.4 ml/h glycerol (Gly group) or triacylglycerol emulsion (Intralipid; Travenol, Sydney, Australia) combined with heparin (40 units/ml; Lip group) was commenced at a constant rate of 1.3 ml/h via the jugular cannula (10). Glycerol infusion functions as a control for triacylglycerol-heparin infusion (7,9,33). After 3 h of infusion, a bolus injection of 2-deoxy-D-[2,6-^{3}H]glucose (Amersham, Buckinghamshire, U.K.) was administered through the carotid line, and blood samples were collected at 2, 5, 10, 15, 20, 30, and 45 min subsequently for determination of plasma glucose and tracer concentrations. At the end of each study, rats were killed and tissues were collected as above.

Hyperinsulinemic-euglycemic clamping. Hyperinsulinemic-euglycemic clamping (HEC) was performed as previously described (32), commencing after acclimatization and 3 h of lipid infusion. Fasted conscious rats were infused with insulin (70 pmol/l, Actrapid; Novo Nordisk, Bagsvaerd, Denmark) via the jugular cannula at 0.5–0.6 units · kg^{-1} · h^{-1}, together with a variable rate of 50% glucose to maintain euglycemia, based on regular blood glucose measurements. Withdrawn erythrocytes were resuspended in sterile 0.9% saline and returned to the animal. ^{3}H-2-deoxyglucose tracer was administered as above once euglycemia was achieved, commencing ~75 min after initiation of the insulin infusion. Collection of subsequent blood and tissue samples and killing were conducted as described above. Plasma tracer disappearance was used to calculate rate of whole-body glucose disposal (R_d), and HGO was estimated from the difference between glucose infusion rate (GIR) and R_d.

Metabolite and hormone assays. Plasma glucose was determined immediately using a glucose analyser (YSI 2300; Yellow Springs Intruments, Yellow Springs, OH), and the remaining plasma was snap frozen. Plasma FFAs were determined spectrophotometrically using an acyl-CoA oxidase–based colorimetric kit (NEFA-C; WAKO Pure Chemical Industries, Osaka, Japan). Plasma triacylglycerol concentrations were measured using enzymatic colorimetric methods (Triglyceride INT, procedure 336 and GPO Trinder; Sigma, St. Louis, MO). Plasma adiponectin and insulin were determined by radioimmunoassay (Linco Research, St. Charles, MO). Plasma lactate (34) and tissue glycogen content (35) were measured as previously described. Tissue triglycerides were extracted (36) and measured using a Peridochrom Triglyceride GPO-PAP kit (Boehringer Mannheim, Indianapolis, IN).

AMPK activity assay. AMPK activity was determined in liver, muscle, and fat using the method of Davies et al. (37), modified to utilize AMARA peptide as substrate, as previously described (38). Tissues were homogenized using a Polytron PT10-35 (Kinematica, Littau-Lucerne, Switzerland) in buffer that contained 100 mmol/l mannitol, 50 mmol/l NaF, 10 mmol/l Tris (pH 7), 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml anti-trypsin and centrifuged at 12,000*g* for 15 min. Protein content of supernatants was measured using Bradford assay (Protein Assay kit; Bio-Rad, Regents Park, NSW, Australia). Five-microliter aliquots of supernatant were reacted in 35 μl of buffer that contained 40 mmol/l HEPES, 80 mmol/l NaCl, 5 mmol/l $MgCl_2$, 8% glycerol, 800 μmol/l EDTA (pH 7), 200 μmol/l cold AMP, 200 μmol/l AMARA peptide, 200 μmol/l ATP, 800 μmol/l dithiothreitol, and 0.148 MBq [γ-^{32}P]ATP (Amersham) for 4 min at 30°C, then reaction was stopped using 10 μl of 40% trichloroacetic acid. Twenty-microliter aliquots of each reaction were spotted onto pieces of P81 Whatman photocellulose paper (Whatman Bioscience, Clifton, NJ), which were washed three times in H_3PO_4 and once in ethanol before scintillation counting in Ultima Gold XR (Packard Bioscience, Meriden, CT).

