Extracellular Cyclic AMP and Adenosine Appearance in Adipose Tissue of *Sus scrofa:* Effects of Exercise

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Cyclic AMP (cAMP) appears extracellularly in a variety of tissues including brain, liver, and kidney; whether it appears in adipose tissue and responds to physiological perturbation is unknown. The purpose of this study was to examine adipose tissue extracellular cAMP appearance and metabolism *in situ* and *in vitro* in physiologically challenged animals. Littermate swine were either sedentary or exercise trained on a treadmill for 3 months and subjected to acute exercise on experiment day. *In situ,* microdialysis probes in subcutaneous back fat were perfused before, during, and after animals performed 20 mins of acute exercise, and dialysate was analyzed for cAMP and adenosine. *In vitro,* isolated adipocytes were hormonally stimulated to provoke cAMP synthesis and efflux, and plasma membrane phosphodiesterase and 5′-nucleotidase activities were measured. Extracellular cAMP and adenosine levels in adipose tissue of sedentary swine averaged 5.2 ± 1.7 and 863 ± 278 n*M*, respectively. Exercise training tended to increase extracellular cAMP (11.3 ± 1.7 n*M*) and reduce extracellular adenosine (438 ± 303 n*M*), although neither change was statistically significant. Acute exercise caused a significant 3-fold and 16-fold increase in extracellular cAMP and adenosine, respectively, compared to rest. These changes occurred despite a 2- to 3-fold increase in adipose tissue blood flow during acute exercise. *In vitro,* cAMP efflux from exercise-trained swine was 42% greater than that from adipocytes of sedentary swine, yet adipocyte plasma membranes from exercise-trained and sedentary swine did not differ in maximal phosphodiesterase and 5′-nucleotidase activities. We conclude that cAMP appears extracellularly in swine adipose tissue and that the levels of extracellular cAMP and adenosine in intact swine adipose tissue are influenced by both acute and chronic exercise. The subsequent impact of the changes in these biochemicals on local cellular metabolism and growth remains to be determined.

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Cyclic AMP (cAMP) appears extracellularly in a variety of tissues in the body including brain, liver, and kidney (1, 2, 3). Work from our laboratory has shown that in isolated swine adipocytes, cAMP is transported to the extracellular medium in a time-dependent fashion by an energy-dependent transporter that is sensitive to probenecid, an anion transport blocker (4). However, the presence of cAMP in extracellular fluid of intact adipose tissue has not been investigated.

One fate of extracellular cAMP in kidney, brain, and liver is metabolism to AMP and adenosine (3). When the kidney is perfused with increasing levels of cAMP, the appearance of AMP, adenosine, and inosine is increased. (3, 5). Likewise, when cultured astrocytes are stimulated with isoproterenol, extracellular cAMP and adenosine appearance increases (6).

The fate of extracellular cAMP in adipose tissue is unknown. Previous work from our laboratory has documented that isolated adipocyte plasma membranes are capable of metabolizing cAMP to AMP by phosphodiesterase (PDE) and AMP to adenosine by 5′-nucleotidase (5′-NT) (7). If this capacity exists in intact adipose tissue, extracellular metabolism of cAMP to adenosine would have the potential to provide the adipocyte with a negative feedback mechanism to regulate lipolysis: extracellular adenosine would bind to the A_1 adenosine receptor, which inhibits adenylyl cyclase activity, reducing intracellular cAMP levels, and inhibiting lipolysis (8). This "extracellular cAMP–adenosine pathway" was first discovered in the kidney by Jackson in 1991 (9).

Acute and chronic aerobic exercise promote lipolysis, which makes fatty acids available as fuel for working muscle.

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Previous work from our laboratory has shown that adenosine inhibition of lipolysis is blunted in adipocytes isolated from exercise-trained swine compared to sedentary controls (10) because of a reduction in adenosine A_1 receptor number. This adaptation would facilitate lipolysis. In addition, extracellular adenosine could influence growth of neighboring cells: adenosine has been shown to stimulate growth of endothelial cells (11), while it inhibits vascular smooth muscle and cardiac fibroblast cell growth (12, 13). Thus, exercise-induced changes in extracellular adenosine levels have the potential to influence local adipose tissue metabolism. However, the ability of adipose tissue to generate extracellular adenosine from extracellular cAMP is unknown.

