Skeletal muscle basal AMP-activated protein kinase activity is chronically elevated in alloxan-diabetic dogs: impact of exercise

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Christopher, Michael J., Zhi-Ping Chen, Christian Rantzau, Bruce E. Kemp, and Frank P. Alford. Skeletal muscle basal AMP-activated protein kinase activity is chronically elevated in alloxan-diabetic dogs: impact of exercise. J Appl Physiol 95: 1523–1530, 2003. First published June 27, 2003; 10.1152/japplphysiol.00199.2003.—The effect of diabetes and exercise on skeletal muscle (SkM) AMP-activated protein kinase (AMPKα1 and -α2 activities and site-specific phosphorylation of acetyl-CoA carboxylase was examined in the same six dogs before alloxan (35 mg/kg)-induced diabetes (C) and after 4–5 wk of suboptimally controlled hyperglycemic and hypoinsulinemic diabetes (DHG) in the presence and absence of 300-min phlorizin (50 μg·kg⁻¹·min⁻¹)-induced “normoglycemia” (DNG). In each study, the dog underwent a 150-min [³H]glucose infusion period, followed by a 30-min treadmill exercise test (60–70% maximal oxygen capacity) to measure the rate of glucose disposal into peripheral tissues (Rd(tissue)). SkM biopsies were taken from the thigh (vastus lateralis) before and immediately after exercise. In the C and DHG states, the rise in plasma free fatty acids (FFA) with exercise was similar. However, the exercise-induced FFA were significantly higher, but the absolute rise in FFA (10.220.33.3 on July 11, 2017 http://jap.physiology.org/ Downloaded from

THE REGULATION OF GLUCOSE and fatty acid (FA) metabolism in skeletal muscle (SkM) during exercise is currently the focus of intense investigation (21, 41). Transmembrane GLUT-4-mediated glucose transport is a major rate-determining site for exercise and insulin-stimulated SkM glucose uptake (48), although several other factors, including enhanced capillary recruitment (19) and intracellular enzyme activities (such as pyruvate dehydrogenase) (31), also contribute to the increased glucose uptake and processing observed in exercising SkM. In addition, as the energy demanded by the exercising muscle rises, FA uptake and oxidation are also increased (34, 37). Thus heavily exercising SkM is potentially at risk of outstripping its rising energy needs and becoming “metabolically” stressed (21, 41). AMP-activated protein kinase (AMPK) (21, 41) senses intracellular energy balance and is activated in SkM during intense exercise, both in rodents (17, 33) and humans (14, 36, 44). The magnitude of the AMPK response to exercise correlates inversely with the glycogen content of the SkM (10, 43), suggesting that high glycogen raises the threshold for AMPK activation during metabolic stress (42). Recent in vitro (17, 23) and in vivo (1) studies also provide evidence that activated AMPK activity promotes SkM glucose uptake independently of insulin and regulates intracellular FA metabolism (26, 27). Moreover, AMPK-stimulated SkM glucose uptake occurs via a phosphatidylinositol 3-kinase-independent GLUT-4-mediated pathway (1, 17, 23).

SkM AMPK is activated both covalently via phosphorylation on site Thr-172 by AMPK kinase (21, 41) and allosterically via rising AMP-to-ATP and possibly creatine-to-phosphocreatine ratios (21, 41). Importantly, AMPK is a heterotrimer possessing one α-catalytic subunit that exists in two isoforms, α1 and α2, and two regulating subunits (β and γ) (21, 41). The SkM-α2 isoform is activated in humans during moderately intense exercise (14, 36, 44), although with more extreme exercise the AMPKα1 isoform can also be

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activated (3). Exercise-induced activation of SkM AMPKα2 results in phosphorylation (on site Ser-221) and subsequent inactivation of acetyl-CoA carboxylase (ACC) (21, 29). This, in turn, possibly via a decrease in malonyl CoA concentration (9, 21, 34), leads to the activation of SkM carnitine palmitoyltransferase-1 (26), which enhances the transfer of long-chain FA across the mitochondrial membrane for subsequent intramitochondrial β-oxidation and ATP generation (26). Thus the site-specific phosphorylation of SkM ACC acts as a reporter for in vivo-activated AMPK (both by covalent and allosteric mechanisms) (41) and, potentially, for intracellular FA oxidation (21, 26, 29).