Tissue lysates, SDS-PAGE, and Western blotting. Total or phosphorylated hepatic signaling molecules were quantified by SDS-PAGE and Western blotting of liver homogenates. Whole-tissue lysates were prepared from dismembranated muscle (Mikro-dismembranator II; B. Braun Biotech, Melsungen, Germany) in radioimmunoprecipitation assay buffer (65 mmol/l Tris, 150 mmol/l NaCl, 5 mmol/l EDTA [pH 7.4], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10% glycerol that contained 1 μg/ml aprotinin and leupeptin, 10 mmol/l NaF, 1 mmol/l sodium orthovanadate, and 1 mmol/l polymethylsulphonyl fluoride) and centrifuged for 15 min at 12,000*g*. Protein content of supernatants was quantified as above, and aliquots that contained 20–50 μg of protein were diluted 1:1 in 2× Laemmli buffer and denatured at 95°C (excepting lysates used for acetyl CoA carboxylase [ACC] quantification). Proteins were resolved by SDS-PAGE in Running buffer (25 mmol/l Tris, 200 mmol/l glycine, and 3.5 mmol/l SDS) on 6–10% separating and 4% stacking gels, typically in duplicate, and electrotransferred onto polyvinylidene fluoride membranes (Hybond-P; Amersham) in transfer buffer (25 mmol/l Tris, 200 mmol/l glycine, and 20% methanol, with 0.01% SDS in the case of ACC). Membranes were blocked in Tris-buffered saline (TBS) that contained 7.5% milk powder or 1% BSA, 0.1% Tween-20, 0.02% sodium azide, and 0.0025% phenol red. Dilutions (1:1,000) of primary antibody (AMPK-α, Ser473-phosphorylated Akt, total Akt, IκBα, JNK, Tyr612-phosphorylated IRS-1, or Ser79-phosphorylated ACC; Cell Signaling Technology, Beverly, MA; total ACC or total IRS-1, Upstate Cell Signaling Solutions, Waltham, MA) in blocking buffer were applied, the blots subsequently were washed, then 1:10,000 dilutions of donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech) were applied, followed by washes in TBS-Tween and TBS alone. Specific protein bands were detected using Western Lightning Chemiluminescence Reagents Plus (Perkin-Elmer Life Sciences, Boston, MA) and visualized on Super RX film (Fujifilm, Bedford, U.K.). Images were captured using a densitometer (Molecular Dynamics Personal Densitometer SI; Amersham), and relative band intensities were quantified using the IP Lab Gel H program (Signal Analytics, Vienna, VA). When membranes were reprobed with a second primary antibody, they were first stripped using 62.5 mmol/l Tris (pH 6.7), 2% SDS, and 100 mmol/l 2-mercaptoethanol and washed extensively.

Real-time RT-PCR. Real-time RT-PCR was used to quantify relative expression of mRNAs for PEPCK and fructose 1,6-bisphosphatase (FBP) in liver. Total RNA was extracted using Tri-Reagent (Sigma-Aldrich, Sydney, Australia). Aliquots of RNA underwent reverse transcription for 60 min at 37°C using the Omniscript RT kit (Qiagen, Clifton Hill, Victoria, Australia) 1× buffer that contained 0.5 μmol/l dNTPs, 1 μmol/l OligodT, 0.5 units/μl RNase inhibitor (Promega, Annandale, NSW, Australia), and 0.2 units/μl RT enzyme and then diluted to 20 ng/μl cDNA. Primers for rat PEPCK, FBP, and cyclophilin were designed to yield trans-intronic amplicons of 211, 236, and 189 bp, respectively, and obtained from PROLIGO (Lismore, NSW, Australia; PEPCK, CCCAGGAAGTGAGGAAGTTT and ATGACCGTCTTGCTTTCGAT; FBP, TC CCTCCGGATAATTCAGC and GATCTCAGTGGGGACGATGT; cyclophilin as published [39]). These were used in 35 cycles of conventional PCR, annealing at 58–60°C, to synthesize DNA standards for relative quantification of targets. PCR was carried out on 0.4 μg/μl total cDNA in 1× buffer that contained 0.03 units/μl AmpliTaq Gold (Roche Diagnostics, Basel, Switzerland), 2 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, and 1.2 pmol/μl of each primer. Agarose electrophoresis was conducted subsequently to demonstrate single DNA species of the expected length. Target mRNAs were quantified using a Lightcycler and the LC fast Start DNA Master SYBR Green I kit (Roche Diagnostics) and compared with that of cyclophilin. For each gene, 1 μl of cDNA or DNA

TABLE 1
Glucose turnover and metabolite concentrations after lipid infusion

Parameter	Con-Gly	Con-Lip	Met-Lip
Insulin (mU/l)	54.8 ± 6.5	68.8 ± 18.4	53.2 ± 2.1
Glucose (mmol/l)	7.98 ± 0.17*	7.13 ± 0.17	7.61 ± 0.15†
R_d or HGO (mg · kg^{-1} · min^{-1})	11.7 ± 2.2	13.8 ± 1.5	13.9 ± 1.2
FFAs (mmol/l)	0.61 ± 0.03	3.76 ± 0.34‡	3.24 ± 0.42‡
TAGs (mmol/l)	0.46 ± 0.04	2.00 ± 0.22‡	1.81 ± 0.27‡
Adiponectin (μg/ml)	5.4 ± 0.7	5.2 ± 0.7	5.8 ± 0.4

Data are means ± SE. Plasma parameters after 3.75 h of lipid (Lip) or glycerol (Gly) infusion into rats preadministered with metformin (Met) or vehicle (Con) and net R_d or HGO during the final 45 min of this period. By ANOVA, values for plasma glucose ($P < 0.01$), FFAs ($P < 0.001$), and TAG ($P < 0.001$) measured at the end of the infusion differed between groups. Post hoc: *$P < 0.001$ and †$P < 0.05$ vs. Con-Lip; ‡$P < 0.05$ vs. Con-Gly. n = 10–13 for plasma metabolites and n = 5–9 for adiponectin and R_d or HGO per group.

standard dilution (10^{-2}-10^{-7} × [PCR product]) was diluted in 10 μl total that contained 2.5 mmol/l $MgCl_2$, 0.6 pmol/μl each primer, and 1 μl of a 1:1 mixture of enzyme/dye mixture and subjected to 40 amplification cycles, with annealing at 55°C. Amplification and melting curves were followed in each case to confirm that profiles were consistent with the production of the expected amplicon, and relative quantification was achieved with reference to curves of crossing point versus dilution of standard with respect to each mRNA species.

