

# Removal of Adenosine Decreases the Responsiveness of Muscle Glucose Transport to Insulin and Contractions

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**Adenosine in the extracellular space modulates stimulated glucose transport in striated muscle. In the heart and in adipocytes, adenosine potentiates insulin-stimulated glucose transport. There is controversy regarding the effect of adenosine in skeletal muscle, with reports of both an inhibitory effect and no effect, on insulin-stimulated glucose transport. We found that, in rat epitrochlearis and soleus muscles, removing adenosine with adenosine deaminase or blocking its action with the adenosine receptor blocker CPDPX markedly reduces the responsiveness of glucose transport to stimulation by 1) insulin alone, 2) contractions alone, and 3) insulin and contractions in combination. Measurement of the increase in GLUT4 at the cell surface in response to a maximally effective insulin stimulus in the epitrochlearis muscle, using the exofacial label ATB-[<sup>3</sup>H]BMPA, showed that adenosine deaminase treatment markedly reduces cell-surface GLUT4 labeling. The reduction in cell-surface GLUT4 labeling was similar in magnitude to the decrease in maximally insulin-stimulated glucose transport activity in adenosine deaminase-treated muscles. These results show that adenosine potentiates insulin- and contraction-stimulated glucose transport in skeletal muscle by enhancing the increase in GLUT4 at the cell surface and raise the possibility that decreased adenosine production or action could play a causative role in insulin resistance. *Diabetes* 47:1671–1675, 1998**

**S**ome of the adenosine that is produced intracellularly is released into the extracellular space, where it interacts with adenosine receptors to regulate various physiological processes in an autocrine manner (1). One such action of adenosine is to modulate insulin-stimulated glucose transport in striated muscle and adipocytes. In both adipocytes (2,3) and cardiac muscle

(4–6), adenosine enhances the stimulation of glucose uptake by insulin, and removing adenosine with adenosine deaminase or blocking adenosine receptors results in a decrease in insulin-stimulated glucose uptake (2–5).

In striated muscle, glucose transport is stimulated not only by insulin but also by a separate, insulin-independent pathway activated by contractile activity (7). The maximal effects of insulin and contractions on glucose transport are additive (8–10). In perfused, beating hearts, glucose uptake is increased by adenosine in the absence of insulin, suggesting that adenosine may also potentiate the effect of contractions on glucose transport (5,6).

While the potentiating effect of adenosine on insulin-stimulated glucose transport in heart muscle and adipocytes is well documented, the literature on the role of adenosine in regulating glucose transport in skeletal muscle is confusing. In contrast with the findings in heart muscle, reports from Newsholme and colleagues (11–14) have described an inhibitory effect of adenosine on insulin-stimulated glucose uptake and metabolism in rat soleus and extensor digitorum longus muscles. In those studies, removing adenosine with adenosine deaminase or blocking its action with adenosine receptor antagonists increased muscle insulin sensitivity with no effect on insulin responsiveness (11–14). Vergauwen et al. (15), working with a perfused rat hindquarter preparation, were unable to confirm the finding of Newsholme's group that blocking adenosine receptors enhances the sensitivity of muscle glucose uptake to insulin. In contrast with the findings on heart muscle, Vergauwen et al. (15) did not find an inhibitory effect of adenosine receptor blockade on either insulin- or contraction-stimulated glucose transport. They did report, however, that adenosine receptor blockade had an inhibitory effect on the combined effect of insulin and contractions. The present study was undertaken to reinvestigate the effect of removing extracellular adenosine with adenosine deaminase or blocking adenosine receptors with the adenosine receptor antagonist CPDPX (8-cyclopentyl-1,3-dipropylxanthine) on insulin- and contraction-stimulated glucose transport in skeletal muscle.