In hyperinsulinemic, insulin-resistant Type 2 diabetes, exercise-stimulated SkM GLUT-4-mediated glucose uptake is thought to be normal (22, 25), despite the defects seen in insulin-stimulated GLUT-4-mediated glucose transport and processing (47). However, SkM glucose uptake is reduced in exercising insulin-deficient Type 1 diabetes (30, 32). Exercise-induced glucose uptake is also reduced in the insulin-deficient pancreatectomized diabetic dog (39) but not in the alloxan-induced hyperglycemic-hypoinsulinemic (40) or basal insulin-replaced pancreatectomized (39) diabetic dog models. Because of the potential favorable impact of exercise-stimulated AMPK activity on SkM GLUT-4-mediated glucose uptake and FA metabolism, much interest has recently focused on the potential regulation of glucose and FA metabolism by AMPK in diabetes (41). In addition, the antidiabetic drugs metformin and rosiglitazone have been found to activate AMPK in diabetic dog models. Because of the potential favorable impact of exercise-stimulated AMPK activity on SkM GLUT-4-mediated glucose uptake and FA metabolism, much interest has recently focused on the potential regulation of glucose and FA metabolism by AMPK in diabetes (41). In addition, the antidiabetic drugs metformin and rosiglitazone have been found to activate AMPK in diabetic dog models. Because of the potential favorable impact of exercise-stimulated AMPK activity on SkM GLUT-4-mediated glucose uptake and FA metabolism, much interest has recently focused on the potential regulation of glucose and FA metabolism by AMPK in diabetes (41).

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The purpose of the present study was to investigate in vivo glucose uptake, activation of SkM AMPK, and phosphorylation of ACC during moderately intense [60–70% of maximal oxygen uptake (VO2 max)] exercise in dogs before low-dose (35 mg/kg) alloxan-induced diabetes (prealloxan) and after 4–5 wk of low-dose insulin-treated diabetes. We hypothesized that, in the metabolically compromised, suboptimally controlled diabetic state, the exercise-induced activation of the SkM AMPK-ACC cascade pathway would be more intense than that seen in the matched prealloxan state, thereby leading to a greater stimulation of AMPK activity and increased phosphorylation of ACC in the diabetic dogs. We also examined whether reducing and “normalizing” the hyperglycemia in the diabetic dogs with the noninsulin-mediated glycosuric agent phlorizin (45) modified the basal and exercise-stimulated SkM AMPK activity and phosphorylation of ACC. We report that basal SkM AMPKα1 and α2 activities and phosphorylation of ACC are raised in the hypoinsulinemic diabetic state, both in the presence and absence of hyperglycemia. With exercise, the activation of both AMPK isoforms was blunted, but the stimulation of ACC phosphorylation was normal in both diabetic treatment groups. However, exercise-stimulated SkM glucose uptake was significantly reduced in the phlorizin-induced “normoglycemic” diabetic state only.