Statistics. All data are shown as mean ± SE. Comparisons between two treatment groups were made using student's t test or the Mann-Whitney rank-sum test as appropriate. Comparisons between data from three treatment groups were made using one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks, followed by Holm-Sidak or Dunn post hoc analysis, respectively, where appropriate. Analyses were conducted using Sigma Stat v3.00 (SPSS, Chicago, IL), with significance taken at $P < 0.05$.

RESULTS

Basal parameters in drug-treated rats. There was a small (4%) reduction in body mass of rats that were administered with Met for 1 week (Con 377 ± 3 g [n = 29] vs. Met 362 ± 5 g [n = 14]; $P = 0.005$), accompanied by a 22% reduction in the epididymal fat pad expressed as a percentage of body mass (Con 1.27 ± 0.07% [n = 11] vs. Met 1.04 ± 0.08% [n = 6]; $P = 0.047$). Plasma insulin was reduced in Met-treated rats (Con 55.3 ± 4.4 vs. Met 40.6 ± 2.4 mU/l; $P < 0.05$, n = 10–20), but Met administration had no effect on basal levels of plasma glucose, FFAs, triacylglycerols (TAGs), or adiponectin (data not shown).

Postlipid infusion biochemistry. After 3.75 h of lipid infusion, plasma TAGs and FFAs were elevated relative to glycerol-infused controls, but Met had no effect on the extent of hyperlipidemia (Table 1). Basal glucose turnover, equated to net R_d and HGO, was not affected overall by lipid infusion or metformin, despite a minor reduction in plasma glucose, which was reversed by metformin. Neither lipid infusion nor metformin preadministration affected plasma adiponectin (Table 1).

Posthyperinsulinemic-euglycemic clamp biochemistry. During the 2 h of HEC, plasma insulin levels reached ~450–500 mU/l, with no difference among groups (Table 2). Similarly, plasma glucose was maintained between 8 and 8.5 mmol/l in all groups throughout the clamps by varying GIR (Table 2). The GIR required to maintain glycemia was lower in the Con-Lip group than in the Con-Gly, but this reduction was substantially normalized in the Met-Lip group (Fig. 1*A*). There was a trend for lipid infusion to reduce R_d and for Met to restore it (ANOVA $P = 0.084$; Fig. 1*B*), but the more striking effect of metformin was to normalize the elevated HGO generated by lipid infusion (Fig. 1*C*). Consistent with this effect, metformin treatment resulted in increased storage of glycogen in liver but not red quadriceps under clamp conditions. Lipid infusion significantly raised both plasma lipids and liver TAGs, but previous metformin administration had no effect on these parameters (Table 2). There was a trend for metformin to raise plasma lactate ($P = 0.082$), in the absence of any effect of lipid infusion (Table 2), consistent with previously published findings in high-fat–fed rats (11).

Liver gluconeogenic gene expression. In view of the effects of lipid infusion and metformin pretreatment on HGO, mRNA expression of FBP and PEPCK was examined in the liver of lipid-infused rats. No changes in expression of FBP (Con-Gly 2.18 ± 0.15, Con-Lip 1.93 ± 0.17, Met-Lip 2.60 ± 0.36 arbitrary units), PEPCK (Con-Gly 1.19 ± 0.24, Con-Lip 1.20 ± 0.26, Met-Lip 1.35 ± 0.21 arbitrary units), or

TABLE 2
Metabolite measurements during hyperinsulinemic-euglycemic clamp

Parameter	Con-Gly	Con-Lip	Met-Lip
Insulin (mU/l)	447 ± 53	497 ± 56	452 ± 19
Glucose (mmol/l)	8.04 ± 0.25	8.26 ± 0.12	8.44 ± 0.18
FFAs (mmol/l)	0.23 ± 0.03	2.82 ± 0.45*	2.92 ± 0.45*
TAGs (mmol/l)	0.39 ± 0.05	1.21 ± 0.14†	1.32 ± 0.12†
Lactate (mmol/l)	1.58 ± 0.08	1.46 ± 0.12	2.22 ± 0.27
Liver glycogen (nmol/mg)	89 ± 13	84 ± 17	159 ± 31*‡
RQ glycogen (nmol/mg)	58 ± 8	54 ± 4	61 ± 4
Liver triacylglycerol (μmol/g)	22.2 ± 2.3	33.3 ± 3.3*	34.3 ± 2.9*

Data are means ± SE. Metabolite concentrations after 5 h lipid (Lip) or glycerol (Gly) infusion plus 2 h clamp in rats pre-administered with metformin (Met) or vehicle (Con). By ANOVA, plasma FFAs ($P < 0.001$) plasma TAG ($P < 0.001$), liver glycogen ($P < 0.05$), and liver triacylglycerol ($P < 0.05$) differed between groups. Post hoc: *$P < 0.05$ and †$P < 0.001$ vs. Con-Gly; ‡$P < 0.05$ vs. Con-Lip. n = 6–8 per group. RQ, red quadriceps.

