Impact of in vivo fatty acid oxidation blockade on glucose turnover and muscle glucose metabolism during low-dose AICAR infusion

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Submitted 26 October 2005; accepted in final form 28 April 2006

AMPK ACTIVATION PLAYS A CENTRAL ROLE in the regulation of intracellular metabolism as the master switch regulating cellular ATP energy supplies by shutting down high energy-consuming pathways, such as fatty acid synthesis and cholesterol synthesis, and by activating ATP-generating pathways such as fatty acid oxidation (42, 58). Thus, through these actions, AMPK influences fuel usage and selection in and to various tissues (42). Endogenous AMPK can be activated pharmacologically by 5-aminomidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR), which is phosphorylated in vivo to the AMP analog AICAR monophosphate (ZMP) (18). However, many of those studies employed high doses of AICAR, resulting in high tissue levels of ZMP (5, 6, 25, 26), which might have allosterically activated glycogen phosphorylase (7), certainly in cardiac muscle (32) and in the liver (45). Nevertheless, when skeletal muscle (SkM) AMPK is activated by upstream kinases and/or AMP allosteric effects (60), the downstream substrate acetyl-CoA carboxylase (ACC) is phosphorylated in SkM (37, 58), at Ser221 (ACC-pSer221) (11, 52) by AICAR. AICAR-phosphorylated ACC (ACC-pSer221) (11, 52) is increased by 50%.

In the AICAR + MP studies, the metabolic responses were modified: the glucose was lower over 120 min, only minor changes occurred with insulin and FFA, and HGP and Rd tissue responses were markedly attenuated, but MCRg and GF increased significantly. SkM substrates were unchanged, but ACC-pSer221 rose by 80%. Thus low-dose AICAR leads to increases in HGP and SkM glucose uptake, which are attenuated, but MCRg and GF increased significantly. SkM substrates were unchanged, but ACC-pSer221 rose by 80%. Thus low-dose AICAR influences fuel usage and selection in and to various tissues (42). Endogenous AMPK can be activated pharmacologically by 5-aminimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR), which is phosphorylated in vivo to the AMP analog AICAR monophosphate (ZMP) (18). However, many of those studies employed high doses of AICAR, resulting in high tissue levels of ZMP (5, 6, 25, 26), which might have allosterically activated glycogen phosphorylase (7), certainly in cardiac muscle (32) and in the liver (45). Nevertheless, when skeletal muscle (SkM) AMPK is activated by upstream kinases and/or AMP allosteric effects (60), the downstream substrate acetyl-CoA carboxylase (ACC) is phosphorylated in SkM (37, 58), at Ser221 (ACC-pSer221) (11, 52) and thereby inactivated (37, 58). Therefore, the formation of ACC-pSer221 represents a key sensitive marker of biological activation of AMPK in vivo (3, 58). Note that the corresponding SkM site (Ser221) recognized by the ACC phosphospecific rat antibody used in the assay is raised against ACCα-Ser79 (11, 36, 52). The inactivation of ACC leads to reduced malonyl-CoA levels and the activation of mitochondrial carnitine palmitoyltransferase I (CPT 1), which promotes the entry of free fatty acids (FFA) into mitochondria for ATP generation by beta-oxidation (34). In addition to the aforementioned AMPK stimulation by AICAR of SkM glucose transport (34, 42) and FFA oxidation (34, 42), activation of AMPK in SkM induces marked glycolgenolysis via phosphorylation and activation of phosphorylase (65), although the phosphorylase kinase is not the direct substrate for AMPK (7). Glycogen synthase activity is also inhibited in SkM by AICAR via AMPK phosphorylation of Ser7 (site 2) on glycogen synthase (10). In contrast, in liver, AMPK activation inhibits gluconeogenesis (31, 57), glycogen (56), lipogenesis, and cholesterol synthesis (23, 35), but hepatic glycolgenolysis is stimulated (26, 38, 45), mainly by an allosteric mechanism (8).

Previous studies employing high-dose AICAR infusions have concluded that hepatic glucose production (HGP) was suppressed by AICAR through enhancing the action of insulin to inhibit HGP (5, 6, 25, 26). These conclusions were based on the observation that high-dose AICAR and raised ZMP may directly inhibit hepatic gluconeogenesis by downregulating several key gluconeogenic enzymes (31). In addition, hepatic...
glycogen is markedly reduced during high-dose AICAR administration in rats (26), and glycogen phosphorylase is activated in AICAR-treated hepatocytes (45). However, more recent studies (8, 9, 38) indicate that, during an infusion of low-dose AICAR, insulin’s action on the liver is opposed, which leads to the increase in HGP by increasing net hepatic glycogenolysis via allosteric mechanisms (8). To date, all studies employing either low-dose or high-dose AICAR infusion in vivo have simultaneously confounded varying amounts of glucose, insulin, and somatostatin with or without basal replacement of glucagon (6, 9, 25, 26, 38), which, given the independent effect of the glucose infusion on HGP (46), may compound the interpretation of the in vivo glucose turnover data.

The aims of the present studies were therefore to examine the impact of low-dose AICAR infusion alone in normal dogs on 1) basal in vivo glucose turnover (HGP), glucose disposal (Rd (tissue), glycolytic flux (GF), and metabolic clearance rate of glucose (MCR)); 2) SkM glucose metabolism; and 3) SkM AMPK activity and ACC→pSer21\textsuperscript{1} formation. These studies were then repeated in the same dogs after a 48-h FA oxidation blockade by methylpalmoxirate (MP) (54) to isolate the glucose turnover and SkM glucose metabolic responses to the AMPK activation. Finally, the above-mentioned studies were repeated in a limited number of these same dogs after induction of chronic low-dose alloxan diabetes (12, 13).