MATERIALS AND METHODS

Animals. Studies were carried out on six male dogs of mixed breed (18–26 kg body wt), with the permission of the Experimental Medical and Surgical Research Ethics Committee at St. Vincent’s Hospital Melbourne. At least 14 days before the first study, each dog had an arteriovenous shunt fashioned in the saphenous vein of the foreleg for arterialized blood sampling and a jugular vein catheterized with radiopaque silicone rubber tubing connected to a subcutaneously fastened stainless steel port (Port-A-Cath, Pharmacia Deltec, St. Paul, MN) located behind the dog’s neck for infusion of solutions (5, 6). Exercise, training, and health monitoring of dogs was as previously described (5, 6). The daily diet for the prealloxan dogs consisted of ~600 g of Pedigree Pal casserole (Uncle Ben’s of Australia, Wodonga, Australia) (7% protein, 4% fat, 8% carbohydrate), ~500 g of Meatybits dry food (Uncle Ben’s of Australia) (23% protein, 13% fat, 48% carbohydrate), and ~50 g of red meat (48% protein, 8% fat, 6% carbohydrate). Therefore, the total diet consisted of ~2,800 calories/day (28% energy from protein, 30% from fat, 42% from carbohydrate). Dogs were acclimated to the treadmill stepwise exercise program for at least 2 wk before the first study. After the prealloxan exercise study, dogs were rendered diabetic (after a 24-h fast) by intravenous (iv) injection of alloxan monohydrate (35 mg/kg) (Sigma Chemical, St. Louis, MO). Within 24 h of the alloxan injection, the dogs had a fasting blood glucose (BG) concentration of >10 mM. Within 2–3 days, the diabetic dogs began receiving twice daily subcutaneous injections of low-dose insulin (human Monotard, Novo Nordisk, Sydney, Australia) with their food for 4–5 wk, aimed at producing chronic hyperglycemia (target premeal BG range of 15–20 mM). The diabetic dogs required (on average) ~40% more dog chow per day to maintain body weight (prealloxan: 22.1 ± 1.2 kg vs. diabetic: 21.3 ± 1.2 kg; P = not significant), thereby compensating for the urinary loss of calories due to glycosuria. The diabetic dogs were tested twice daily for premeal BG concentrations at 8:00 to 9:00 AM (14.6 ± 0.4 mM) and 5:00 to 6:00 PM (18.3 ± 0.3 mM) by using a portable BG meter (Companion II, Medisense-Abbot, Melbourne, Australia) and required 10 ± 1 units of Monotard per day. Therefore, these hyperglycemic and hypoinsulinemic alloxan-diabetic dogs represent a model of chronic (30 ± 2 days) suboptimally controlled diabetes.

Experiments. Dogs were fasted for at least 15 h before each study and had free access to water at all times. Before fasting, the diabetic dogs received a 20–30% lower dose of Monotard insulin with their usual food ration to reduce the risk of insulin-induced nocturnal fasting hypoglycemia. On the morning of each study, an 18-gauge blood sampling catheter was inserted in the dog’s foreleg arteriovenous fistula, and a 19-gauge infusion set was inserted in the subcutaneous venous access port connected to the jugular vein (5,
AMP-ACTIVATED PROTEIN KINASE ACTIVITY IN DIABETIC DOGS

Collection of blood, urine, and SkM biopsy samples. Throughout all experiments, regular blood samples were collected 10–15 min apart during the preexercise periods and every 5 min during the exercise test, placed into tubes containing appropriate anticoagulants and preservatives, and centrifuged at 4°C within 2 h, and the separated plasma was stored frozen at −20°C until assayed for plasma glucose, total insulin, FFA, lactate, [3-3H]glucose, and tritiated water, as previously described (5). At the commencement and completion of the DHG and DNG periods, and at the completion of exercise in the diabetic studies, the bladder was emptied by using a urinary catheter coated with 1% lignocaine gel. The total volume of urine produced throughout each period was measured, and the urine was analyzed for both glucose concentration and specific activity of [3-3H]glucose to precisely quantify urinary glucose and [3-3H]glucose loss throughout the basal and exercise periods (12). Furthermore, within 2–4 min of the completion of all exercise tests, SkM biopsy samples were taken from the thigh (vastus lateralis) after rapid induction of anaesthesia with a 4.2 mg/kg iv bolus and 1.8 mg/kg titration of Diprivan (Propofol; 2,6-diisopropylphenol, Abbott). Each biopsy sample was immediately frozen in liquid nitrogen within 7 s of biopsy and stored at −70°C until assayed (6, 7). In addition, SkM biopsies were taken from each dog on separate days after a 180-min normal saline infusion in the fasting prealloxan and hyperglycemic diabetic states, and after a 330-min phlorizin infusion in the diabetic state, with the opposite back leg used for each subsequent muscle biopsy procedure. SkM biopsy samples were analyzed for AMPKα1 and -α2 activities, and site-specific phosphorylation of AMPKα1 and -α2, and ACC.