This study examined for the appearance of extracellular cAMP in adipose tissue and tested the hypothesis that adipose tissue extracellular cAMP and adenosine appearance *in situ* and *in vitro* are increased by acute and chronic exercise, using the miniature swine animal model. Swine are an excellent model for studying the metabolic effects of exercise because their size, physiology, and metabolism are similar to humans (14, 15). Our results demonstrate that (i) cAMP and adenosine appear extracellularly in intact adipose tissue and their appearance is enhanced by an acute bout of exercise, (ii) exercise training enhances cAMP efflux *in vitro* and *in situ*, and (iii) exercise training does not enhance adenosine levels *in situ*, nor does it alter maximal velocity of plasma membrane–bound PDE and 5′-NT. Whether or not extracellular cAMP and adenosine play a role in optimizing the responsiveness of adipose tissue to acute and chronic exercise remains to be determined.

Materials and Methods

Animals. Thirty-eight swine (17 littermate pairs of female swine and 2 littermate pairs of male swine), aged 10 to 12 weeks, were used in this 3-month study. One littermate was assigned to the endurance-exercise group, while its sibling remained sedentary. Although adipose tissue from male and female swine are equally sensitive to the antilipolytic effects of adenosine *in vitro* (10), primarily female swine were used in this study because they demonstrate better compliance to the exercise regimen and they possess more subcutaneous adipose tissue than males.

Swine were housed four to six per 18-m^2 pen at the Burley-Demeritt swine facility in Lee, New Hampshire. All swine were fed CRL Big Pels-Pig Maintenance Ration twice daily (Blue Seal Feeds, Bow, NH). The ration is formulated to meet the nutritional needs of growing miniature swine and has a caloric density of 10.21 kJ/g. Swine had free access to water except during exercise sessions. Swine body weights were recorded at the beginning and end of the study. All procedures were approved by the University of New Hampshire Institutional Animal Care and Use Committee (IACUC 000202, 011105, 020301, and 012202).

Endurance Exercise. Swine assigned to the endurance-exercise group followed a 3-month training regimen described by Carey and Sidmore (10). This regimen gradually adapts the swine to run on motor-driven treadmills with rubberized belts so that by the end of 2 months, they run 45 mins/day, 5 days/week, at 9 km/hr under climate-controlled conditions. They continued to train for an additional month at this intensity.

Experimental Design. Seven pair of swine were used for *in vitro* experiments using intact adipocytes and adipocyte plasma membranes. For *in situ* experiments, microdialysis probes were implanted into adipose tissue of exercise-trained and sedentary overnight-fasted swine at the end of the 3-month training regimen. Microdialysate was collected and blood flow was measured while swine were at rest, during an acute exercise bout, and postexercise. Six pair of swine were used for *in situ* cAMP measurements, and another six pair of swine were used for *in situ* adenosine measurements.

***In Vitro* Experiments.** At the end of the 3-month training regimen, swine were fasted overnight and anesthetized with isoflurane. Adipose tissue was biopsied, and intact adipocytes were prepared. Experiments were conducted on freshly isolated intact adipocytes to measure cAMP efflux, and on the plasma membrane fraction of these adipocytes to measure PDE and 5′-NT activity.

Fat Biopsy and Adipocyte Isolation. Approximately 8 g of subcutaneous adipose tissue from the shoulder region was removed surgically and transported to the laboratory in warm saline. Tissue was minced into 1-mm^3 pieces, rinsed with warm saline, dissociated with collagenase, and used to isolate intact adipocytes as described previously (10).

Cyclic AMP Efflux Experiments. Cyclic AMP efflux was measured in 600-μl aliquots of intact adipocytes as described previously (4). Cells were incubated at 38°C in a shaking water bath for 30 mins in Krebs-Ringer bicarbonate buffer without or with either 10^{-4} *M* forskolin or 10^{-4} *M* epinephrine (both are cAMP efflux stimulators). Extracellular and total cAMP were measured via enzyme immunoassay (4). Intracellular cAMP was calculated by difference. Two 600-μl aliquots of isolated cells were fixed in osmium tetroxide (10 g/l) for 48 hrs, filtered, and resuspended for adipocyte sizing using computerized image analysis and counting via hemacytometer (10).