## RESEARCH DESIGN AND METHODS

**Materials.** 3MG (3-*O*-methyl-D-[<sup>3</sup>H]glucose) and [U-<sup>14</sup>C]mannitol were purchased from New England Nuclear (Boston, MA). 2DG (2-deoxy-D-[1,2-<sup>3</sup>H]glucose) was obtained from American Radiolabeled Chemicals (St. Louis, MO). CPDPX and CPA (*N*<sup>6</sup>-cyclopentyl-adenosine) were obtained from Research Biochemicals International (Natick, MA). Adenosine deaminase was purchased from Boehringer Mannheim (Indianapolis, IN). The exofacial label ATB-[2-<sup>3</sup>H]BMPA [2-*N*-4(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis-(mannose-4-yloxy)-2-propylamine; BSA, bovine serum albumin; CPA, *N*<sup>6</sup>-cyclopentyl-adenosine; CPDPX, 8-cyclopentyl-1,3-dipropylxanthine; 2DG, 2-deoxy-D-[1,2-<sup>3</sup>H]glucose; KHBB, Krebs-Henseleit bicarbonate buffer; 3MG, 3-*O*-methyl-D-[<sup>3</sup>H]glucose.

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ATB-[2-<sup>3</sup>H]BMPA, 2-*N*-4(1-azi-2,2,2-trifluoroethyl)-benzoyl-1,3-bis-(mannose-4-yloxy)-2-propylamine; BSA, bovine serum albumin; CPA, *N*<sup>6</sup>-cyclopentyl-adenosine; CPDPX, 8-cyclopentyl-1,3-dipropylxanthine; 2DG, 2-deoxy-D-[1,2-<sup>3</sup>H]glucose; KHBB, Krebs-Henseleit bicarbonate buffer; 3MG, 3-*O*-methyl-D-[<sup>3</sup>H]glucose.

g to the COOH-terminal 16 amino acids of rat GLUT4 was a gift of Dr. Mike Ueckler. The cAMP assay kit was obtained from Cayman Chemical (Ann Arbor, MI). Purified porcine insulin (Iletin II) was obtained from Eli Lilly (Indianapolis, IN). All other reagents were purchased from Sigma (St. Louis, MO).

**reatment of animals and muscle preparation.** Colony-bred male Wistar rats were maintained on a diet of Purina rodent chow and water. On the day before the experiment, food was restricted to 4 g per animal after 5:00 P.M. At the time of the experiment, animals weighed ~100 g. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and epitrochlearis and soleus muscles were removed. The epitrochlearis is a small, thin muscle of the forelimb, which has been shown to be suitable for in vitro measurement of glucose transport (17). The intact soleus muscle, which is too thick for accurate measurement of glucose transport in vitro, was split longitudinally into strips having an average weight of 15–20 mg (18).

**Muscle incubation.** Immediately after dissection, muscles were placed in 2 ml oxygenated Krebs-Henseleit bicarbonate buffer (KHBB) containing 8 mmol/l glucose, 32 mmol/l mannitol, and 0.1% radioimmunoassay grade bovine serum albumin (BSA) and allowed to recover for 30 min at 30°C. Muscles were then transferred to 2 ml of identical medium with or without 5 U/ml adenosine deaminase, with 1 or 70  $\mu\text{mol/l}$  CPA or CPDPX, and with or without either 60  $\mu\text{U/ml}$  or 2 mU/ml insulin for 30 min at 30°C. The flasks were gassed with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$ , and taken continuously in a Dubnoff incubator (Precision Scientific, Chicago).

**Electrical stimulation.** After the initial incubations, muscles were attached to vertical Lucite rod containing two platinum electrodes and immersed in KHBB containing 8 mmol/l glucose, 32 mmol/l mannitol, 0.1% BSA, and the same additions as the preceding incubation. The muscles were stimulated with supramaximal square-wave pulses of 0.2 ms duration with a Grass SII stimulator (Grass Instruments, Quincy, MA). Ten tetanic contractions were produced by stimulating at 100 Hz for 10 s at a rate of 1 contraction per minute for 10 min. This stimulation protocol elicits a maximal effect on glucose transport activity (19).

**Measurement of glucose transport activity.** After pretreatment, muscles were transferred to a flask containing 2 ml KHBB with 40 mmol/l mannitol and incubated with shaking for 10 min to remove glucose from the extracellular space. Muscles were then incubated at 30°C in 1.5 ml KHBB containing 4 mmol/l 2DG (1.5  $\mu\text{Ci/ml}$ ), 36 mmol/l mannitol (0.2  $\mu\text{Ci/ml}$ ), or 8 mmol/l 3MG (2.2  $\mu\text{Ci/ml}$ ) and 32 mmol/l mannitol (0.2  $\mu\text{Ci/ml}$ ), 0.1% BSA, and the same additions as the preceding incubation. Initial rates of 3MG transport were measured over 10 min; during that time period, intracellular accumulation of 3MG is linear under the experimental conditions used in this study (17). 2DG uptake was measured over a 30-min period; 2DG uptake is linear for 60 min or longer under our experimental conditions (20).