Laboratory analyses and calculations. Plasma levels of glucose, total insulin, FFA, and lactate, and specific activities of [3-3H]glucose in plasma and urine samples were measured as previously described (5, 6). The rate of total glucose disposal (Rdtotal) during the 150-min preexercise periods were determined from plasma [3-3H]glucose-specific activities obtained at steady state (5), as defined by Høther-Nielsen et al. (20). During the exercise period, Rdtotal was calculated from [3-3H]glucose data by using the non-steady-state Steele equation (11, 12, 34), assuming a glucose pool size of 0.65 and a glucose volume of distribution of 200 ml/kg. Any errors in the calculation of the rate of appearance and disappearance of glucose (Rdtotal) with the use of these assumptions are minimized by the regularity of sampling (5 min) and the lack of exercise-induced changes in plasma glucose levels and [3-3H]glucose-specific activities (due to the increasing [3-3H]glucose infusion rates). Rdtotal in the diabetic studies was corrected for urinary glucose loss to reflect the actual rate of glucose disposal by tissues (Rdissue). SkM glycogen content was determined by using a fluorometric enzymatic assay, as previously described from our laboratory (7).

AMPK activity assay. Approximately 100–150 mg of each frozen muscle biopsy sample was homogenized in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride), as previously detailed (3, 36). The homogenates were incubated with the AMPKα1 or -α2 antibody-bound A Sepharose beads for 2 h at 4°C. Immuno complexes were washed with PBS plus 2% Triton X-100 and then suspended in 50 mM Tris-HCl buffer (pH 7.5) for the AMPK activity assay (4). Briefly, a 40-µl reaction comprising 50 mM HEPES, pH 7.5, 10 mM MgCl2, 0.5% glycerol, 0.005% Triton X-100 with 1 mM DTT, 0.25 mM ATP with
AMP-Activated Protein Kinase Activity in Diabetic Dogs

[γ-32P]ATP (500 cpm/μmol), and 100 μM rat ACC (73–87)A77 (SAMS) peptide. After incubation for 6–7 min at 30°C, the reactions were stopped by withdrawing 30-μl aliquots and applying them to P81 papers. The AMPK activities in the immune complexes were measured in either the presence or absence of 200 μM AMP. The polyclonal antibodies to AMPKα1 and -α2 were raised to nonconserved regions of the AMPK isoforms α1 (373–390 CARHTLDELNPQKESKHQG) and α2 (490–516 CSAAGLHRPRSSDVSTAEHNLSSG). There is no cross reactivity between the polyclonal antibodies (2). Activities were calculated as pmol of phosphate incorporated into the SAMS peptide per minute per milligram of total protein subjected to immunoprecipitation.

**ACC phosphorylation assay.** Muscle homogenates were incubated with immobilized monomeric Avidin agarose beads to affinity purify ACC. The bound ACC fraction was subjected to SDS-PAGE, and site-specific phosphorylated ACC was detected by immunoblotting with anti- phospho-ACCα-Ser79 (Amersham Biosciences). All data are expressed in quantitative densitometric arbitrary units (3, 36).

**Statistical analysis.** Data are presented as means ± SE. One-way analysis of variance was used for repeated measures, with differences within and/or between groups determined by using Wilcoxon’s matched-pairs signed-rank test. Correlation analyses were performed by using the Spearman rank correlation coefficient (r values).

**RESULTS**

After 4–5 wk of low-dose insulin-treated diabetic therapy, the fasting diabetic dogs exhibited hyperglycemia and modest hypoinsulinemia compared with the corresponding prealloxan preexercise period (Table 1). During the 30-min treadmill exercise test (60–70% \( \text{VO}_{2\text{max}} \)), plasma glucose and insulin levels did not change in the prealloxan or hyperglycemic diabetic states (Table 1). In addition, the rise in plasma FFA (~40%) and lactate (~180%) with exercise was similar in the prealloxan and hyperglycemic diabetic dogs. Notably, despite the fasting hypoinsulinemia, both pre-exercise \( \text{Rd}_{\text{tissue}} \) and increment in \( \text{Rd}_{\text{tissue}} \) with exercise (~150–170%) were also similar in the prealloxan and hyperglycemic diabetic states (Table 1).

