5-Aminooimidazole-4-carboxamide-1-β-D-ribofuranoside renders glucose output by the liver of the dog insensitive to a pharmacological increment in insulin

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The compound 5-aminooimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) is taken up by cells and phosphorylated to become the AMP analog, 5-aminooimidazole-4-carboxamide-1-β-D-ribofyl-5-monophosphate (ZMP). Intraportal AICAR infusion (1 mg·kg⁻¹·min⁻¹) stimulates hepatic glycogenolysis and glucose output by the liver in the presence of basal intraportal glucagon, high physiological insulin, and either euglycemia or hypoglycemia (6). Moreover, it causes a dose-dependent stimulation of hepatic glucose output (NHGO) when the liver is in what normally would be a glucose uptake mode (in the presence of hyperinsulinemia, a negative arterial-portal venous glucose gradient, and hyperglycemia; see Ref. 25). In light of the prevalence of insulin-induced hypoglycemia, it is notable that AICAR is effective despite high physiological levels of insulin. The hypothesis tested in the present study was that AICAR renders NHGO insensitive to a pharmacological increase in insulin. To this end, AICAR was infused intraportally at rates 10% of those used in rodent models (to minimize extrahepatic effects; see Refs. 3, 4, 29) during euglycemic clamps in which somatostatin (SRIF) was infused, glucagon was replaced at basal rates, and insulin was replaced at high physiological or pharmacological rates.

RESEARCH DESIGN AND METHODS

Animals and surgical procedures. Experiments were performed on a total of 19 overnight-fasted mongrel dogs (mean weight 22.2 ± 0.5 kg) of either sex that had been fed a standard diet (7). Protocols were approved by the Vanderbilt University Animal Care Committee. Surgical procedures used in this study have been previously described (7). Dogs meeting the established health criteria (7) were studied after an 18-h overnight fast.

Experimental procedures. Experiments consisted of tracer equilibration (−130 to −30 min) and basal (−30 to 0 min), and hyperinsulinemic-euglycemic clamp (0–150 min) periods. At time (t) = 0 min, somatostatin was infused, and basal glucagon was replaced via the portal vein. Insulin was infused in the portal vein at rates 10% of those used in rodent models (to minimize extrahepatic effects; see Refs. 3, 4, 29) during euglycemic clamps in which somatostatin (SRIF) was infused, glucagon was replaced at basal rates, and insulin was replaced at high physiological or pharmacological rates.

IATROGENIC HYPOGLYCEMIA due to hyperinsulinemia is the leading short-term complication for both type 1 and type 2 diabetic patients striving to maintain tight glycemic control (10). Current treatments for hypoglycemia include carbohydrate ingestion or, if necessary (i.e., during unconsciousness) administration of exogenous glucagon. However, depending on the degree of hyperinsulinemia, neither of these treatments may be sufficient. A more optimal treatment would be one that is effective in the presence of hyperinsulinemia.

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animals were killed with pentobarbital sodium and liver biopsies were frozen in liquid nitrogen. An autopsy was performed to confirm catheter placement.

**Blood sample and tissue collection and processing.** Arterial, portal vein, and hepatic vein blood was drawn at \( t = -30, -15, 0, 15, 30, 45, 60, 75, 90, 120, \) and 150 min. Arterial samples were taken every 10 min for the duration of plasma glucose specific activity. Plasma glucose, glucose radioactivity, free fatty acids, cortisol, catecholamines, and immunoreactive insulin and glucagon were determined as previously described (7). Blood was deproteinized in perchloric acid and incubated with \( ^{3} \text{H} \)-hydroxybutyrate, glycerol, and lactate, using standard enzymatic methods (18). Hepatic glycogen and purine nucleotide concentrations were determined as previously described (6).

**Western blotting.** Protein from liver lysates (30 \( \mu \)g; see Ref. 6) was run on a SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane. Membranes were treated with rabbit \( \alpha \)-phospho-AMP-activated protein kinase (AMPK, Thr\(^{172} \)) or \( \alpha \)-phospho-acetyl-CoA carboxylase (ACC, Ser\(^{79} \)) antibodies (Cell Signaling, Beverly, MA) and then incubated with \( \alpha \)-rabbit-horseradish peroxidase (Promega, Madison, WI). Chemiluminescence was detected with a WesternBlot kit from Invitrogen (Carlsbad, CA). Densitometry was performed using Scion Image software (Frederick, MD).

**Calculations.** Endogenous glucose production \( (R_{p}) \) and utilization \( (R_{d}) \) were determined by isotope dilution (20).