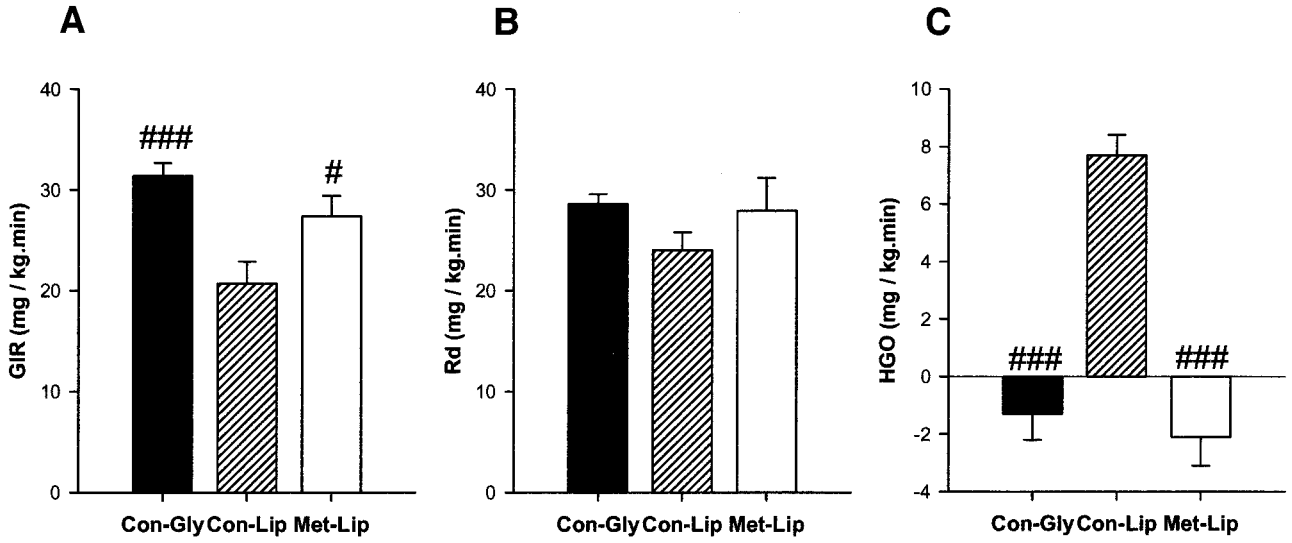

FIG. 1. Whole-body glucose turnover during HED. GIR, whole-body R_d, and HGO during HEC carried out during hours 3–5 of lipid (Lip) or glycerol (Gly) infusions in rats that were pretreated with metformin (Met) or vehicle (Con). By ANOVA, there were significant differences between groups in both GIR ($P < 0.05$) and HGO ($P < 0.001$). Post hoc testing revealed #$P < 0.05$ and ###$P < 0.001$ vs. Con-Lip; $n = 4–8$ per group.

the housekeeping gene cyclophilin (Con-Gly 6.08 ± 0.48, Con-Lip 5.77 ± 0.63, Met-Lip 7.47 ± 0.83 arbitrary units) were detected.

Basal liver signaling. Western blotting was used to characterize the expression and/or activation of several molecules of importance in the insulin signaling cascade in tissue lysates, and AMPK activity was measured in liver, red and white quadriceps muscle, and epididymal fat removed from rats at the completion of a 1-week dosing with metformin. There was a pronounced elevation in activity in the liver after drug treatment (Fig. 2*A*), accompanied by an increase in phosphorylation at Ser79 of a principal downstream target of AMPK in the liver, ACC (Fig. 2*D*). These changes occurred in the absence of any change in protein expression of the AMPK α subunit (Fig. 2*B*) or total ACC (Fig. 2*E*) in liver or of any change in AMPK activity in either red or white muscle or in epididymal fat (Fig. 2*C*). The basal level of IκBα in the liver of drug-treated rats was dramatically increased in Met rats versus controls (Fig. 3*A*), suggesting reduced activity of IKK-β. In addition, protein expression of both JNK-1 and JNK-2 isoforms was reduced after this treatment period (Fig. 3*B* and *C*), although Thr183/Tyr185 phosphorylation of JNK was undetectable under these conditions. There were no changes in either activating tyrosine phosphorylation (Fig. 3*D*) or expression (Fig. 3*E*) of IRS-1 in Met-treated liver.

Liver signaling after lipid infusion and clamp. In lipid-infused animals that underwent HEC, metformin increased Ser473-phosphorylation of Akt/PKB in the liver (Fig. 4*A*), denoting increased levels of activation of this intermediate, but no effect of lipid infusion per se was detected. This activation by metformin occurred in the absence of any change in total Akt/PKB protein expression in the liver and without any change in expression or phosphorylation in red quadriceps (data not shown) under the same circumstances. Although the effect did not reach significance under conditions of insulin stimulation, there was a similar trend for metformin to increase AMPK activity to that seen under basal conditions ($P = 0.11$; Fig. 4*B*). Furthermore and consistent with our basal findings, no changes in Tyr612-phosphorylation (Fig. 4*C*) or total protein expression of IRS-1 (Fig. 4*D*) were detected in liver.