In contrast, during the 300-min phlorizin infusion in the diabetic dogs, the preexercise plasma glucose fell significantly (by ~10 mM), and the magnitude of the fall in plasma insulin levels (~3 mU/l) was similar to that seen when the same hyperglycemic diabetic dogs were fasted for 5 h (data not shown). With exercise, there was no change in either plasma glucose or insulin levels in the phlorizin-infused diabetic dogs (Table 1). Although the preexercise plasma FFA concentration in the phlorizin-infused diabetic dogs was significantly higher than that obtained in the prealloxan and hyperglycemic diabetic states, the absolute rise in FFA with exercise was similar to that found in the prealloxan and hyperglycemic diabetic states. Preexercise plasma lactate was unchanged during the phlorizin infusion in the diabetic dogs, but the increment in lactate with exercise was significantly greater than that observed in the other two states. In contrast, both the absolute \( \text{Rd}_{\text{tissue}} \) and increment in \( \text{Rd}_{\text{tissue}} \) with exercise were significantly blunted (by ~30–50%) in the phlorizin-treated normoglycemic diabetic dogs compared with the prealloxan and hyperglycemic diabetic states (Table 1). Importantly, there was a strong positive correlation between preexercise plasma glucose concentration and the increment in \( \text{Rd}_{\text{tissue}} \) with exercise for the two diabetic states (\( r = 0.811, P = 0.001 \)).

**Activity of AMPKα1 and -α2 isoforms: responses to exercise and diabetes.** In the prealloxan state, AMPKα1 activity (in the presence of AMP) rose significantly with exercise (by <15%) (Fig. 1A). In both the hyperglycemic and phlorizin-induced normoglycemic diabetic states, the preexercise AMPKα1 activities were significantly raised (by ~60 and ~125%, respectively) compared with the corresponding prealloxan state, but did not rise with exercise. However, the AMPKα1 activities both before and during exercise in the hyperglycemic and phlorizin-infused diabetic states were greater than those observed in the prealloxan group with exercise (Fig. 1A).

Although there was a ~70% rise in the mean activity of AMPKα2 with exercise (in the presence of AMP) in the prealloxan state, this value did not reach signifi-

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Table 1. Plasma levels of substrates and hormones and the rates of glucose disposal into peripheral tissues before and after 30-min treadmill exercise at 60–70% \( \text{VO}_{2\text{max}} \) in the same 6 dogs in the prealloxan state and after 4–5 wk of suboptimally controlled diabetes in the absence (hyperglycemia) and presence of phlorizin-induced normoglycemia

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Prealloxan</th>
<th>Hyperglycemia</th>
<th>+ Phlorizin infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preexercise</td>
<td>Exercise</td>
<td>ΔExercise</td>
</tr>
<tr>
<td>Glucose, mM</td>
<td>5.1±0.1</td>
<td>5.0±0.1</td>
<td>−0.1±0.1</td>
</tr>
<tr>
<td>Insulin, mU/l</td>
<td>9.2±1.4</td>
<td>10.9±2.6</td>
<td>1.7±1.7</td>
</tr>
<tr>
<td>FFA, mM</td>
<td>0.87±0.07</td>
<td>1.24±0.09*</td>
<td>0.36±0.13</td>
</tr>
<tr>
<td>Lactate, mM</td>
<td>0.7±0.1</td>
<td>1.9±0.8*</td>
<td>1.3±0.6</td>
</tr>
<tr>
<td>( \text{Rd}_{\text{tissue}} ) ( \mu\text{molkg}^{-1}\text{min}^{-1} )</td>
<td>11.9±0.6</td>
<td>32.3±1.4*</td>
<td>20.4±1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. FFA, free fatty acid; \( \text{Rd}_{\text{tissue}} \), rate of glucose disposal into peripheral tissues; Δexercise, response induced by exercise; \( \text{VO}_{2\text{max}} \), maximal oxygen consumption. *\( P < 0.05 \) vs. corresponding preexercise value. †\( P < 0.05 \) vs. corresponding prealloxan value. ‡\( P < 0.05 \) vs. corresponding hyperglycemic diabetic value.
response prealloxan state but did not rise significantly. Exercise AMPK activities were significantly raised (by ∼70% and ∼90%) in both the hyperglycemic and phlorizin-induced normoglycemic diabetic states, the preexercise AMPKα activities were significantly raised (by ∼70 and ∼100% respectively) compared with the corresponding prealloxan state but did not rise significantly with exercise. Moreover, the AMPKα activities both before and during exercise in the hyperglycemic and phlorizin-infused diabetic states were not different from the value observed in the prealloxan group with exercise (Fig. 1B). When the three treatment states were combined, significant negative correlations were found between both preexercise AMPKα1 and -α2 activities vs. Rd_{issue} during exercise (r = -0.585, P = 0.011; and r = -0.569, P = 0.014, respectively).