**Statistical analysis.** Statistical comparisons between groups and over time were made using ANOVA designed to account for repeated measures. Specific time points were examined for significance using contrasts solved by univariate repeated measures. Liver purine nucleotides were compared using ANOVA designed to account for repeated measures. Liver glycogen was compared using \( t \)-tests. AMPK and ACC phosphorylation was compared using one-tailed \( t \)-tests. Statistics are reported in Table 1 and Figs. 1–3. Data are presented as means \( \pm \) SE. Statistical significance was defined as \( P < 0.05 \).

**RESULTS**

**Arterial plasma glucose and hormones.** Arterial plasma glucose was similar during the basal period and was clamped in both groups (107 ± 1 mg/dl in INS2 and 104 ± 1 mg/dl in INS5; Table 1). The glucose infusions required to clamp at euglycemia during the last 90 min were 0.4 ± 0.5 mg·kg\(^{-1} \)·min\(^{-1} \) and 16.4 ± 0.4 mg·kg\(^{-1} \)·min\(^{-1} \) in INS2 and INS5, respectively (Table 1). Arterial insulin rose to 52 ± 7 and 213 ± 26 \( \mu \)U/ml in INS2 and INS5, respectively (Table 1). Arterial plasma glucagon, cortisol, epinephrine, and norepinephrine were similar between groups and did not change during the study (data not shown).

**Glucose kinetics.** NHGO was similar during the basal period and fell similarly during the first 60 min of hyperinsulinemia in each group (Fig. 1). AICAR increased NHGO similarly in INS2 (\( -0.2 \pm 0.2 \) at \( t = 60 \) min to 1.8 ± 0.6 mg·kg\(^{-1} \)·min\(^{-1} \) at \( t = 150 \) min, \( P < 0.05 \)) and INS5 (\( -0.9 \pm 0.3 \) at \( t = 60 \) min to 1.1 ± 0.6 mg·kg\(^{-1} \)·min\(^{-1} \) at \( t = 150 \) min, \( P < 0.05 \)). In contrast, NHGO remained suppressed when saline was infused instead of AICAR (Fig. 1).

Endogenous \( R_{a} \) was similar during the basal period and fell during the first 60 min of hyperinsulinemia in each group (Fig. 2A). AICAR increased \( R_{a} \) in both INS2 (0.9 ± 0.2 at \( t = 60 \) min to 2.1 ± 0.7 mg·kg\(^{-1} \)·min\(^{-1} \) at \( t = 140 \) min) and INS5 (1.7 ± 0.4 at \( t = 60 \) min to 1.4 ± 0.6 mg·kg\(^{-1} \)·min\(^{-1} \) at \( t = 140 \) min, \( P < 0.05 \)). \( R_{d} \) was similar during the basal period and rose similarly during the first 30 min of hyperinsulinemia in each group (Fig. 2B). \( R_{d} \) was significantly greater in INS5 compared with INS2 from \( t = 40 \)–140 min.

**Metabolite concentrations and net hepatic balances.** Arterial concentrations and net hepatic balances of alanine, \( \beta \)-hydroxybutyrate, free fatty acids, glycerol, and lactate were similar between groups during the basal period and hyperinsulinemia periods (data not shown).

**Tissue analyses.** At \( t = 150 \) min, liver glycogen was 47 ± 4, 41 ± 3, and 35 ± 4 mg/g liver in a portal venous saline-infused group (from Ref. 6), INS2, and INS5, respectively (\( P < 0.05 \), INS5 vs. saline). Liver ZMP was not detectable, 3.51 ± 0.38, and 4.34 ± 0.46 \( \mu \)mol/g liver in saline-infused dogs (from Ref. 6), INS2, and INS5, respectively (\( P < 0.05 \), INS2 and INS5 vs. saline). Phosphorylation of hepatic AMPK (Thr\(^{172} \)) was similar in all groups (data not shown). Phosphorylation of hepatic ACC (Ser\(^{79} \)) was significantly greater in INS2 vs. INS5 and saline-infused dogs (\( P < 0.05 \), Fig. 3).