MATERIALS AND METHODS

Animals

The studies were performed on eight male dogs of mixed breed (20.1 ± 1.6 kg body wt, means ± SE), with permission from the Experimental and Surgical Research Ethics Committee, St. Vincent’s Hospital, Melbourne, Australia. Surgical preparation, exercising program, dietary intake, health monitoring and training of dogs, and blood sampling schedules on the day of experiments were as previously detailed (12–15). A subsequent set of experiments was undertaken in three of these eight dogs following induction of diabetes by a low intravenous dose of alloxan (35 mg/kg body wt; Sigma, St. Louis, MO) (12, 13). Within 2–3 days, these dogs received, with their food, twice-daily, low-dose, intermediate-acting insulin (human Monotard; Novo Nordisk, Sydney, Australia) 14 ± 0.5 units daily over the next 4–5 wk. This produced chronic hyperglycemia [premeal blood glucose (BG): AM 17.2 ± 0.3 mM, PM 18.8 ± 0.3 mM], with weight maintained by increasing food intake by 40% (12, 13). All dogs remained active and healthy.

Experiments

After the overnight 15-h fast (with free access to water) and rest period of ≥30 min, fasting blood samples were taken for measurement of plasma glucose, insulin, FFA, glycerol, glucagon, lactate, [3-\textsuperscript{3}H]glucose, and \textsuperscript{3}H\textsuperscript{2}O. The following experiments were performed on eight control dogs and the subset of three suboptimally controlled diabetic dogs. In experiment 1, determination of glucose tolerance and total body water (TBW) content, a standard 120-min intravenous glucose tolerance test performed at the dog’s prevailing fasting glycemia and insulinemia, commencing with an intravenous bolus of 50% glucose (0.3 g/kg) and 50 μCi of highly purified tritiated water (\textsuperscript{3}H\textsuperscript{2}O; NEN Life Science Products, Boston, MA) given over 30 s followed by frequent blood samples from 0–60 min for measurement of plasma glucose and from 60–120 min for measurement of plasma \textsuperscript{3}H\textsuperscript{2}O specific activity (13–15, 20). These data were used to determine glucose tolerance (\textsuperscript{Kg}) and TBW content (20).

In experiment 2, baseline + AICAR infusion, the study was performed at the dog’s prevailing fasting glycemia and insulinemia and involved a 150-min baseline equilibration period consisting of a primed (control 20 μCi vs. diabetic 30–50 μCi) continuous infusion (10 μCi/h) of highly purified tritiated glucose ([3-\textsuperscript{3}H]glucose; NEN Life Science Products) (13–15). This was followed by a 120-min infusion of AICAR (Toronto Research Chemicals, Toronto, ON, Canada), commencing with an intravenous priming dose of 7.5 mg/kg AICAR dissolved in 10 ml of saline given over 30 s followed by the continuous infusion (1.5–2.0 μg·kg\textsuperscript{-1}·min\textsuperscript{-1}) of AICAR, which was given in parallel with the [3-\textsuperscript{3}H]glucose infusion by using separate infusion pumps (Fig. 1) (14, 15). The AICAR infusion was aimed at producing plasma AICAR levels of ~100–150 mM (64).

Experiment 3, AICAR + MP infusion, involved the same protocol as experiment 2 but was preceded by the administration of five oral doses (each 10 mg/kg) of the fatty acid (FA) oxidation inhibitor methylpalmoxirate (methyl-2-tetradecylglycidate; R. W. Johnson Pharmaceutical Research Institute, Spring House, PA) given twice daily for 2 days before and on the morning of the experiment (Fig. 1) (47, 63). MP suppresses mitochondria long-chain fatty acid oxidation, mainly in liver but also in skeletal muscle, by irreversible binding to the active site of CPT I (4, 39, 54). For experiments 2 and 3, which were carried out in random order, regular blood samples were taken throughout the 270-min study period for measurement of plasma glucose, insulin, [3-\textsuperscript{3}H]glucose, and \textsuperscript{3}H\textsuperscript{2}O. Blood samples were also taken at 60, 150, 210, and 270 min for measurement of plasma FFA, glycerol, glucagon, and lactate. Throughout the AICAR infusion with or without MP periods (150–270 min), the BG concentration was measured on a Companion 2 portable blood glucose analyzer (Medisense, Balwyn, Australia) every 10–15 min to monitor for any experimentally induced hypoglycemia. If the BG level fell below 3.5 mmol/l, as occurred in only two of the eight control dogs during the AICAR + MP infusion period, a variable infusion rate of 10% glucose, averaging 8.0 and 12.1 μmol·kg\textsuperscript{-1}·min\textsuperscript{-1}, prelabelled with 2.0 μCi/g [3-\textsuperscript{3}H]glucose (\textsuperscript{3}H-GINS), was commenced to maintain the BG level at ~4.5–5.0 mmol/l, thereby preventing the confounding effects induced by counterregulatory responses associated with hypoglycemia and avoiding rapid dilution of the labeled-glucose pool (13, 24). The blood samples were collected into tubes containing appropriate anticoagulants and preservatives, placed on ice, and centrifuged at 4°C within 2 h, and the separated plasma was frozen and stored at −20°C until assayed (13–15).

At the completion of all experiments, SkM biopsy samples (4–5 passes) were taken from the thigh (vastus lateralis) after rapid induc-
tion of anesthesia [4.2 mg/kg iv bolus followed by a 1.8 mg/kg iv titration of Diprivan (Propofol, 2,6-disopropylphenol); AstraZeneca, North Ryde, Australia], as previously described (12, 13) (Fig. 1). Each SkM biopsy sample was immediately frozen in liquid nitrogen (within 7 s of biopsy) and stored at −70°C until assayed (12, 13, 16). For each dog, the opposite hindleg was used for subsequent muscle biopsy procedure.