**hotolabeling of GLUT4 in epitrochlearis muscle.** The ATB-[ $^3\text{H}$ ]BMPA exocytic photolabeling technique was used to measure GLUT4 at the cell surface in epitrochlearis muscle as described (21) except that the labeled GLUT4 was immunoprecipitated with G4829.

**cAMP analysis.** cAMP concentrations were measured with a Cayman cAMP assay kit.

**Statistical analysis.** Data are means  $\pm$  SE. Statistical analyses were performed using Student's *t* test at a significant  $\alpha$  level of  $P < 0.05$ .

## RESULTS

**Effect of adenosine deaminase on glucose transport activity.** Addition of adenosine deaminase to the incubation medium had no significant effect on basal 2DG uptake but markedly decreased the effects of a maximally effective concentration of insulin alone, of contractions alone, and of contractions plus insulin (Fig. 1). The effect of adenosine deaminase on glucose transport activity was also examined in the soleus muscle. In the soleus, glucose transport is more than twice as responsive to insulin as to contractions; in the epitrochlearis, insulin and contractions induce similar increases in glucose transport. As shown in Fig. 2, treatment with adenosine deaminase also resulted in large decreases in the stimulation of 2DG uptake by insulin, contractions, and insulin plus contractions in the soleus.

Under the conditions of these experiments, 2DG can normally be used to measure glucose transport (20). However, to make certain that treatment with adenosine deaminase was not acting via an inhibition of hexokinase, we did an experiment in which 3MG was used to measure glucose transport activity. Incubation of muscle with adenosine deaminase

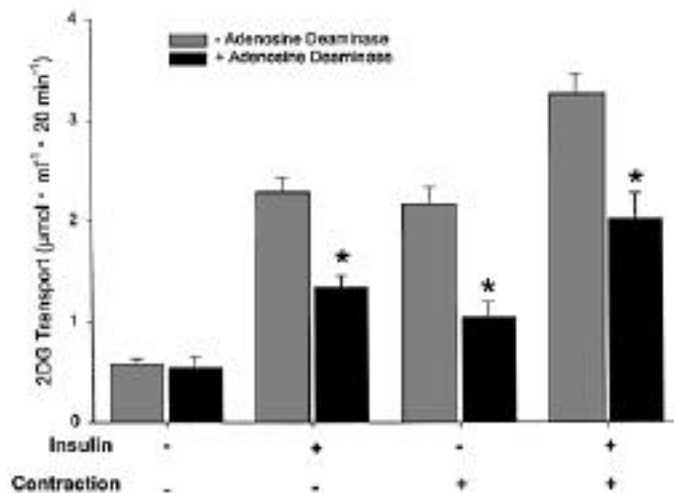


FIG. 1. Effect of adenosine deaminase on basal, maximally insulin-stimulated, contraction-stimulated, and insulin plus contraction-stimulated 2-deoxyglucose transport in epitrochlearis muscle. Values are means  $\pm$  SE for 10–12 muscles per group. \*Adenosine deaminase-treated versus control,  $P < 0.01$ .

caused a large decrease in maximally insulin-stimulated 3MG transport in epitrochlearis that was similar in magnitude to that observed when 2DG was used to measure glucose transport (3MG transport rate averaged [means  $\pm$  SE]  $1.27 \pm 0.12 \mu\text{mol} \cdot \text{ml}^{-1} \cdot 10 \text{ min}^{-1}$  in 12 control muscles vs.  $0.75 \pm 0.07 \mu\text{mol} \cdot \text{ml}^{-1} \cdot 10 \text{ min}^{-1}$  in 12 muscles treated with adenosine deaminase,  $P < 0.001$ ).