Plasma Membrane Experiments. A portion of the isolated adipocyte fraction was homogenized and used to prepare an enriched plasma membrane fraction, as described by Zacher and Carey (7). PDE was assayed with ^{3}H-cAMP substrate followed by ion-exchange chromatography as described by Thompson *et al.* (16). 5′-NT was assayed with ^{3}H-AMP substrate followed by ion-exchange chromatography, as described previously (7). Plasma membrane protein was assayed by the method of Lowry *et al.* (17).

***In Situ* Experiments.** Each swine was familiarized with resting in a Panepinto sling once for ~15 mins within 5 days of performing the microdialysis experiment. This

precaution reduces swine agitation caused by an abrupt change in routine. Likewise, sedentary swine were accustomed to exercise by placing them on the treadmill for 5 mins at 3–4 k/hr once within 3 days of performing an experiment. On the day of the experiment, microdialysate and blood flow measurements were made for three 20-min periods: before, during, and after acute exercise.

Microdialysis. On the day of the experiment, swine were placed in the sling and anesthetized with inhaled isoflurane (3% in medical grade oxygen) during the probe insertion. A CMA 20 microdialysis probe was inserted into subcutaneous adipose tissue in the scapula region of the shoulder at a 45 degree angle into a split guide cannula, to a depth of 0.5 in. (CMA, Winchester, MA). The probe was attached to a CMA 107 portable microdialysis pump. The split guide was removed, the probe and pump were taped in place on the swine's back, and the anesthesia was discontinued. The experiment began after a 45-min recovery period, during which the probe was perfused with Ringer solution at 2 μl/min and the dialysate was discarded. For the first experimental period, swine remained in the sling, the probe was perfused at 2 μl/min and all dialysate was collected. For the second period, swine were placed on the treadmill for acute exercise at 5 km/hr and all dialysate was collected. For the third period, swine were returned to the sling, and all dialysate was collected. Dialysate samples were stored at –80°C until being assayed for cAMP using a commercial enzyme immunoassay kit (acetylated protocol, Amersham Life Sciences, Piscataway, NJ) or for adenosine using a double antibody radioimmunoassay kit (Yamasa, Corp., Tokyo, Japan).

Cyclic AMP and Adenosine Recovery. The internal reference technique was used to measure recovery of cAMP and adenosine *in situ* (18) using 0.5 μCi/ml ^{3}H-cAMP and ^{3}H-adenosine, respectively. Results from three experiments were averaged to calculate cAMP and adenosine recovery before, during, and after acute exercise.

Adipose Tissue Blood Flow. The ^{133}xenon washout technique was used to measure local adipose tissue blood flow on the contralateral side from the probe insertion site, as described by Moher and Carey (19) before, during, and after acute exercise.

Statistical Analyses. *In situ* experiments were analyzed using a repeated measures analysis of variance (ANOVA) blocked by litter, testing for main effects of exercise training and acute exercise. *In vitro* experiments were analyzed using one-way ANOVA, blocked by litter, testing for main effects of exercise training and *in vitro* treatment, followed by pairwise comparison using Tukey's test. For both *in situ* and *in vitro* analyses, when main effects were significant without interaction, results are shown for each main effect. Enzyme activities were analyzed via Student's *t* test. Systat software (version 7.0.1) was used for all statistical analyses.