**DISCUSSION**

These studies show that intraportal infusion of AICAR at a rate of 1 mg·kg\(^{-1} \)·min\(^{-1} \) renders hepatic glucose output blind.
to the suppressive effects of pharmacological insulin. Although it has previously been shown that intraportal AICAR stimulates hepatic glucose output (6, 25), it was unknown if the effect was because of AMPK activation [and the subsequent activation of glycogen-phosphorylase kinase (8) and phosphorylase (31)] and/or allosteric activation of glycogen phosphorylase by ZMP (12, 19, 28). These studies show that

1) AICAR is equally effective in stimulating hepatic glucose output at physiological and pharmacological insulin levels and that

2) AICAR does not require an increase in AMPK activity (as reflected by ACC phosphorylation) to exert this effect.

Several studies have examined the hepatic effects of administering AICAR in vivo. Intracerebroventricular infusion of AICAR increases endogenous Ra in 5-h-fasted mice (26) and overnight-fasted rats (21). Iglesias et al. (16) showed that a 250 mg/kg intraperitoneal injection of AICAR caused a marked increment in net hepatic glycogen breakdown in the 5-h-fasted

Fig. 1. Net hepatic glucose output (NHGO) in the presence of a 2 mU·kg⁻¹·min⁻¹ (INS2, n = 7, ●) and 5 mU·kg⁻¹·min⁻¹ (INS5, n = 12, ○) hyperinsulinemic insulin infusion during the basal period (−30 to 0 min) and hyperinsulinemic euglycemic clamp period (0 to 150 min) in the presence of intraportal 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) from 60 to 150 min. The dotted line represents the normal response of NHGO in the presence of hyperinsulinemia alone (data from Ref. 6). Inset: area under the curve (AUC) of NHGO after time (t) = 60 min. Data are means ± SE. *Significantly different from saline (P < 0.05).

Fig. 2. Endogenous Ra (A) and Rd (B) in the presence of a 2 mU·kg⁻¹·min⁻¹ (n = 7, ●) and 5 mU·kg⁻¹·min⁻¹ (n = 12, ○) hyperinsulinemic infusion during the basal period (−30 to 0 min) and hyperinsulinemic euglycemic clamp period (0 to 150 min) in the presence of intraportal AICAR from 60 to 150 min. Data are means ± SE. *Significantly different from 5 mU·kg⁻¹·min⁻¹ (P < 0.05).

Fig. 3. Quantification of the phosphorylated form of acetyl-CoA carboxylase (ACC, Ser79) from livers after a 2 mU·kg⁻¹·min⁻¹ hyperinsulinemic infusion with a portal venous saline (Sal) infusion (n = 5, gray bar, from Ref. 6), and portal venous AICAR-treated dogs in the presence of 2 mU·kg⁻¹·min⁻¹ (n = 7, filled bar) and 5 mU·kg⁻¹·min⁻¹ (n = 5, open bar) insulin. Data are means ± SE. *Significantly different from saline and INS5 (P < 0.05).
Our laboratory has shown that, in the presence of hyperinsulinemia, basal glucagon, and either hypoglycemia, euglycemia (6), or hyperglycemia (25), an intraportal AICAR infusion (1 mg·kg⁻¹·min⁻¹) increases hepatic glucose output in the 18-h-fasted dog. This AICAR infusion results in hepatic ZMP concentrations of ~3 µmol/g liver, and an increase in hepatic AMPK activity, reflected by an increase in hepatic ACC phosphorylation (6, 25). These studies were in contrast to those of Bergeron et al., who showed that primed (100 mg/kg) systemic AICAR infusions of 7.5 (4) and 10 (3) mg·kg⁻¹·min⁻¹ suppressed R₆ in overnight-fasted rats. The differences between the studies of Bergeron et al. and the previously mentioned studies showing an AICAR stimulation of glucose production can be attributed to differences in glycogen content, since an overnight fast virtually glycogen depletes rats. Consistent with differences in hepatic glycogen levels being responsible for these differences is the fact that the marked stimulation of NHGO from an intraportal AICAR infusion was lost when given to a 42-h-fasted dog in which liver glycogen was markedly reduced (Camacho and Wasserman, unpublished observation).

Intraportal AICAR infusion was associated with significant decreases in hepatic glycogen (9 ± 4 and 12 ± 4 mg/g tissue in INS2 and INS5, respectively). This coupled with the fact that there were no differences in net hepatic alanine, glycerol, or lactate uptake indicates that an increase in glycogenolysis was responsible for the rise in hepatic glucose output. The differences in hepatic glycogen correspond to rates of net hepatic glycogenolysis of 3.3 ± 1.2 and 4.7 ± 1.5 mg·kg⁻¹·min⁻¹ in INS2 and INS5, respectively. Because endogenous R₆ tends to be underestimated (as reflected by negative rates) under hyperinsulinemic euglycemic clamp conditions (14), we focused on NHGO. However, it is noteworthy that intraportal AICAR stimulated endogenous R₆ to rise to a similar rate (~2 mg·kg⁻¹·min⁻¹) in both INS2 and INS5. Net hepatic glycogenolysis exceeded NHGO by twofold, indicating that ~50% of the glucose 6-phosphate formed by glycogenolysis has a fate other than glucose release. Because insulin stimulates pyruvate dehydrogenase, pyruvate oxidation is likely to be an alternative fate (24).