DISCUSSION

This study demonstrates that metformin administration to normal rats is capable of preventing the development of acute lipid-induced insulin resistance. The primary effect of metformin was amelioration of the lipid-impaired insulin suppressibility of HGO; thus, the liver would seem to be the principal target tissue for this prophylactic effect. Furthermore, we have implicated enhancement of liver signaling in the mechanism of this effect, as drug-induced increases in AMPK activity and Akt phosphorylation were identified, along with reduced JNK and increased amounts of IκBα protein. To our knowledge, metformin-induced enhancement of insulin signaling in the liver has not previously been identified in any in vivo model of insulin resistance.

Previous studies of lipid-induced insulin resistance in a few species have demonstrated that accelerated gluconeogenesis is primarily responsible for the increased HGO observed (4,7,9,20,21); however, not all studies have detected a net effect on HGO, because a concurrent effect of lipid to suppress glycogenolysis has been observed (4,9). In this study, we quantified the expression of two key gluconeogenic enzymes (FBP and PEPCK) in the liver after lipid infusion but did not detect differences in expression of either enzyme as a result of lipid infusion or metformin. Although an effect of metformin to reduce expression of FBP and PEPCK has been implicated in its action to increase insulin suppressibility of HGO in high-fat–fed rodents (20), our acute studies agree with that of Samuel et al. (15) in suggesting that these changes are not a requirement for its effect on HGO. Effects of metformin on gluconeogenesis may be mediated instead through effects on other important gluconeogenic enzymes, such