Site-specific phosphorylation of ACC: response to exercise and diabetes. Site-specific phosphorylation of ACC increased markedly with exercise (by ∼270%) in the prealloxan dogs (Fig. 2). In the hyperglycemic and phlorizin-induced normoglycemic diabetic states, the preexercise phosphorylation of ACC was elevated (by ∼80 and ∼70%, respectively) compared with the corresponding prealloxan state, but statistical significance was only reached in the former group. With exercise, the phosphorylation of ACC increased by ∼110 and ∼90%, respectively, in the hyperglycemic and phlorizin-infused diabetic states, but again statistical significance was only reached in the former group. There was no statistical difference in either the absolute value or increment in the phosphorylation of ACC with exercise between the three groups (Fig. 2). No association was found between preexercise ACC phosphorylation and Rd_{issue} during exercise.

Finally, preexercise SkM glycogen content was not different between the three groups (C: 273 ± 23 vs. DHG: 270 ± 31 vs. DNG: 288 ± 19 vs. mmol/kg dry weight muscle). Preexercise SkM glycogen content did not correlate with AMPKα1 or -α2 activities either basally or during exercise but did negatively correlate with both the absolute and incremental ACC phosphorylation during exercise for the combined groups (r = -0.695, P = 0.001; and r = -0.608, P = 0.007, respectively) and the two diabetic states (r = -0.783, P = 0.003; and r = -0.650, P = 0.022, respectively).

DISCUSSION

The glucose and FA metabolic responses to moderately severe exercise in decompensated hyperglycemic and hypoinsulinemic diabetes are complex and depend on the metabolic state of the individual and the duration and intensity of the exercise (30, 33, 37, 39). To date, there is no data on the activation of AMPK and the importance of its metabolic role in decompensated hypoinsulinemic diabetes. This is of particular interest, given the potential role for AMPK activation on the stimulation of glucose transport (1, 18, 23) and FA metabolism (26, 27) in the diabetic state, both at rest and during exercise.

The key findings in the present study were, first, that the basal (preexercise) activities of SkM AMPKα1 and -α2 isofoms were significantly raised in both the hyperglycemic and phlorizin-induced normoglycemic diabetic states, the preexercise AMPKα2 activities were significantly raised (by ∼70 and ∼100% respectively) compared with the corresponding prealloxan state but did not rise significantly with exercise. Moreover, the AMPKα2 activities both before and during exercise in the hyperglycemic and phlorizin-infused diabetic states were not different from the value observed in the prealloxan group with exercise (Fig. 1B). When the three treatment states were combined, significant negative correlations were found between both preexercise AMPKα1 and -α2 activities vs. Rd_{issue} during exercise (r = -0.585, P = 0.011; and r = -0.569, P = 0.014, respectively).

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The key findings in the present study were, first, that the basal (preexercise) activities of SkM AMPKα1 and -α2 isofoms were significantly raised in both the hyperglycemic and phlorizin-induced normoglycemic diabetic states, the preexercise AMPKα2 activities were significantly raised (by ∼70 and ∼100% respectively) compared with the corresponding prealloxan state but did not rise significantly with exercise. Moreover, the AMPKα2 activities both before and during exercise in the hyperglycemic and phlorizin-infused diabetic states were not different from the value observed in the prealloxan group with exercise (Fig. 1B). When the three treatment states were combined, significant negative correlations were found between both preexercise AMPKα1 and -α2 activities vs. Rd_{issue} during exercise (r = -0.585, P = 0.011; and r = -0.569, P = 0.014, respectively).