Metabolite Measurements

Plasma levels of glucose, total insulin, FFA, glucagon, and lactate and specific activities of plasma [3-3H]glucose and \( ^3\text{H}_2\text{O} \) were measured as previously described (13–15). Plasma glycerol was determined in 40-μl samples measured in triplicate in a total volume of 1.75 ml (10 mm light path) using a fluorometric enzymatic assay (0.1 M Tris-2.5 mM MgCl₂, pH 7.6 buffer; pyruvate kinase-lactate dehydrogenase-glycerokinase-coupled enzyme system), as modified from the method described by Eggstein and Kuhlmann (21). The average rates of total glucose appearance (Ra, total) and total glucose disposal (Rd, tissue) calculated from 120–150 min (pre-AICAR infusion period) and from 240–270 min (AICAR infusion period) in experiments 2 and 4 were determined from plasma [3-3H]glucose specific activities using steady-state equations (14, 15, 24) or, if not at steady state, employing Steele’s non-steady-state equations (50), assuming a glucose pool steady-state equations (14, 15, 24) or, if not at steady state, employing Steele’s non-steady-state equations (50), assuming a glucose pool fraction of 0.65 and a glucose volume of distribution of 200 ml/kg (15). The measurement of the rate of in vivo total glycolysis (GF) from extracellularly derived glucose due to the linear generation of plasma \( ^3\text{H}_2\text{O} \) from the [3-3H]glucose infusion (41) was estimated from a previously described formula from our laboratory (14, 15).

SkM concentrations of intracellular glucose (ICglucose), glucose 6-phosphate (G-6-P), glycogen, and lactate were determined as previously described from our laboratory (13, 16) using a fluorometric enzyme-coupled assay as modified from the method of Schalin-Jantti et al. (44). Concentrations of SkM G-6-P are expressed as millimoles per kilogram dry weight. Glycogen content is expressed as millimoles of glucose residues per kilogram dry weight. Concentrations of SkM ICglucose and lactate are expressed as millimoles per liter of IC water, assuming an extracellular water content in the biopsies of 0.3 l/kg dry wt and an IC water content in the biopsies of 2.8 l/kg dry wt, as discussed previously (13, 28, 48, 49, 55).

AMPK Activity and ACC Phosphorylation Assays

These assays were carried out as detailed previously from our laboratory (11, 12, 52). Activities of AMPKα1 and -α2 were calculated as picomoles of phosphate incorporated into the SAMS peptide per minute per milligram of total protein subjected to immunoprecipitation. Specific phosphorylation of SkM Ser211 in ACCβ (human sequence) was detected by employing a rat anti-phospho-ACCα-Ser79 polyclonal antibody (11, 12, 52). ACC-pSer211 are expressed in quantitative densitometric arbitrary units (11, 12, 52).

SkM ZMP, AMP, ADP, ATP, and ACC concentrations were measured using a reversed-phase high-performance liquid chromatography (HPLC) technique as previously described (51, 61). Separation was achieved using a Merck Hībar Lichrosphere 100 CH-18/2 250 × 4 mm column with a Waters chromatography work station.

Statistical Analysis

Data are presented as means ± SE. Between-group differences for paired data were compared using Student’s paired t-test and, where not all data are matched, Wilcoxon’s rank sum test (see Tables 2 and 3). For analyses within and between groups, one-way ANOVA was used for assessment for changes with time for substrate, hormone, and glucose turnover data; two-way ANOVA was employed for comparison between study groups. Correlation analyses were performed using the Spearman rank correlation coefficient (r values). All computations were carried out using the Minitab II program (release 13, Minitab).

RESULTS

Acute AICAR Infusion in Control Dogs

Effects of AICAR infusion on plasma substrates and hormones. Plasma glucose concentration was reduced after 30 min and 60–75 min of the AICAR infusion but returned to normal by 90–120 min (P < 0.01; Fig. 2 and Table 1). After the commencement of the AICAR infusion, plasma insulin levels rose by 30 min and remained elevated after 60–75 min but returned to normal by 90–120 min (P = 0.06; Fig. 2). Plasma glucagon levels were unchanged during the AICAR infusion (Fig. 2). Plasma FFA concentration fell by 10% after 150 min of the baseline [3-3H]glucose infusion period (0.65 ± 0.08 vs. prestudy, 0.76 ± 0.10 mmol/l). There was a further 30% decrease in plasma FFA level after 60 min of AICAR infusion, which was maintained to 120 min (Fig. 2). Plasma glycerol concentration did not change after the commencement of the AICAR infusion (Fig. 2). Plasma lactate concentration increased twofold after 60 min of AICAR infusion and increased further after 120 min (P < 0.001; Fig. 2).

Effects of AICAR infusion on in vivo Ra total, Rd, MCRg, and GF. Thirty minutes after commencement of the AICAR infusion, dpm/ml [3-3H]glucose fell by ~25% and continued to decrease thereafter (P = 0.06; Table 1). Ra total rose by 60–75 min of AICAR infusion and increased further after 90–120 min of AICAR (P = 0.06; Fig. 3). Rd tissue also rose after 60–75 min of AICAR infusion but did not increase further after 90–120 min of AICAR (P = 0.06; Fig. 3). Although MCRg rose after 60–75 min of AICAR infusion, the rise was attenuated after 90–120 min of AICAR infusion (Fig. 3). However, the early rises in Rd tissue and MCRg were greater than the corresponding increase in Ra total, which accounted for the transient lower plasma glucose observed during the AICAR infusion (Fig. 2). The rate of GF was significantly increased by the end of the AICAR infusion, but there was no statistical difference in GF expressed as a percentage of Rd tissue (%GF; Table 2).

Effects of AICAR infusion alone on SkM metabolites (ICglucose, G-6-P, glycogen, lactate, ATP, AMP, ZMP, and AICAR concentrations), AMPK-α1 and -α2 activities, and ACC-pSer211 ex vivo. SkM ICglucose, G-6-P, and glycogen did not change during the AICAR infusion (Table 3). However, SkM lactate rose by ~50%, but this failed to reach significance at 60 min (P = 0.08) or 120 min (P = 0.06; Table 3). AMP, ADP, and ATP did not change during the AICAR infusion, but ZMP levels increased significantly to 0.37 ± 0.07 μmol/g wet wt (Table 3). AICAR concentration was raised to 0.13 ± 0.03 μmol/g wet wt. AMPKα1 and -α2 activities did not change during the AICAR infusion. Importantly, ACC-pSer211 increased significantly by ~50% (Table 3).

Oral MP with or without Acute AICAR Infusion in Control Dogs

Effects of oral MP alone on plasma substrates and hormones. After the administration of the five oral doses of MP twice daily, the plasma glucose concentration after the 120- to 150-min baseline period was slightly reduced compared with the 120- to 150-min baseline (saline) period in experiment 1 (Fig. 2). Oral MP alone had no effect on basal plasma insulin,
but plasma glucagon doubled after oral MP (Fig. 1). Basal FFA, glycerol, and lactate did not change (Fig. 2).