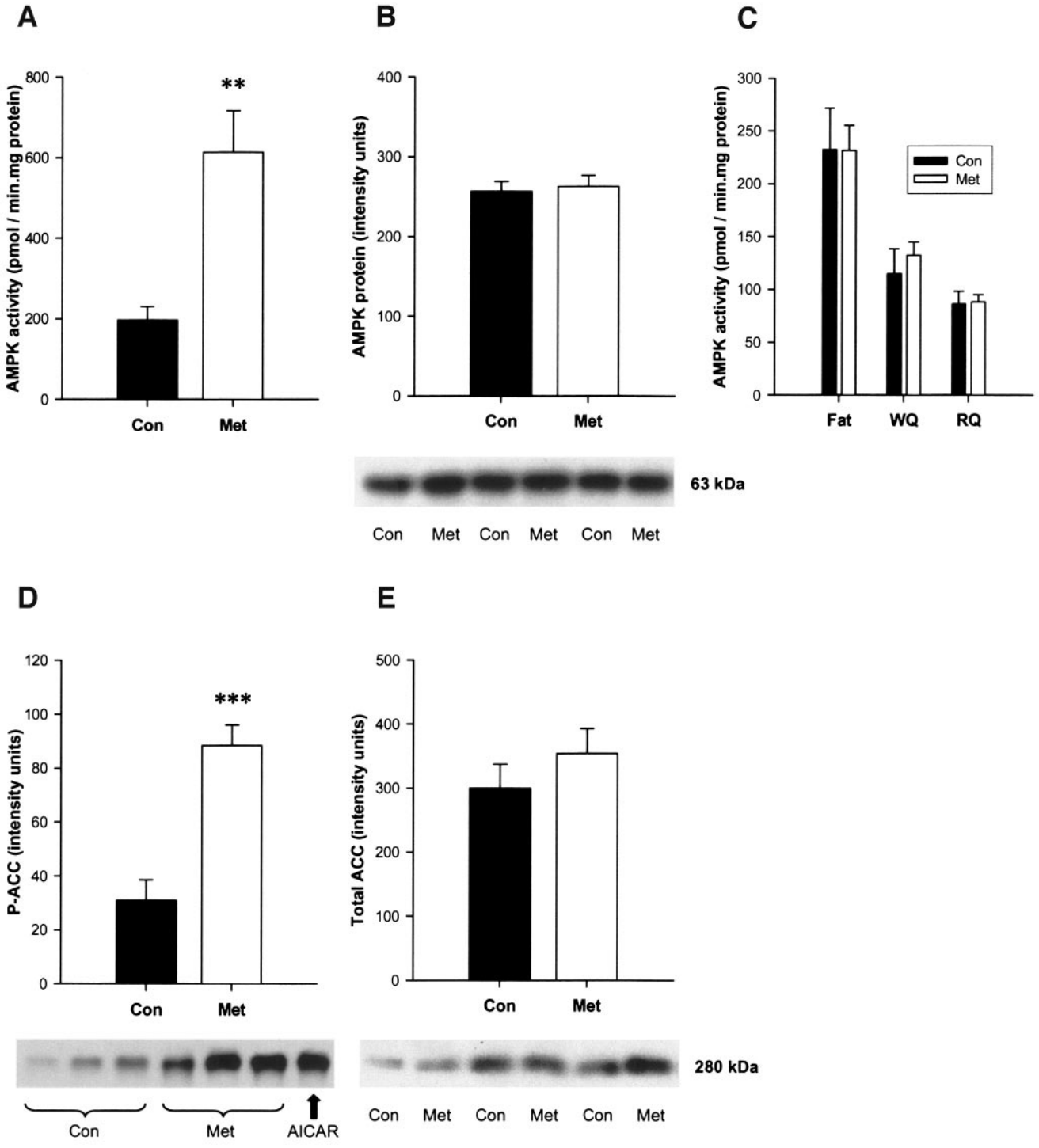

FIG. 2. AMPK activity after metformin administration. AMPK activity (*A*) and AMPK protein (*B*) in liver; AMPK activity in epididymal fat, white quadriceps (WQ), and red quadriceps (RQ); and Ser79-phosphorylated (P-ACC) and total ACC in liver from rats that were given metformin (Met) or vehicle (Con) for 1 week. Representative blots are shown in each panel, indicating specific protein bands at the expected sizes, the P-ACC blot showing 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)-treated liver as positive control. **$P < 0.01$, ***$P < 0.001$ vs. Con; $n = 4–6$ per group.

as pyruvate carboxylase, pyruvate kinase (40), or glucose 6-phosphatase (11).

The effect of metformin to protect against the onset of hepatic insulin resistance is consistent with previous reports of the therapeutic effect of the drug in established insulin-resistant states (11,20–22,41). Under HEC conditions, metformin pretreatment resulted in increased storage of liver glycogen, as observed previously in high-fat–fed rats (11), suggesting that metformin attenuated glycogenolysis or enhanced glycogen synthesis, perhaps from gluconeogenic substrates. There was a tendency in this study for lipid infusion to reduce clamp R_d, but this did not reach significance. This finding is consistent with our previous findings using a similar protocol (8), although other studies have demonstrated a significant effect (41,42). Furthermore, previous work examining chroni-