**Effect of CPA on insulin-stimulated glucose transport.** To evaluate the possibility that endogenously produced adenosine was insufficient to maximally potentiate stimulated glucose transport activity, we examined the effect of adding the adenosine analog CPA to the incubation medium. A maximally effective insulin stimulus (2 mU/ml) induced an

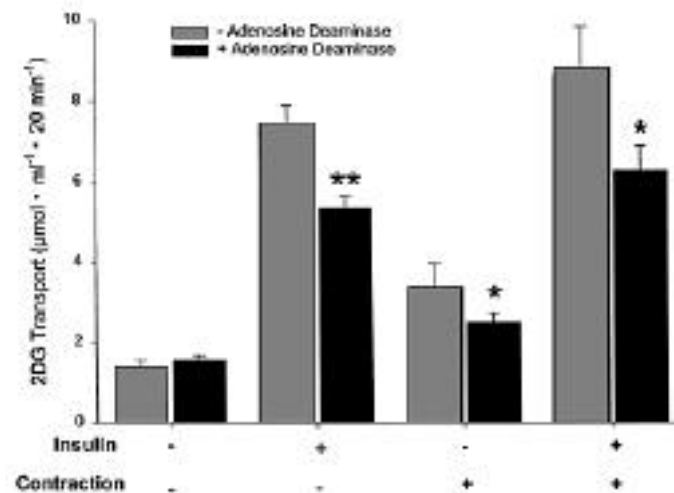


FIG. 2. Effect of adenosine deaminase on basal, maximally insulin-stimulated, contraction-stimulated, and insulin plus contraction-stimulated 2-deoxyglucose transport in soleus muscle. Values are means  $\pm$  SE for 9–12 muscles per group. \*Adenosine deaminase-treated versus control,  $P < 0.05$ .

increase in 3MG transport of  $1.04 \pm 0.14 \mu\text{mol} \cdot \text{ml}^{-1} \cdot 10 \text{ min}^{-1}$  in the absence of the adenosine receptor agonist, compared with an increase of  $1.03 \pm 0.17 \mu\text{mol} \cdot \text{ml}^{-1} \cdot 10 \text{ min}^{-1}$  in the presence of  $1 \mu\text{mol/l}$  CPA. This finding provides evidence that the endogenously produced adenosine provides a maximally potentiating effect on stimulated transport in our muscle preparation.

**Effect of CPDPX on glucose transport activity.** We also examined the effect of the adenosine receptor blocker CPDPX, at a concentration similar to that used by Vergauwen et al. (15), on stimulated glucose transport. As shown in Fig. 3, incubation of epitrochlearis muscles with CPDPX resulted in a large decrease in insulin-stimulated 2DG transport ( $\sim 50\%$ ), both at a maximally effective insulin concentration ( $2 \text{ mU/ml}$ ) and at a concentration of insulin ( $60 \mu\text{U/ml}$ ) that produces approximately a half-maximal effect in this muscle preparation. Thus, the decrease in glucose transport activity at the lower insulin concentration was proportional to that seen at the maximally effective insulin concentration. This finding, that the increases in glucose transport activity induced by  $2 \text{ mU/ml}$  and  $60 \mu\text{U/ml}$  of insulin were both reduced by  $\sim 50\%$ , provides evidence that the entire effect of adenosine is on insulin responsiveness without a separate effect on insulin sensitivity. Contraction-stimulated glucose transport activity was similarly reduced, by  $\sim 50\%$ , in the epitrochlearis by exposure to CPDPX. Insulin-stimulated 2DG transport was also reduced by exposure to CPDPX in the soleus, by  $\sim 30\%$  (data not shown).

**Effect of adenosine deaminase on insulin-stimulated increase in GLUT4 at the cell surface.** The effect of removing adenosine with adenosine deaminase on GLUT4 at the cell surface was assessed using the exofacial label ATB- $[\text{^3H}]$ BMPA. As shown in Fig. 4, treatment of muscles with adenosine deaminase resulted in a marked reduction in the magnitude of the increase in GLUT4 labeling induced by a maximally effective insulin stimulus. The decrease in cell surface GLUT4 labeling was similar in magnitude to the

reduction in maximally insulin-stimulated 3MG transport caused by treatment with adenosine deaminase.