Site-specific phosphorylation of ACC: response to exercise and diabetes. Site-specific phosphorylation of ACC increased markedly with exercise (by ∼270%) in the prealloxan dogs (Fig. 2). In the hyperglycemic and phlorizin-induced normoglycemic diabetic states, the preexercise phosphorylation of ACC was elevated (by ∼80 and ∼70%, respectively) compared with the corresponding prealloxan state, but statistical significance was only reached in the former group. With exercise, the phosphorylation of ACC increased by ∼110 and ∼90%, respectively, in the hyperglycemic and phlorizin-infused diabetic states, but again statistical significance was only reached in the former group. There was no statistical difference in either the absolute value or increment in the phosphorylation of ACC with exercise between the three groups (Fig. 2). No association was found between preexercise ACC phosphorylation and Rd_{issue} during exercise.

Finally, preexercise SkM glycogen content was not different between the three groups (C: 273 ± 23 vs. DHG: 270 ± 31 vs. DNG: 288 ± 19 vs. mmol/kg dry weight muscle). Preexercise SkM glycogen content did not correlate with AMPKα1 or -α2 activities either basally or during exercise but did negatively correlate with both the absolute and incremental ACC phosphorylation during exercise for the combined groups (r = -0.695, P = 0.001; and r = -0.608, P = 0.007, respectively) and the two diabetic states (r = -0.783, P = 0.003; and r = -0.650, P = 0.022, respectively).
and α2 isoforms (in the presence of AMP) and site-specific phosphorylation of ACC were elevated in the hypoinsulinemic suboptimally controlled diabetic dogs compared with the prealloxan state. Moreover, during the period of relative hypoglycemia produced by acute phlorizin-induced normoglycemia, preexercise activities of AMPKα1 and α2 activities and the phosphorylation of ACC were similar to those seen in the hyperglycemic diabetic state. Second, in both the hyperglycemic and normoglycemic diabetic states, AMPKα1 and α2 activities failed to significantly rise further with exercise, but the increases in phosphorylation of ACC with exercise were similar to that seen in the prealloxan state. Thus it appears that the basal SkM AMPK-ACC phosphorylation cascade is chronically elevated by decompensated hypoinsulinemic diabetes and that this reflects substantial stimulation of AMPK by upstream kinases (AMPK kinase) (21, 41). We interpret the exercise-induced elevation of ACC phosphorylation in diabetes as indicating that the AMPK-ACC phosphorylation cascade is very tightly coupled. The measurement of AMPK isoform activities in SkM biopsy extracts reflects the extent of activation of the AMPK due to Thr-172 phosphorylation in the activation loop (21, 36, 41, 44). However, the extent of AMPK allosteric activation in vivo due to AMP is not measured in the in vitro assay. Therefore, our finding of increased phosphorylation of ACC in vivo with exercise in the diabetic states may be due to AMP-mediated allosteric activation of AMPK without an apparent increase in AMPK activity (21, 41). If this phenomenon was not taken into account, the apparent lack of correlation between AMPK activity measured in vitro and ACC phosphorylation measured in vivo in the present study may falsely be interpreted to mean that the regulation of ACC was being mediated by one or more other protein kinases.

In addition, the activity of SkM AMPKα1 at rest in our prealloxan dogs was approximately sixfold higher than the levels seen in both rats (17, 18, 21, 41) and humans (3, 14, 28, 36, 44). Furthermore, the stimulation of AMPKα1 activity in rats (17) and humans (3) occurs only during more extreme exercise or muscle contraction. Whether the increased levels of AMPKα1 in dogs indicates that SkM AMPKα1 plays a greater physiological role than AMPKα2 in the inhibition of ACC and stimulation of glucose transport is unknown.

It is important to speculate what metabolic impact the elevated preexercise constitutive AMPKα1 and α2 activities may have in our decompensated diabetic dogs. For example, preexercise Rd\textsubscript{tissue}, when corrected for urinary loss of glucose, was normal in both the hyperglycemic and phlorizin-induced normoglycemic hypoinsulinemic states. It is possible that the elevation of both constitutive AMPKα1 and α2 activities was required to maintain normal preexercise Rd\textsubscript{tissue} in the diabetic states. However, during the added metabolic stress of exercise in both diabetic states, there was no further significant activation of AMPKα1 and α2 isoforms, and exercise-induced Rd\textsubscript{tissue} (a measure of glucose uptake from plasma by SkM) was significantly reduced in the phlorizin-infused diabetic dogs. It is recognized that the increase in glucose uptake by 5-aminoimidazole-4-carboxamide riboside (AICAR) in perfused rat hindlimb muscles is due to AMPK-activated translocation of GLUT-4 to the cell membranes (23). Thus one could postulate that the raised preexercise AMPKα1 and α2 activities in the diabetic dogs may play a permissive role in the metabolic stress response to exercise, but this cannot be determined from the present data. However, the hyperglycemic pump is likely to be a significant factor in maintaining the normal increment in Rd\textsubscript{tissue} with exercise in hyperglycemic diabetes (12), given that we found a strong positive correlation between preexercise plasma glucose concentration and the increment in Rd\textsubscript{tissue} with exercise in the two diabetic states.