Effects of oral MP alone on in vivo $R_a$ total, $R_d$ tissue, MCR$_g$, and GF. Basal $R_a$ total and $R_d$ tissue did not change after oral MP alone in the control dogs, but MCR$_g$ was significantly higher ($P < 0.05$) compared with saline alone (Fig. 3). GF also increased significantly after oral MP alone, with the contribution of GF to basal $R_d$ tissue rising sharply to $\sim 95\%$ (Table 2).

Effects of acute AICAR infusion in the presence of oral MP on plasma substrates and hormones. In the eight control dogs, the plasma glucose concentrations showed a different pattern to that of AICAR infusion alone, with plasma glucose being significantly reduced after 30 and 60–75 min with AICAR + MP and remaining low at 120 min compared with AICAR infusion alone (Fig. 2). There was no significant rise in insulin during the AICAR + MP infusion, but the overall insulin profile was significantly lower compared with the AICAR-alone study ($P < 0.001$; Fig. 2 and Table 1). Plasma glucagon did not rise significantly with AICAR + MP infusion but was significantly elevated during the AICAR + MP study ($P < 0.001$; Fig. 2). The decrease in FFA was blunted with the AICAR + MP infusion, but FFA was significantly higher compared with the AICAR-alone study ($P < 0.02$; Fig. 2). In contrast, glycerol was unchanged during the AICAR + MP infusion (Fig. 2). Plasma lactate rose similarly with the AICAR + MP infusion (Fig. 2).

Low-dose GINF, which was given to two of the eight dogs, did not influence any of the hormone or substrate patterns listed above (data not shown).

Table 1. Plasma glucose, insulin, and [3-3H]glucose during AICAR and AICAR + MP infusions in normal dog

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AICAR Alone, min</th>
<th>AICAR + MP, min</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>P. Glucose, mmol/l</td>
<td>5.1±0.1</td>
<td>4.4±0.2</td>
</tr>
<tr>
<td>P. Insulin, mU/l</td>
<td>5.9±1.0</td>
<td>14.5±4.3</td>
</tr>
<tr>
<td>[3-3H]glucose, dpm/ml</td>
<td>6,137±762</td>
<td>4,565±586</td>
</tr>
</tbody>
</table>

Values are means ± SE. AICAR, 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside; MP, methylpalmoxirate; P., plasma. Glucose and insulin statistical data are provided in the legend to Fig. 2. For dpm/ml changes over time, *$P = 0.06$ for differences between AICAR and AICAR + MP; †$P < 0.01$. 

Fig. 2. Plasma (P.) glucose (A), insulin (B), glucagon (C), FFA (D), glycerol (E), and lactate (F) concentrations before and during AICAR (●) and AICAR + MP (○) infusions in normal dogs. Note: from −20 to 0 min are data with or without prior 48-h MP; from 0 to 120 min are data of AICAR alone or AICAR + MP. *$P = 0.06$, **$P < 0.01$, ***$P < 0.001$ vs. basal (over time); †$P < 0.02$, ††$P < 0.001$ for AICAR vs. AICAR + MP profiles. 

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AICAR STIMULATES HEPATIC AND MUSCLE GLUCOSE TURNOVER

Table 3. Skeletal muscle intracellular substrate concentrations of glucose, G-6-P, glycogen, AMP, ADP, ATP, and ZMP, AICAR and ACC−pSer2121 and activities of AMPKα1 and -α2 in control dogs (n = 8) before and during AICAR infusion alone (1.5−2.0 mg·kg−1·min−1) or AICAR + MP

<table>
<thead>
<tr>
<th>Study</th>
<th>Fasting</th>
<th>AICAR</th>
<th>AICAR + MP</th>
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<tbody>
<tr>
<td>ICglucose, mmol/l</td>
<td>0.29±0.14</td>
<td>0.21±0.08</td>
<td>0.50±0.17</td>
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<tr>
<td>G-6-P, mg/kg wet wt</td>
<td>3.2±0.5</td>
<td>3.4±0.6</td>
<td>4.1±0.7</td>
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<tr>
<td>Glycogen, mg/kg wet wt</td>
<td>285±28</td>
<td>314±35</td>
<td>271±31</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>6.7±1.1</td>
<td>9.6±1.6</td>
<td>11.3±2.2†</td>
</tr>
<tr>
<td>AMPKα1, pmoles·mg−1·min−1</td>
<td>10.2±1.0</td>
<td>10.9±1.3</td>
<td>9.0±2.0</td>
</tr>
<tr>
<td>AMPKα2, pmoles·mg−1·min−1</td>
<td>1.4±0.2</td>
<td>1.6±0.3</td>
<td>1.4±0.2</td>
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<tr>
<td>ACC−pSer2121, AU</td>
<td>1.0±0.1</td>
<td>1.5±0.3*</td>
<td>1.8±0.2*</td>
</tr>
<tr>
<td>ATP, μmol/g wet wt</td>
<td>8.76±0.34</td>
<td>9.76±0.56</td>
<td></td>
</tr>
<tr>
<td>ADP, μmol/g wet wt</td>
<td>0.68±0.03</td>
<td>0.77±0.04</td>
<td></td>
</tr>
<tr>
<td>AMP, μmol/g wet wt</td>
<td>0.03±0.00</td>
<td>0.03±0.00</td>
<td></td>
</tr>
<tr>
<td>ZMP, μmol/g wet wt</td>
<td>0.00</td>
<td>0.02±0.04</td>
<td></td>
</tr>
<tr>
<td>AICAR, μmol/g wet wt</td>
<td>0.00</td>
<td>0.13±0.03</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. G-6-P, glucose 6-phosphate; ACC, acetyl-CoA carboxylase; IC, intracellular. †P = 0.06 vs. fasting state.