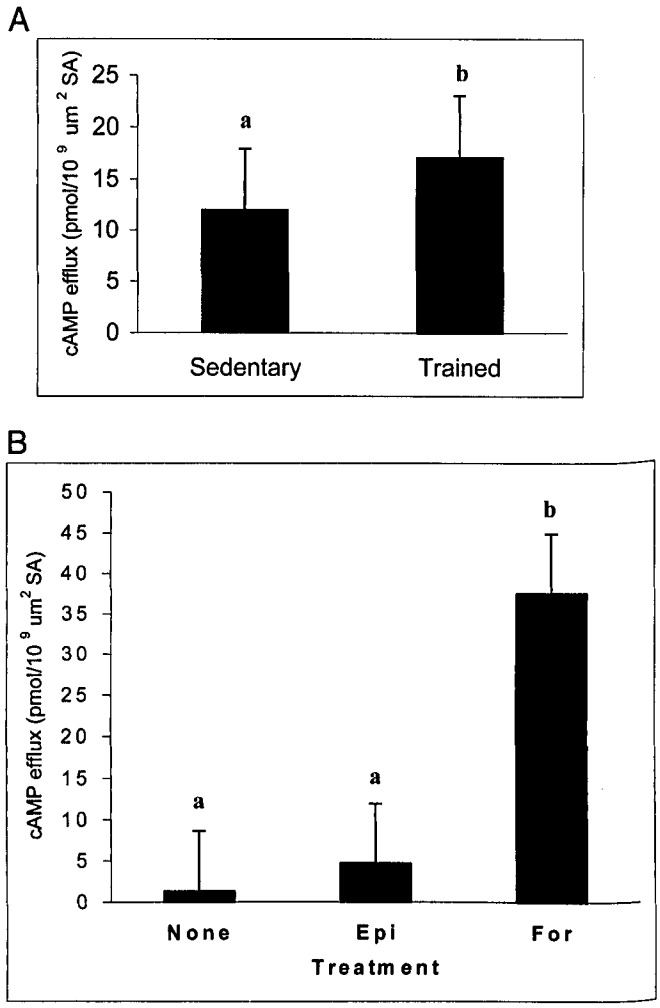

Figure 1. (A) Effect of exercise training on cAMP efflux from isolated swine adipocytes. Adipocytes were incubated for 30 mins; efflux is expressed per unit adipocyte surface area (SA). Values are means ± SEM averaged across three *in vitro* treatments (n = 18). (B) Effect of *in vitro* treatment on cAMP efflux from isolated swine adipocytes. Adipocytes were incubated for 30 mins with either no addition (None), 10^{-4} *M* epinephrine (Epi), or 10^{-4} *M* forskolin (For), and cAMP efflux was measured. Values are means ± SEM averaged across both chronic treatments (n = 12). For both figures, values with different superscripts are significantly different at $P < 0.05$.

Results

Swine Characteristics. Swine were between 5.0 and 5.7 months old and weighed from 42 to 59 kg on experiment day. No difference in body weight was found between sedentary and exercise-trained swine within an experiment. Adipocytes isolated from the exercise-trained swine tended to be smaller than adipocytes isolated from sedentary controls (100 vs. 104 μ*M*, respectively, P = 0.066).

In Vitro. The main effects of exercise training (Fig. 1A) and hormone treatment (Fig. 1B) on cAMP efflux from isolated adipocytes, with no interaction between treatments, are shown in Figure 1. Exercise training significantly increased cAMP efflux from isolated adipocytes across all treatments by 42%, expressed per unit adipocyte surface

Table 1. Adipocyte Plasma Membrane PDE and 5′NT Activities[a]

	Sedentary	Exercise trained
5′NT (pmol/min/mg protein)	34.5 ± 10.4	27.2 ± 3.3
+ 1 m*M* AMPCP[b]	0	0
PDE (pmol/min/mg protein)	90.1 ± 13.8	80.1 ± 5.6
+ 5 μ*M* cilostamide[c]	29.2 ± 7.3	24.2 ± 2.5

[a] Adipocytes were isolated from subcutaneous adipose tissue biopsies of sedentary and exercise-trained swine. Plasma membranes were prepared using differential centrifugation. Phosphodiesterase (PDE) and 5′-nucleotidase (5′NT) activities were assayed using 5 μg membrane protein. Values are mean ± SEM (n = 5).
[b] AMPCP = α,β-methylene adenosine 5′-diphosphate, an inhibitor of 5′-NT.
[c] Cilostamide inhibits microsomal PDE3B nearly 100% (7).

area ($P < 0.05$, Fig. 1A). Extracellular cAMP efflux from adipocytes was nearly zero with no treatment; addition of 100 μ*M* epinephrine did not significantly increase cAMP efflux, but addition of 100 μ*M* forskolin increased cAMP efflux 29-fold ($P < 0.05$) over no treatment (Fig. 1B). No difference was found in intracellular cAMP levels between exercise-trained and sedentary swine in the unstimulated, epinephrine-incubated, or forskolin-incubated conditions (data not shown).

No differences were found in maximal PDE or 5′-NT activities in adipocyte plasma membranes of exercise-trained versus sedentary controls, with or without inhibitors (Table 1). 5′-NT activity was completely inhibited by 1 m*M* α,β-methylene adenosine 5′-diphosphate (AMPCP), a known 5′-NT inhibitor. PDE activity was reduced to an average of 27 pmol/min/mg protein from 85 pmol/min/mg protein by 5 μ*M* cilostamide, which completely inhibits contaminating microsomal PDE activity from the plasma membrane fraction (7).