The liver of the AICAR-infused dog appears to be resistant to the suppressive effects of high physiological concentrations of insulin on hepatic glucose output (6, 25). This suggests that a compound with similar liver actions could be effectively exploited to combat insulin-induced hypoglycemia, which is the single most frequent complication in diabetic patients (10). Both increased AMP/ZMP concentrations (12, 19, 28) and increased AMPK activity (8, 31) have been shown to result in activation of glycogen phosphorylase. Even small increments (11) in insulin potently inhibit glycogen phosphorylase in vivo (5) via its regulation of cAMP (13). This is one of the few studies to investigate insulin’s regulation of hepatic AMPK.

Intracerebroventricular infusion of insulin reduces α₂-AMPK activity in all hypothalamic regions in mice (22). Chronic insulin treatment normalizes hypothalamic α₂-AMPK hyperactivity in streptozotocin-induced diabetic rats (23). Beauloye et al. (2) showed that insulin antagonizes AMPK activity (Thr₁⁷² phosphorylation) induced by ischemia or anoxia in rat hearts. Gamble and Lospaschuk (15), as well as Witters and Kemp (30), have shown that AMPK inhibition and ACC activation are equally sensitive to insulin and that AMPK may be the major target for insulin regulation of ACC. One way in which AMPK is activated is by phosphorylation of the Thr₁⁷² residue by an upstream kinase, AMPKK, or LKB1 (17). However, another study showed that AICAR does not activate AMPK by stimulating LKB1 (27). Given that AMPK activity is regulated by both allosteric interactions and phosphorylation of ACC, it is likely that phosphorylation of ACC is a more sensitive marker of AMPK activity in vivo (1, 6, 25). Although there was no apparent increase in phosphorylation of the Thr₁⁷² residue of AMPK, the downstream target of AMPK, ACC (Ser⁷⁹), showed increased phosphorylation in the presence of intraportal (physiological) insulin and AICAR infusions (Fig. 3). This notion is supported by other work that showed that AICAR treatment resulted in a clear increase in the phosphorylation of ACC, but not in that of AMPK (19). The low AICAR dose used in this study may not be high enough to cause covalent modification (phosphorylation) of AMPK, yet may be sufficient to cause allosteric activation (19).

When the intraportal insulin infusion was increased to 5 mU·kg⁻¹·min⁻¹, ACC (Ser⁷⁹) phosphorylation was not different from that of saline-infused dogs, confirming that insulin inhibits AMPK activation (2, 15, 22, 30). Because AMPK was not activated in the presence of a 5 mU·kg⁻¹·min⁻¹ intraportal insulin infusion and the increase in glucose output remained, it is clear that AICAR is stimulating net hepatic glycogen breakdown via an AMPK-independent mechanism. This is consistent with the assertion that AICAR increases hepatic glucose output in vivo (6, 25) via allosteric activation of glycogen phosphorylase (12, 19, 28).

In summary, results show that, in the presence of basal glucagon, an intraportal infusion of AICAR stimulates endogenous glucose output equally well regardless of whether insulin concentrations are in the high physiological or pharmacological range. The present results combined with earlier studies (6, 25) have numerous important implications. First, because purine nucleotides are a natural constituent of all cells, including hepatocytes, they may comprise part of an endogenous pathway involved in physiological activation of hepatic glucose output. Second, understanding hepatic insulin resistance caused by AICAR may provide insight into basic mechanisms underlying this liver pathology. Third, AMPK-independent actions of AICAR are a potent component of its gluco-regulatory actions. Fourth, mechanisms that increase hepatic purine nucleotide concentrations may be effective in countering insulin-induced hypoglycemia. The fact that AICAR counters even pharmacological levels of insulin, and that hypoglycemia potentiates the glycogenolytic effect of AICAR (6), adds further weight to this last point. This suggests that a compound with similar hepatic actions could effectively be exploited to combat insulin-induced hypoglycemia, which is the single most frequently occurring complication in diabetes.

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