AICAR + MP infusion, which contrasted with the significant 30 and 32% rises, respectively, observed with AICAR infusion alone (Fig. 3). On the other hand, MCRg rose (P = 0.06) from an already raised basal level, and the rise in MCRg was sustained. This, together with the minor response in Ra, total led to the sustained lower plasma glucose (P < 0.001; Fig. 2). The increased basal GF with MP alone was not maintained during the AICAR + MP infusion, with both absolute and percent GF of Rd tissue falling by 120 min of the AICAR + MP infusion (Table 2). The low-dose Glu did not influence these results (data not shown).

Effects of oral MP + AICAR infusion on ex vivo SkM substrates (ICglucose, G-6-P, glycogen, and lactate concentrations), AMPKα1 and -α2 activities, and ACC−pSer2121. There was no effect of AICAR + MP infusion on SkM ICglucose G-6-P, or glycogen concentrations, but a 70% rise (P = 0.06) in SkM lactate was observed (Table 3). AMPKα1 and -α2 were again not changed by this treatment, but ACC−pSer2121 phosphorylation was markedly stimulated by 80% (Table 3).

Acute AICAR ± MP Infusion in Poorly Controlled Diabetic Dogs

Effects of AICAR infusion on plasma substrates and hormones. In the three hyperglycemic alloxan-diabetic dogs, plasma glucose concentration fell slightly during the AICAR infusion by ~6% (Table 4), and there was no change in plasma insulin (Table 4). Plasma glucagon was elevated at baseline compared with their prealloxan state but did not alter during the 120 min of AICAR infusion (Table 4). However, similar to the prealloxan state, there was a sustained and significant drop in plasma FFA concentration at 60 and 120 min of AICAR infusion by 55 and ~60%, respectively (Table 4). Plasma glycerol concentration did not change significantly (Table 4). Plasma lactate concentration increased 2.6-fold by 60 min and 4-fold (P = 0.06) after 120 min of AICAR infusion (Table 4).

Effects of AICAR infusion on in vivo Ra, total Rd tissue, MCRg, GF, and UrGloss. As expected (12, 13), basal Ra, total in the three poorly controlled alloxan-diabetic dogs was markedly raised, about twofold, compared with their paired nondiabetic dogs.

Table 2. GF and %GF in the basal state with or without prior 48-h MP exposure and at the end of AICAR infusion with or without MP

<table>
<thead>
<tr>
<th>State</th>
<th>GF, μmol/kg−1·min−1</th>
<th>%GF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>10.2±1.3</td>
<td>76±5</td>
</tr>
<tr>
<td>MP alone</td>
<td>14.2±1.1††</td>
<td>96±2†</td>
</tr>
<tr>
<td>AICAR</td>
<td>12.2±0.7*</td>
<td>72±3</td>
</tr>
<tr>
<td>AICAR + MP</td>
<td>12.0±2.1</td>
<td>72±10</td>
</tr>
</tbody>
</table>

Values are means ± SE. GF, glycolytic flux. *P < 0.05 vs. basal; †P < 0.01 vs. basal.

Fig. 3. Total glucose appearance (Ra, total; A), tissue glucose disappearance (Rd, tissue; B), and metabolic clearance of glucose (MCRg, C) before and after 48-h MP in the basal state and during AICAR infusion. *P = 0.06 vs. basal (over time); †P = 0.04, ††P = 0.01, †††P < 0.001 for AICAR vs. AICAR + MP profiles.
The AICAR infusion had no effect on these values in the over the 120 min of AICAR infusion, nor did Rd tissue change lactate (Table 4).

In the three alloxan-diabetic dogs, plasma glucose concentration now fell continuously by 15% after AICAR + MP infusion, and there was no change in plasma insulin throughout the AICAR + MP infusion (Table 4). Plasma glucagon also did not change after the AICAR + MP infusion (Table 4). However, there was a 50% fall in FFA at 60 min, which was less evident by 120 min of the AICAR + MP infusion, reminiscent of the pattern observed for FFA in the prealloxan AICAR + MP-infused studies (Table 4). There was no change in plasma glycerol during the AICAR + MP infusion (Table 4). Plasma lactate again rose two- to threefold after the AICAR + MP infusion (Table 4).

### Effects of acute AICAR infusion in the presence of oral MP (vs. MP alone) on in vivo Ra total, Rd total, Rd tissue, MCRg, GF, and UrGloss.

Table 4. Plasma substrates, hormone concentrations, and glucose turnover data in 3 alloxan-diabetic dogs before and during acute AICAR infusion alone and during AICAR + MP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline Basal</th>
<th>AICAR Infusion</th>
<th>MP Alone</th>
<th>AICAR + MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. Glucose, mmol/l</td>
<td>19.1±1.7</td>
<td>17.9±1.0</td>
<td>17.3±1.0</td>
<td>16.0±1.0</td>
</tr>
<tr>
<td>P. Insulin, μU/l</td>
<td>5.9±0.4</td>
<td>7.1±0.7</td>
<td>5.8±0.1</td>
<td>5.0±0.9</td>
</tr>
<tr>
<td>P. Glucagon, ng/l</td>
<td>184±49</td>
<td>497±5</td>
<td>97±5</td>
<td>108±9</td>
</tr>
<tr>
<td>P. FFA, mmol/l</td>
<td>0.77±0.03</td>
<td>0.35±0.01</td>
<td>0.29±0.02</td>
<td>0.78±0.03</td>
</tr>
<tr>
<td>P. Glycerol, μmol/l</td>
<td>117±8</td>
<td>84±16</td>
<td>97±5</td>
<td>108±9</td>
</tr>
<tr>
<td>Rₐ total, μmol·kg⁻¹·min⁻¹</td>
<td>32.4±2.0</td>
<td>33.3±4.6</td>
<td>31.6±3.7</td>
<td>23.6±2.6</td>
</tr>
<tr>
<td>Rₐ tissue, μmol·kg⁻¹·min⁻¹</td>
<td>21.1±1.0</td>
<td>22.1±2.4</td>
<td>20.4±1.5</td>
<td>20.9±2.8</td>
</tr>
<tr>
<td>MCRg, mgl⁻¹·min⁻¹</td>
<td>1.1±0.1</td>
<td>1.3±0.2</td>
<td>1.3±0.1</td>
<td>1.5±1.0</td>
</tr>
<tr>
<td>GF, μmol·kg⁻¹·min⁻¹</td>
<td>0.7±0.2</td>
<td>0.7±0.2</td>
<td>0.7±0.2</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>%GF of Ra</td>
<td>84±13</td>
<td>79±9</td>
<td>90±10</td>
<td>85±7</td>
</tr>
<tr>
<td>UrGloss, μmol·kg⁻¹·min⁻¹</td>
<td>12±3</td>
<td>11±2</td>
<td>3±1</td>
<td>2±1</td>
</tr>
</tbody>
</table>

Values are means ± SE. FFA, free fatty acid; MCRg, metabolic clearance rate of glucose.