In Situ. Recovery of extracellular metabolites using the microdialysis technique is a function of perfusion rate, probe design, perfusate tonicity, and tissue temperature (20). Consistent with published reports for other metabolites under similar conditions, recovery of cAMP and adenosine from the microdialysis probes was low (21–23). Cyclic AMP recovery before, during, and after exercise averaged 4.9%, 6.9%, and 6.8%, respectively, while adenosine recovery before, during, and after exercise averaged 10.4%, 18%, and 18.4%, respectively. Extracellular concentrations of both metabolites were corrected for recovery.

The main effects of exercise training (Fig. 2A) and acute exercise (Fig. 2B) on extracellular cAMP levels, with no interaction between treatments, are shown in Figure 2. Exercise training tended to increase adipose tissue extracellular cAMP level (P = 0.13, Fig. 2A) compared to sedentary swine. An acute bout of exercise significantly increased extracellular cAMP levels 3-fold and this increase persisted into the postexercise period ($P < 0.05$, Fig. 2B). The during and postexercise increase in extracellular cAMP occurred despite an increase in adipose tissue blood flow during both periods ($P < 0.05$, Fig. 3). An increase in

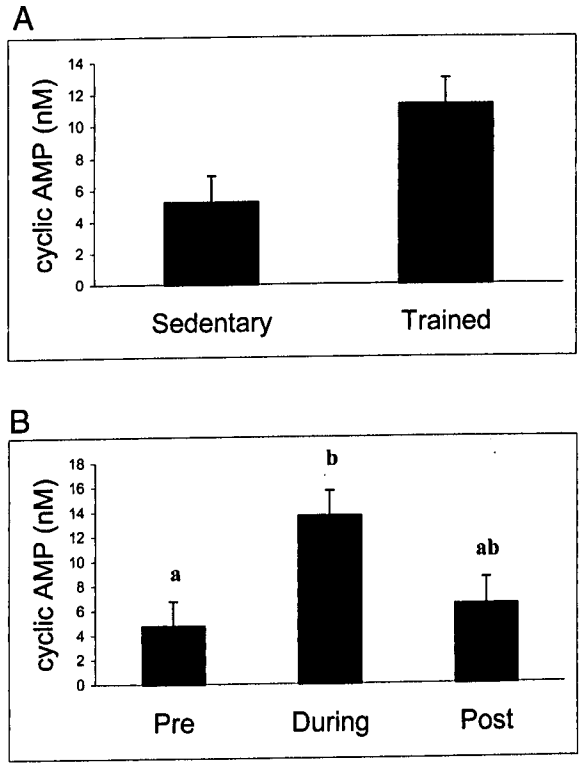

Figure 2. (A) Effect of exercise training on *in situ* cAMP appearance in microdialysate from adipose tissue. Values are means ± SEM averaged across the three 20-min acute treatments (n = 16). (B) Cyclic AMP level in adipose tissue microdialysate before, during, and after an acute bout of exercise. Values are means ± SEM averaged across both chronic treatments (n = 10–11). Values with different superscripts are significantly different at $P < 0.05$.

adipose tissue blood flow with acute exercise has been previously reported from this laboratory (19).

Although exercise training caused a 50% decrease in extracellular adenosine level, this was not significantly different from sedentary controls (Fig. 4A). However, acute exercise caused a significant increase in extracellular

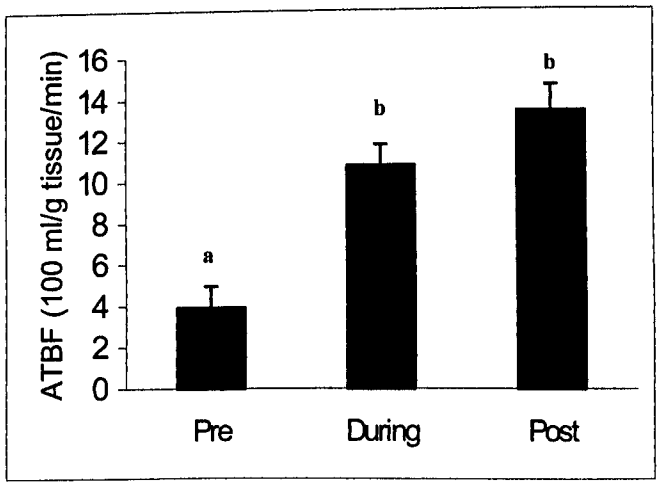

Figure 3. *In situ* adipose tissue blood flow before, during, and after acute exercise. Values are means ± SEM averaged across both chronic treatments for each of the 20-min periods (n = 18–22). Values with different superscripts are significantly different at $P < 0.05$.