**cAMP levels.** One of the actions mediated by interaction of adenosine with the  $A_1$  receptor is inhibition of adenylate cyclase activity. As a consequence, some of the metabolic effects of adenosine have been attributed to a decrease in cAMP concentration. cAMP levels were, therefore, measured in epitrochlearis muscles incubated with  $2 \text{ mU/ml}$  insulin with or without adenosine deaminase. The concentration of cAMP was low in the control muscles, and incubation with adenosine deaminase had no significant effect on cAMP level (control  $126 \pm 12$  vs. adenosine deaminase-treated  $113 \pm 14 \text{ pmol/g}$  muscle wet wt, 12 muscles per group).

## DISCUSSION

The results of this study show that removing adenosine with adenosine deaminase or blocking its action with an adenosine receptor antagonist causes a large decrease in stimulated glucose transport activity in skeletal muscle. Both insulin- and contraction-stimulated glucose transport are reduced, as is the combined effect of insulin plus contractions. This decrease in stimulated glucose transport could be due to either a reduction in the number of GLUT4 at the cell surface or a decrease in GLUT4 intrinsic glucose transport activity. We found that adenosine removal with adenosine deaminase results in a reduction in the number of GLUT4 at the cell surface in maximally insulin-stimulated muscles. That decrease in GLUT4 at the cell surface closely matches the decrease in the insulin responsiveness of muscle glucose transport. This finding provides evidence that the entire effect of adenosine removal on insulin-stimulated muscle glucose transport is attributable to a reduction in the number of GLUT4 accessible to glucose or to the ATB- $[\text{^3H}]$ BMPA photolabel at the cell surface.

Although there are important differences in the regulation of glucose uptake between striated muscle and adipocytes, the mechanisms involved in the stimulation of glucose transport by insulin appear to be similar in those tissues (7). It is, there-

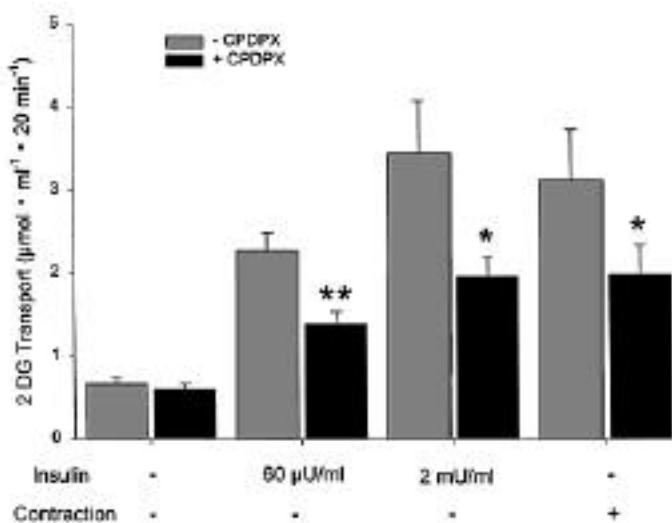


FIG. 3. Effect of CPDPX on basal, submaximally insulin-stimulated ( $60 \mu\text{U/ml}$ ), maximally insulin stimulated ( $2 \text{ mU/ml}$ ), and contraction-stimulated 2-deoxyglucose transport in epitrochlearis muscle. Values are means  $\pm$  SE for 6 muscles per group except the  $60 \mu\text{U/ml}$  insulin group, in which there were 12 muscles. CPDPX-treated versus control: \* $P < 0.05$ , \*\* $P < 0.01$ .

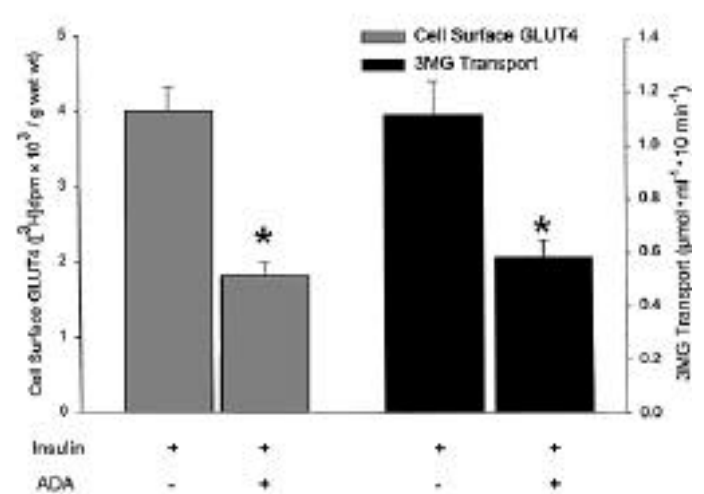


FIG. 4. Effects of a maximally effective insulin stimulus on cell surface GLUT4 and on 3MG transport in the presence or absence of adenosine deaminase. Amount of GLUT4 at the cell surface was determined with the exofacial label ATB- $[\text{^3H}]$ BMPA. Values are means  $\pm$  SE for six muscles per group. \*Adenosine deaminase-treated versus control,  $P < 0.01$ .