Effects of acute AICAR infusion in the presence of oral MP infusion (AMPKα1 and -α2 activities and ACC—pSer221). AMPKα1 and -α2 activities and ACC—pSer221 were not altered by the MP infusion (AMPKα1 14.0 ± 3.9 and AMPKα2 1.7 ± 0.3 pmol·mg⁻¹·min⁻¹, ACC—pSer221 1.6 ± 0.2 AU) compared with their respective diabetic values before or after the AICAR infusion alone.

### DISCUSSION

Activation of AMPK by AICAR in SkM increases glucose uptake (34), FFA oxidation (34), and glycogenolysis (65) and inhibits glycogen synthesize activity (10). In liver, high-dose AICAR stimulates glycogenolysis (25, 26) but also inhibits gluconeogenesis (31, 57), lipogenesis, and cholesterol synthesis (23, 35). However, enhanced glycogenolysis in muscle (32) and liver (8, 22) and suppressed hepatic gluconeogenesis (31) can both be induced allosterically in vivo by raised intracellular levels of ZMP independently of a direct AMPK effect. In the most recent studies employing low-dose AICAR, HGP was enhanced in the presence of low- and high-dose insulin at eu- and hyperglycemia (8, 9, 38). These (8, 9, 38) and the earlier studies (5, 6, 26) necessitated coinusions of insulin and glucose with AICAR, which compound the determination of in vivo glucose turnover and SkM glucose metabolism due to the direct effect of GtG on HGP independently of changes in insulin (46). It is also important to note that, in the overnight-fasted rat as opposed to overnight-fasted dog (8, 9) or 5-h-fasted rat (26), hepatic glycogen is markedly depleted, and HGP depends mainly on gluconeogenesis, a critical factor governing the suppressive effect of AICAR (31) on HGP in rat (5). The novel findings in the present study, in which infusion of low-dose AICAR alone was used, were a transient fall in plasma state (15.1 ± 2.5 μmol·kg⁻¹·min⁻¹). Rₐ tissue total did not change over the 120 min of AICAR infusion, nor did Rₐ tissue change lactate (Table 4). However, MCRg rose by 15% after AICAR (Table 4). However, there were 25 and 35% rises in MCRg after 60–75 and 90–120 min of AICAR + MP infusion (Table 4). However, there were 25 and 35% rises in MCRg after 60–75 and 90–120 min of AICAR + MP infusion, which probably led to the fall in plasma glucose. The AICAR + MP infusion resulted in little change in the rate of GF or %GF (Table 4). With the lower plasma glucose, UrGloss was stable but reduced to ~10% of Rₐ tissue before the AICAR + MP infusion (Table 4).
glucose and a brief rise in insulin associated with lower FFA, which were accompanied by a prompt and sustained increase of HGP, $R_d$ tissue, and GF (Fig. 3). No changes occurred in SkM of IC$_{\text{glucose}}$, G-6-P, or glycolen or AMPKo1 and -o2 activities, but a 50% increase of ACC$\sim$P Ser$^{221}$ was observed (Table 3). Although the level of ZMP achieved is fourfold lower than that reported in SkM of high-dose AICAR-infused rats (5, 6), the SkM ZMP was fivefold the concentration of AMP, sufficient to activate AMPK (18, 58), without any changes in ATP, ADP, or AMP during the AICAR infusion. On the other hand, when FA oxidation was blocked by prior 2-day administration of MP (47, 54, 63), the low-dose AICAR now resulted in a sustained lower plasma glucose profile, with no changes in insulin and FFA. These plasma changes were accompanied by a smaller but significant increase in HGP and $R_{d\text{total}}$ but a sustained increase in MCR$_{\text{G}}$ (Fig. 3). SkM, IC$_{\text{glucose}}$, G-6-P, glycolen, and AMPKo1 and -o2 activities were again unchanged, but ACC$\sim$P Ser$^{221}$ was more markedly raised (Table 3). Thus the present data demonstrate that low-dose AICAR, with or without prior block of FA oxidation, clearly enhances HGP and peripheral glucose disposal. Whereas the hepatic effects of AICAR on HGP are secondary to the allosteric effects of ZMP (8), the changes in peripheral glucose metabolism are likely to be due to in vivo activation of endogenous AMPK, given the specific increase of ACC$\sim$P Ser$^{221}$.

Impact of Low-Dose AICAR

Earlier in vivo studies of AICAR administration have employed high doses of the agent 10–15 times that used in the present studies (5, 6, 26). Such pharmacological doses would raise tissue levels of muscle ZMP to that of a direct ZMP-allosteric stimulation of key intracellular enzymes of glucose metabolism (22, 32, 45), in addition to allosteric activation of AMPK (18). AICAR appears to activate AMPK by an allosteric ZMP-directed mechanism and not directly by activation of its upstream AMPK kinase (60). However, ZMP can increase AMPK$\sim$Thr$^{172}$ phosphorylation, which is a more favorable substrate for phosphorylation and activation by LKB1 (60). The current in vitro assays for AMPK activity reflect only in situ endogenous activated AMPK (58). Thus the phosphorylation status of ACC$\sim$Ser$^{221}$ is a more sensitive biological marker of in vivo AICAR-stimulated AMPK activity (3, 58). In the present low-dose AICAR infusion studies, AMPK was activated allosterically in SkM in vivo, as shown by the significant increases in SkM ACC$\sim$P Ser$^{221}$, despite the failure to demonstrate a change in assay AMPKo1 and -o2 activities in vitro. A similar impact of low-dose AICAR (1–2 mg·kg$^{-1}$·min$^{-1}$·ip) was recently noted in dogs with respect to a failure to observe any increase in assayed hepatic AMPK$\sim$Thr$^{172}$ phosphorylation despite a marked increase in ACC$\sim$Ser$^{79}$ phosphorylation (9, 38), which was accompanied by enhanced HGP in the presence of the concomitant hyperinsulinaemia (9, 38).