Despite our finding of no further increases in AMPKα1 and α2 activities in the diabetic dogs with exercise, the phosphorylation (inhibition) of ACC increased further with exercise in both diabetic states, and in the setting of normoglycemia this increase was associated with heightened circulating FFA and lactate levels.

The mechanism(s) responsible for the chronically elevated preexercise AMPKα1 and α2 activities and site-specific phosphorylation of ACC in our suboptimally controlled hyperglycemic and phlorizin-induced relatively hypoglycemic diabetic dogs warrants further consideration. In a previous study employing our alloxan-induced suboptimally controlled (hyperglycemic) diabetic dog model, we found that fasting SkM glycogen content was significantly reduced and that insulin-stimulated glycogen synthase (GS) fractional activity was severely impaired compared with the fasting prealloxan state (MJ Christopher, C Rantzau, and FP Alford, unpublished observations). It is also known that fasting SkM glycogen content is already lower in healthy dogs than in humans (7, 36). Therefore, the present findings of raised preexercise AMPKα1 and α2 activities and ACC phosphorylation, and negative correlations between preexercise SkM glycogen content and the absolute and increment in ACC phosphorylation with exercise for the two diabetic states, support the observations of Wojtaszewski et al. (43) that SkM AMPKα1 and α2 activities and ACC phosphorylation, both at rest and during exercise, are inversely related to the preexisting SkM glycogen content. Furthermore, in recent studies employing cultured human myoblasts (15) and perfused rat hindlimb muscle (42), strong negative correlations were found between GS fractional activity and AMPK activity during both acute AICAR treatment and glucose starvation (15), and during acute AICAR perfusion in the presence of lowered muscle glycogen content (42). In addition, with the use of gel-shift analysis, it has been shown that AICAR treatment leads to the phosphorylation of GS in perfused SkM, suggesting that AMPK may phosphorylate and inactivate GS in vivo (42).

Recently, it has been suggested that the hormonal response, particularly catecholamines, may be important in the metabolic response to the activation of the
AMPK-ACC phosphorylation cascade (38, 43). Our laboratory has previously observed normal plasma epinephrine and norepinephrine levels in alloxan-diabetic dogs (35). Moreover, after induction of normoglycemia by phlorizin infusion in hypoinsulinemic diabetic dogs, only minor changes in epinephrine and norepinephrine occurred (24). Therefore, it is unlikely that catecholamines play a major role in the regulation of the AMPK-activated metabolic responses in alloxan-induced diabetes or subsequent acute phlorizin-induced relative hypoglycemia.

In conclusion, preexercise SkM AMPKα1 and α2 activities and ACC phosphorylation are chronically elevated in suboptimally controlled hypoinsulinemic diabetes. This suggests an important role for this insulin-independent signaling pathway in ensuring an ongoing basal supply of glucose and FA metabolism in diabetes. However, with the increasing metabolic stress induced by moderate exercise, AMPKα1 and α2 activities failed to further increase in either the hyperglycemic or phlorizin-infused normoglycemic diabetic dogs, and there was an ~50% reduction in the increment in Rδtissue with exercise in the normoglycemic diabetic state. Whether the raised preexercise AMPKα1 and α2 activities play a permissive role with the mass action of glucose in normalizing the metabolic stress response to exercise in the hyperglycemic diabetic state remains to be determined. Furthermore, it would be interesting to examine whether the AMPK-ACC signaling cascade is also altered in metabolically uncompensated hypoinsulinemic human diabetes.

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DISCLOSURES

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