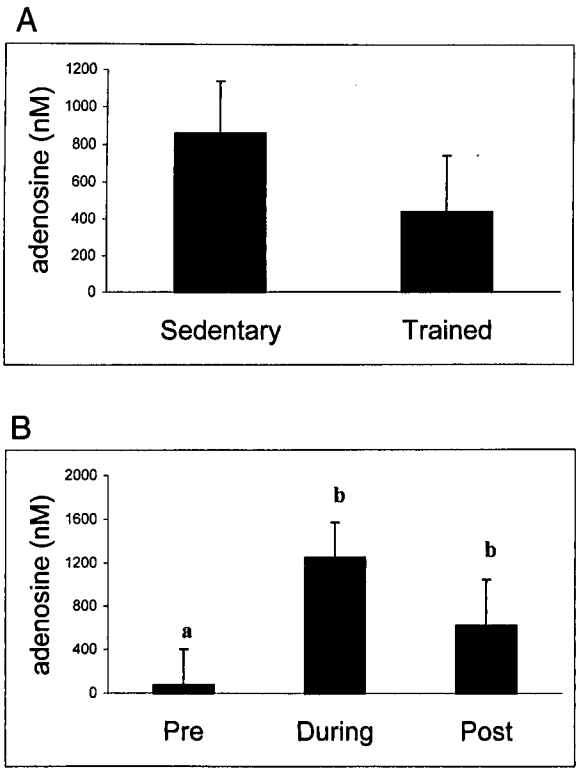

Figure 4. (A) Effect of exercise training on *in situ* adenosine appearance in microdialysate from adipose tissue. Values are means ± SEM averaged across the three 20-min acute treatments (n = 13–14). (B) *In situ* adenosine level in microdialysate from adipose tissue during the 20-min periods before, during, and after exercise. Values are means ± SEM averaged across both chronic treatments. Values with different superscripts are significantly different at $P < 0.05$.

adenosine that persisted into the postexercise period ($P < 0.05$, Fig. 4B), as was observed for cAMP.

Discussion

Our findings demonstrate that cAMP is present in extracellular fluid of swine adipose tissue *in situ*, and that its presence is increased by an acute bout of exercise. This *in situ* finding was mimicked *in vitro* upon β-adrenergic stimulation of isolated adipocytes, suggesting that one source of extracellular cAMP in adipose tissue is the adipocyte. Our lab has shown previously that isolated adipocytes can export cAMP in a time-dependent, probenecid-sensitive manner (4).

We measured the level of extracellular cAMP in adipose tissue, which ranged from an average of 3 n*M* at rest to 14 n*M* during acute exercise. The increase in cAMP level during acute exercise persisted despite an increase in adipose tissue blood flow, which would tend to increase the rate of cAMP removal from the extracellular fluid pool. Thus, the measured value may underestimate actual cAMP appearance in the extracellular pool. Regardless, these levels are strikingly similar to those reported for brain, but far less than that for liver. Microdialysis of the cerebral cortex of conscious rats demonstrated that basal levels of extracellular cAMP averaged 3 n*M*, and this value increased 3-fold to 7-fold upon stimulation with norepinephrine (24). Likewise, in another study, microdialysis of rat cerebral cortex yielded levels of ~2.5 n*M* if cAMP recovery is estimated at 10% (25). In contrast, when rat livers were perfused with 10 n*M* glucagon, cAMP levels reached as high as 1 μ*M* (26).

Once outside the cell, interstitial cAMP could be transferred to the circulation. Plasma cAMP, in concert with glucagon, is proposed to act upon the kidney to regulate glomerular filtration rate and increase sodium and phosphate excretion (1). The liver and kidneys are the primary organs that clear cAMP from the plasma into the urine at a rate averaging 210 nmol/hr. The lower levels of extracellular cAMP in adipose tissue, compared to liver, suggest that adipose tissue may not be a significant source of plasma cAMP, which is reported to be 50 n*M* in anesthetized rats (27) and between 15 and 50 n*M* in healthy humans (1). Indeed, Strange *et al.* demonstrated that inhibition of lipolysis with nicotinic acid did not reduce plasma cAMP levels in humans, nor did it reduce plasma cAMP levels in dogs infused with isoproterenol (28). However, given that human adipose tissue mass can vary widely—from 10% to more than 50% of body weight—it could be a significant contributor to the total extracellular cAMP pool.