There are only a few studies that have examined the effects of AICAR on in vivo glucose turnover. Our observed increased HGP response to low-dose AICAR was initially unexpected, given the marked suppression of HGP noted in earlier studies, which employed 10-fold greater AICAR doses and concomitant glucose and insulin infusions (5, 6, 26). Those authors attributed their findings of AICAR-induced suppression of HGP to a targeted inhibition of the prevailing increased gluconeogenesis present in 18-h-starved (5), insulin-resistant obese (6), or high-fat insulin-resistant rats (26). They also acknowledged the possibility that allosteric inhibition of fructose-1,6-diphosphatase by the raised levels of ZMP (7) may occur (6, 26). In contrast to these findings, in the present study HGP was markedly and rapidly increased over the 120 min of the AICAR infusion despite the small, significant, early increase of insulin over the initial 60 min of the study (Table 1). These data support the recent findings in overnight-fasted dogs, which employed similar low-doses of AICAR (1–2 mg·kg$^{-1}$·min$^{-1}$·ip) during either hypoglycemic and euglycemic or modestly hyperglycemic hyperinsulinemic clamp studies, in which HGP was enhanced due to the direct allosteric effect of the raised ZMP and not the coexisting increased hepatic AMPK (8, 9, 38). Both in these latter (8, 9, 38) and in the present studies, hepatic gluconeogenesis would have represented only 5–25% of net hepatic glucose output (17). Camacho et al. (9) reported that there was no increase in hepatic uptake of gluconeogenic precursors, supporting a failure of these low doses of AICAR to influence in vivo gluconeogenesis. Thus it is likely that the AICAR-induced increase in HGP is due to a direct stimulation of glycolysis via phosphorylation and activation of hepatic glyco- gen phosphorylase (45) and deactivation of hepatic glycogen synthase (10) by possible direct purine nucleotide allosteric activation of hepatic glycogen phosphorylase (22) and indirect activation of phosphorylase kinase (65). Low-dose AICAR infusion also blocks insulin-induced suppression of HGP, thus causing acute hepatic insulin resistance (8, 9, 38). This inhibitory action of AICAR on hepatic insulin action contrasts sharply with that of AICAR stimulation of insulin-induced glucose uptake in SkM (5).

In the present study, stimulation of whole body glucose uptake ($R_d$ tissue) and intracellular glucose metabolism (as reflected by increased GF) occurred over the 120 min of the low-dose AICAR infusion (Fig. 3 and Table 2). This increase in $R_d$ tissue was more rapid than the rise in HGP; therefore, glucose fell over the initial 60 min of the AICAR infusion. Within SkM, no changes of IC$_{\text{glucose}}$, G-6-P, and glycogen concentrations were detected. However, ACC$\sim$P Ser$^{221}$ rose by 50%, consistent with activation of SkM AMPK (3, 58) and FA oxidation (34), which contributed to the lower plasma FFA that we observed during the AICAR infusion, in the presence of steady plasma glycerol levels. These findings are supported by previous studies in rats where in vivo stimulation of whole body (5, 6) and SkM 2-deoxy-d-[3-3H]glucose uptake (5, 6, 25, 26) appeared to occur during high-dose acute AICAR. Similarly, with low-dose AICAR infusion in our dogs, $R_d$ tissue was also enhanced. In addition, we measured for the first time in vivo AICAR-stimulated GF and showed a sustained increase in GF (in proportion to the increased $R_d$ tissue) over the 120 min of AICAR infusion. The AICAR-induced increment of GF was accompanied by an increased SkM lactate and ACC$\sim$Ser$^{221}$ phosphorylation in the presence of constant SkM G-6-P levels (Table 3). Together, these data are consistent with an overall enhancement of intracellular metabolic response down the glycolytic pathway in response to the low-dose AICAR-induced increase of glucose uptake in the possible presence of reduced glycolytic pathway activity induced by the AICAR (10). On the other hand, Bergeron et al. (5) found in their overnight-starved rats large increases in SkM G-6-P and lactate during the combined glucose-insulin-high-dose AICAR infusions. Their metabolic SkM substrate profile, with a 10-fold increase in G-6-P, probably reflected the large infusion-in-
duced increase in glucose uptake (~40% above basal uptake), which exceeded the capacity of the glycolytic flux pathway (5). The high-dose AICAR would have also inhibited the glycogen synthesis pathway (10, 59), which would have further compromised the ability to clear the excessive production of G-6-P.

Thus our results and those of others (8, 9, 38) appear to indicate an appropriate metabolic response to the low-dose AICAR of the liver and peripheral tissues, in which the increase of HGP maintains peripheral glucose homeostasis. However, these AICAR effects are achieved by different mechanisms: allosterically by ZMP alone for the HGP responses (8) and by activation of AMPK for SkM (6, 34, 37).

Impact of AICAR in the FAOX-Blocked Dog

Oral MP, which blocks mitochondrial CPT I activity (54), produces an irreversible intracellular blockade of FAOX (27, 47, 63) in all tissues, predominantly in liver, heart, and diaphragm and to a lesser extent in SkM (27). The accumulated dose of MP employed in the present study (500 mg/kg over 48 h) is similar to that reported previously in dogs (47, 63) but more than the single-dose acute schedules (30–100 mg/kg) used in rats (27). Such doses would be expected to produce ~50–70 and 100% blockade of FAOX in muscle and liver, respectively (27, 54), although the extent of the FAOX blockade on glucose metabolism in the various tissues depends on the length of starvation before study, the SkM fiber type examined (27), and the glycogen status of the SkM (27). In the present study, in 10-h-fasted dogs of 48-h FAOX blockade, HGP and R_d tissue were unchanged by MP, but peripheral MCR_g and GF increased significantly, with the %GF of R_d tissue rising to 96% (Table 2). Fasting glucose was reduced. One explanation for these data is that the whole body metabolic response is due to the Randle glucose-FAOX cycle (40). That is, as a direct effect of the FAOX blockade, MCR_g and glucose oxidation (GF) in SkM are enhanced (40). Alternatively, because the blockade of FAOX would seriously curtail ATP production, there could be a compensatory shift within peripheral glucose metabolism to oxidative glycolysis and, hence, greater ATP production; i.e., the proportion of GF increases but R_d tissue remains constant. This metabolic phenomenon is known as the Pasteur effect (29).