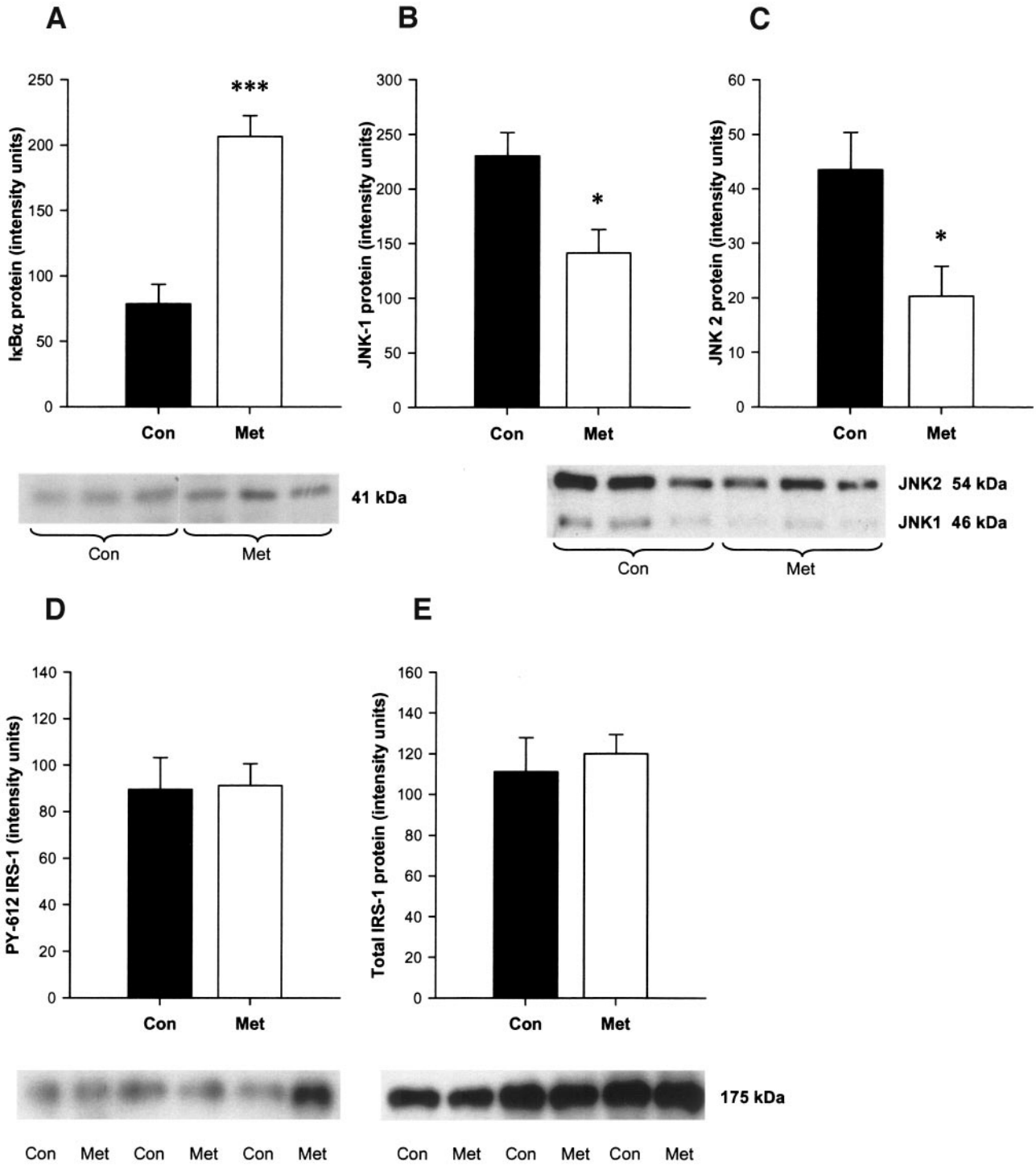

FIG. 3. Liver-signaling molecules after metformin administration. IκBα (*A*), JNK-1 (*B*), JNK-2 (*C*), Tyr612-phosphorylated (*D*), and total IRS-1 (*E*) protein content of liver from rats that were given metformin (Met) or vehicle (Con) for 1 week. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Con; *n* = 5–6 per group.

cally insulin-resistant high-fat–fed rats under HEC conditions demonstrated adverse effects on HGO before the manifestation of attenuated peripheral glucose disposal (11,43,44).

The most novel findings in this study are the metformin-induced changes in expression or activity of several important hepatic signaling molecules, which provide potential mechanisms for the positive effects of the drug in preventing lipid-induced insulin resistance. Enhancement of AMPK activity after metformin treatment has been previously observed in isolated rat hepatocytes (26,27) and in skeletal muscle of individuals with type 2 diabetes after 4 weeks' administration of the drug (28). Here, we detected a threefold increase in liver AMPK activity after 1 week of metformin administration to normal rats, an effect that was manifested downstream in a similar increase in phosphorylated ACC (27), suggesting deactivation of this enzyme that is responsible for malonyl CoA synthesis.