Locally, extracellular cAMP may act directly on cells. Cyclic AMP binds to the plasma membrane of the renal cortex and bone marrow and may inhibit the sodium-phosphate co-transporter; receptors for cAMP have not been identified in adipose tissue (29). Alternatively, cAMP could be metabolized to adenosine. Human adipose tissue adenosine averages 128 n*M*, a level that could modulate lipolysis (30). Adipose tissue adenosine levels also have been shown to transiently rise with epinephrine infusion of swine (31), and a 5-min bout of exercise has been shown to increase extracellular adenosine in human muscle (32).

The mechanism by which acute exercise causes an increase in the extracellular cAMP level in adipose tissue is unknown. One possibility is that intracellular cAMP synthesis is stimulated by local and systemic exercise-induced factors such as epinephrine and norepinephrine. This could result in an increase in intracellular cAMP levels that approaches the cAMP threshold. Once the threshold is exceeded, cAMP could be transported out of the cell. Our results, however, do not demonstrate which cell type may be involved. The adipocyte has been shown to export cAMP, but many cell types are capable of this process (33). A second possibility is that adipose tissue extracellular fluid is merely mirroring an exercise-induced increase in plasma cAMP. This possibility suggests that the origin of extracellular adipose tissue cAMP is not the adipocyte, but another organ such as the liver. It also assumes that adipose tissue endothelial cells are capable of exporting cAMP, or that they become sufficiently leaky to permit cAMP to exit from the capillary lumen. However, the half-life of plasma adenosine is short, ranging from 1 to 10 secs. Whether or not plasma cAMP increases with exercise or can

be transported across endothelial cells into the interstitial fluid of adipose tissue remains to be determined.

Although the increase in extracellular cAMP with exercise training was observed both *in vitro* and *in situ*, the *in situ* findings may or may not reflect increased synthesis coupled with transport to the extracellular space. Other possibilities include a slower removal of cAMP due to a decrease in adipose tissue blood flow, a slower metabolism of cAMP due to allosteric inhibition of PDE (we measured maximal velocity only), or exercise-induced cell damage that released intracellular cAMP. It is striking, however, that the training-induced increase in cAMP efflux both *in vitro* and *in situ* did not lead to an increase in extracellular adenosine. In fact, adenosine levels tend to be lower in adipose tissue extracellular fluid of exercise-trained swine compared to sedentary swine. Again, we found no difference in maximal activities of ecto-PDE and 5′-NT to explain this finding, but this does not preclude the possibility that an allosteric mechanism is invoked that alters the affinity of PDE or 5′-NT or both for their substrates. In fact, a recent study documents an increase in 5′-NT activity with hypoxia, a condition that would be expected to exist in adipose tissue during exercise but one that should increase, not decrease, extracellular adenosine levels (34). One reason for our finding may be in the study design: the bout of acute exercise was set at a treadmill speed and duration that the sedentary swine would tolerate. However, this intensity was a minimal effort for the exercise-trained swine, at nearly half the speed and half the time to which the exercise-trained swine were accustomed. The lower level of extracellular adenosine in adipose tissue of exercise-trained swine confers to them a physiological advantage: adipocytes are exposed to less antilipolytic stimulus, and lipolysis proceeds unencumbered. Indeed, our previous work (10, 35) shows that adipocytes from exercise-trained swine are less sensitive to the antilipolytic effects of adenosine and have a 50% reduction in adenosine A_1 receptor number, compared to adipocytes from sedentary swine. The combination of fewer receptors with less extracellular ligand would translate into less inhibition of lipolysis in adipose tissue of exercise-trained swine—this possibility remains to be confirmed, however.

In summary, cAMP appears extracellularly in adipose tissue of miniature swine; its appearance, as well as that of its metabolite adenosine, is provoked by an acute bout of exercise. This appearance is partly dependent upon the exercise-trained status of the animal but is not a function of maximal ectoenzyme activity.

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