We next examined the metabolic effects of AICAR in 48-h-treated MP dogs to isolate the effect of AICAR on glucose metabolism alone. AICAR + MP studies have not been reported before. The prior blockade of FAOX modified the metabolic responses compared with AICAR alone by 1) enhancing and sustaining the decrement of plasma glucose but attenuating the rise of insulin, 2) reducing the previously observed increments in HGP, R_d tissue, and GF while sustaining an increase in MCR_g, 3) maintaining normal SkM concentrations of I_c glucose, G-6-P, and glycogen, and 4) increasing ACC–pSer221 phosphorylation in the AICAR + MP FAOX-blocked dogs may also indicate a greater degree of intracellular metabolic “stress” due to compromised ATP generation within SkM. An alternative explanation for the failure of HGP to rise during the AICAR infusion in MP-blocked dogs may be due to an inhibition of the expected AICAR-stimulated central hypothalamic FAOX response in the MP-treated dogs (39). A reduced central FA oxidative response might lead to the decreased HGP, as seen in our MP + AICAR-treated dogs (39).

Impact of AICAR and AICAR + MP in Diabetic Dogs

The limited in vivo glucose turnover and in vitro SkM data in the alloxan-diabetic dogs in the present study are similar to our previously reported studies (12, 13). In particular, fasting hyperglycemia and hypoinsulinemia were associated with mildly increased basal HGP and R_d tissue but compensated normal GF in the presence of raised SkM I_c glucose, reduced glycogen, and significantly raised basal SkM AMPKα1 and α2 activities and ACC–pSer221 (12, 13) (Table 4). In this in vivo and in vitro “stressed” metabolic state, increased gluconeogenesis accounts primarily for the increased HGP (53). Hepatic glycogen content is also low, which could further limit a glycogenolytic response to AICAR (8, 9). Thus HGP was essentially not altered by the low-dose AICAR infusion in the hyperglycemic diabetic state. However, the fasting hyperglycemia fell slightly (by 6%) and MCR rose by 15% (Table 4). In contrast, with MP alone and with AICAR + MP, fasting glucose was substantially lower, which was secondary to the 30% decrement of HGP and consequent 30% increase in MCR_g (40), respectively. Here, the 30% decrement in HGP may be due to suppression by low-dose AICAR of the compromised hepatic gluconeogenesis rate (57), as seen during MP blockade of FAOX in these poorly controlled diabetic dogs. It may also reflect the direct inhibitory effect of central hypothalamic FAOX blockage on HGP (39).

Insulin Secretory Responses to AICAR and AICAR + MP

The present pancreatic β-cell responses to low-dose AICAR were unexpected. To date, only in vitro studies in multiple species of derived clonal β-cells have been reported, and the majority of those studies demonstrated inhibition of insulin secretion by AICAR stimulation of islet AMPK activity (for review see Ref. 42). Thus, when AMPK activity is chronically upregulated by adenoviral infection of the islet by constitutively active AMPK or “acutely” by in vitro exposure of clonal islets to high-dose AICAR (~2 mM AICAR), insulin secretion falls (19, 30, 66). However, some in vitro studies have demonstrated stimulation of insulin secretion by AICAR (2, 43). To date, no in vivo studies of the insulin secretory responses to AICAR infusion have been reported. Our novel preliminary observations demonstrate a significant transient early AICAR-induced increase in endogenous insulin secretion with low-dose AICAR alone and an apparent lesser insulin secretory response during the combined AICAR + MP study. These in vivo data are consistent with previous in vitro studies (2, 43, 62) in which AICAR-induced β-cell AMPK activity was increased and β-cell ACC phosphorylation, FAOX, and insulin secretion were enhanced (62). It is of interest to note that both the initial dpm/ml of [3-3H]glucose and R_a total time profiles mirrored the initial time profile response of insulin secretion.
(Table 1). Whether the small initial AICAR-induced increases of $R_{a}$ total and insulin in the presence of a slightly decreasing blood glucose are related cannot be determined from our study. The overall biological impact of these changes of β-cell function with AICAR with or without MP needs further evaluation.

In conclusion, the present study clearly establishes that low-dose AICAR leads to a sustained increase in peripheral glucose uptake, which is matched by the AICAR-induced increase of HGP. The SKM metabolic responses are consequent to stimulation of endogenous AMPK activity and ACC~Pser308, whereas the hepatic response is due mainly to an allosteric effect of ZMP on HGP (8, 9, 38). When FAOx is blocked (by MP), AICAR + MP-induced changes in HGP and peripheral glucose metabolism are attenuated. On the other hand, in the poorly controlled hyperglycemic diabetic state, the low-dose AICAR alone has little effect on glucose turnover or glycemia, whereas AICAR + MP decreases HGP and increases MCRg, leading to a significant reduction in plasma glycemia, whereas AICAR

ACKNOWLEDGMENTS

We are grateful to Sue McKay and Liliana Pepe for performing arteriovenous shunts and venous catheterizations on the dogs and for assistance with muscle biopsies. We thank Selma Gotsbacher and Sally Misfud and the staff of the Experimental Medical and Surgical Unit for care, health monitoring, and exercising of the dogs and Jane Ford for expert secretarial assistance.

GRANTS

This study was supported by grants from the Juvenile Diabetes Foundation International/Australia, the National Health and Medical Research Council of Australia, and the Diabetes Australia Research Trust.

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