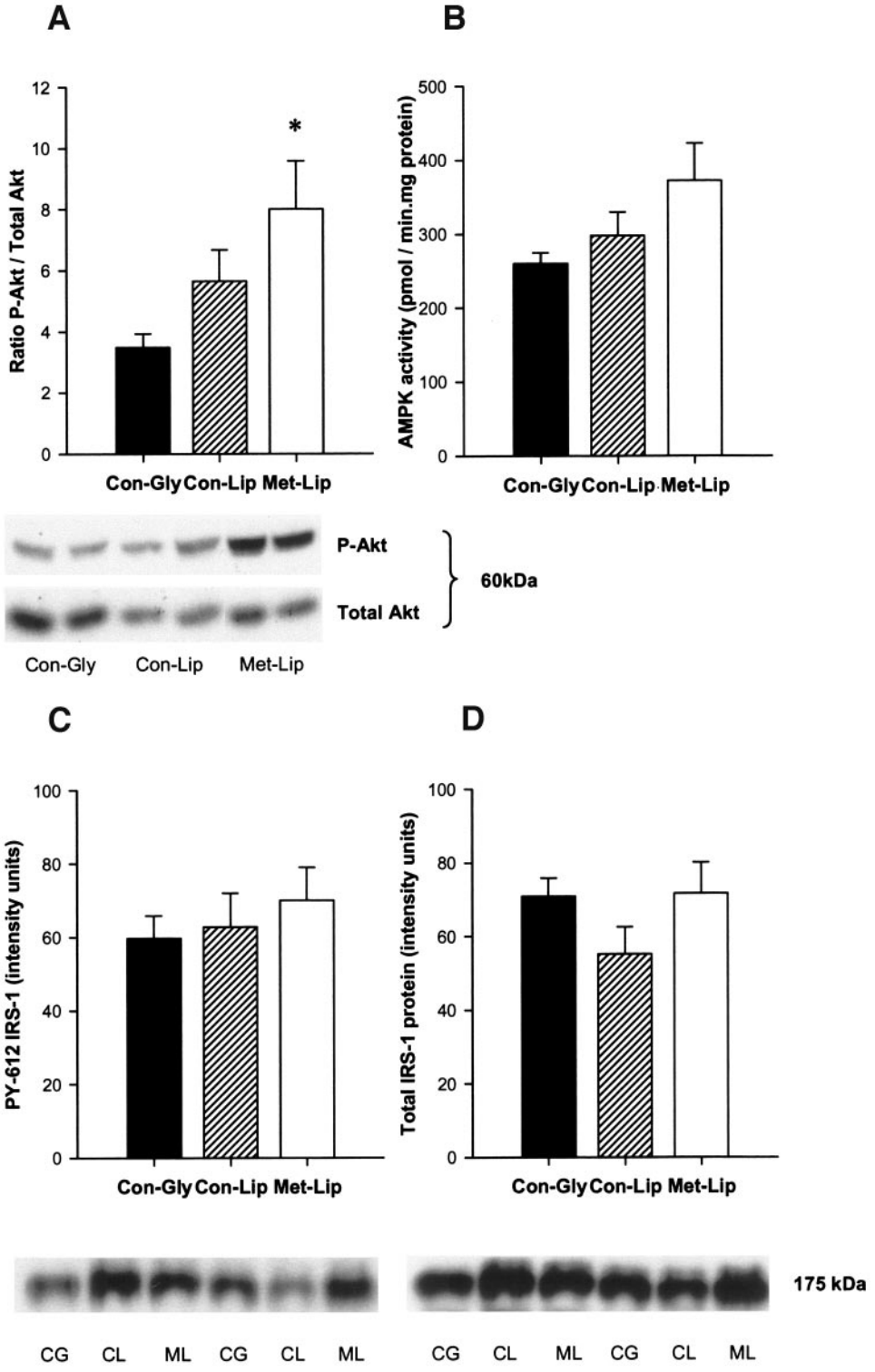

FIG. 4. Liver-signaling molecules after lipid infusion and clamp. Ratio of Ser473-phosphorylated Akt/PKB (P-Akt) over total Akt/PKB protein (*A*), AMPK activity (*B*), tyrosine Tyr612-phosphorylated (*C*), and total IRS-1 (*D*) protein content in liver from rats that were given metformin (Met/M) or vehicle (Con/C) and subsequently infused with lipid (Lip/l) or glycerol (Gly/G) for 5 h, incorporating HEC between 3 and 5 h. By ANOVA, there was a significant effect of treatment on P-Akt/total ($P < 0.05$; post hoc $^*P < 0.05$ vs. Con-Gly); $n = 5–6$ per group.

After lipid infusion and HEC, AMPK activity showed a similar upward trend in liver of metformin-treated animals, although this did not reach significance, possibly because of increased AMPK activation in all groups resulting from the stress of the infusion and sampling protocol. In contrast, no effect of metformin was detected on AMPK activity in red or white muscle or a central fat depot, consistent with a lack of effect of the drug on peripheral glucose disposal. Adiponectin is a recently described activator of liver and muscle AMPK (45); however, the lack of an association in this study between adiponectin and AMPK activation suggests that this is not the mechanism responsible for the effects of metformin in the liver.

Despite the observed increase in phosphorylated ACC and thus attenuation of the inhibitory effect of malonyl CoA on mitochondrial β-oxidation (46), there was no accompanying reduction in triacylglycerol storage in liver after acute lipid infusion and HEC. A longer period of lipid infusion may have revealed a downstream effect of ACC activation in this respect, but the lack of requirement for a

reduction in liver lipid storage suggests that this is not the primary mechanism for the effect of metformin on HGO. Nevertheless, it is possible that AMPK may have increased mitochondrial oxidation of other lipid species, such as diacylglycerols, long-chain acyl CoA, or ceramide, potentially alleviating inhibition of insulin signaling at the level of PKB/Akt (47) or IRSs (48).

Metformin treatment also had positive effects on the insulin signaling pathway in the liver, indicated by an increase in activating serine phosphorylation of PKB/Akt under insulin stimulation, a change that we also observed after treatment of normal rats with pioglitazone (8). Whether this reflects a direct induction of PKB/Akt phosphorylation or an effect of metformin at a more proximal point in the pathway is unclear; however, after metformin administration, we also identified altered expression of kinases shown to have an impact on insulin signaling. JNKs are serine kinases that are activated in obesity, and JNK-1 deficiency alleviates the development of the associated insulin resistance in rodents (18). Levels of IκBα in tissue are normally inversely related to the activity of IKK, which has also been implicated in the mechanism of lipid-induced insulin resistance in muscle (17,49). The observed metformin-induced reductions in JNK-1 and JNK-2 and increase in IκBα thus could be important in mediating its ability to reduce the impact of lipid infusion on insulin action in the liver. Both JNK and IKK have been shown to phosphorylate IRS-1 in vitro at the Ser307 site (16), which results in reduced tyrosine phosphorylation and thus activity of IRS-1. We found no change in IRS-1 expression or phosphorylation in this study as a result of either lipid infusion or metformin administration. However, JNK and/or IKK could be phosphorylating alternative substrates, such as PKB/Akt itself or IRS-2. IRS-2 is quantitatively much more important in insulin signaling in liver than it is in muscle, and metformin has been shown to preferentially activate this isoform in hepatocytes in vitro (50). However, the physiologically significant sites of phosphorylation on this molecule are poorly characterized. Overall, the effects of metformin on these signaling molecules may explain its protective effect on liver insulin sensitivity in the absence of a reduction in lipid levels.

In conclusion, previous treatment with metformin is capable of preventing the development of insulin resistance in rats induced by an acute lipid infusion, primarily through amelioration of impaired insulin suppressibility of HGO. However, in contrast to the effect of pioglitazone (8), its effect is not reliant on altering circulating lipid concentrations. The mechanism for metformin's effect seems to involve improved signaling through both AMPK and the insulin signaling pathway in the liver. These findings may help in explaining the prophylactic effect of metformin to oppose further deterioration in glucoregulation in humans with impaired glucose tolerance or obesity (29,30) and in implicating enhanced liver signaling in the therapeutic mechanism of action of metformin.

ACKNOWLEDGMENTS

This study was funded by grants obtained from GlaxoSmithKline and the National Health and Medical Research Council of Australia.

We thank Dr. Steve Smith, GlaxoSmithKline (Harlow, U.K.), for support; Tracie Reinten and Joanna Edema for excellent technical assistance; and the staff of the Biological Testing Unit for assistance with animal care.

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