ore, of interest relative to our findings that in a number of studies adenosine enhances insulin-stimulated glucose transport in fat cells (2,3,22,23). As in the present study on muscle, Uroda et al. (2) found that the potentiation of insulin-stimulated glucose transport by adenosine in adipocytes is cAMP-dependent. Thus, it appears that the effects of adenosine on insulin-stimulated glucose transport are very similar in adipocytes and skeletal muscle. Of particular interest, relative to the mechanism of action of adenosine, Vannucci et al. (3) found that the quantity of GLUT4 measured by Western blotting was the same in the plasma membrane fraction prepared by differential centrifugation from adipocytes stimulated with insulin in the presence and absence of adenosine. However, cell-surface GLUT4 labeling with the ATB- $^3\text{H}$ BMPA photo-label changed in parallel with glucose transport in response to adenosine. The authors interpreted this finding to indicate that after insulin stimulation, GLUT4 exists in two distinct states in the adipocyte plasma membrane, one that is functional and accessible to extracellular substrate, and one that is nonfunctional and unable to bind extracellular substrate (3). They proposed that adenosine enhances insulin-stimulated glucose transport by promoting an "unoccluded" conformation of GLUT4 in the plasma membrane that is accessible to extracellular substrate (3).

The results of this study showing that adenosine removal decreases insulin action are in marked contrast with the reports by Newsholme's group (11–14) that removing adenosine or blocking its action increases insulin sensitivity of muscle glucose uptake, that is, that adenosine causes insulin resistance. We have no explanation for this discrepancy, which is made even more puzzling by the fact that some of our experiments were done on the split soleus muscle preparation that was also used in most of the studies by Newsholme's group. However, our findings are in keeping with the evidence that adenosine potentiates the effect of insulin on glucose uptake in heart muscle (4–6) and adipocytes (2,3,22,23). The finding that adenosine also increases glucose uptake in beating hearts in the absence of insulin (5,6) suggests that, as we found in skeletal muscle, adenosine potentiates the effect of contractile activity on glucose transport in cardiac muscle.

Vergauwen et al. (15) studied the effect of adenosine receptor blockers in a perfused rat hindquarter preparation. Unlike Newsholme's group, they found no evidence that adenosine inhibits either insulin-stimulated or contraction-stimulated glucose uptake. In contrast to our findings on isolated muscles, they observed no inhibitory effect of the adenosine receptor blocker CPDPX on the stimulation of glucose uptake by either insulin alone or contractions alone. They did, however, find that adenosine receptor blockade resulted in a decrease in the stimulation of glucose uptake by insulin and contractions in combination (15). The reason for their inability to detect the inhibitory effect of adenosine receptor blockade on insulin- or contraction-stimulated muscle glucose transport may relate to the experimental model used. Although the perfused hindlimb model has provided important insights regarding the regulation of muscle glucose uptake (24), it is not ideal for evaluating the effects of inhibitors, because in perfused muscle, for muscle *in vivo*, either muscle cell membrane permeability to glucose or glucose availability can limit the rate of glucose uptake (25,26).

Adenosine deaminase or CPDPX markedly reduced stimulated glucose transport activity in both epitrochlearis and soleus muscles in the present study. The soleus consists predominantly of type I slow-twitch fibers (27), while the epitrochlearis consists almost entirely of type II (mostly type IIb), fast-twitch fibers (28). Thus, our results show that adenosine potentiates the effects of contractions and insulin in both fast-twitch and slow-twitch muscle fibers. Vergauwen et al. (15), however, reported that blocking adenosine receptors with caffeine caused a 42% decrease in 3MG uptake stimulated by insulin plus contractions only in the slow-twitch red fibers, with no significant effect on fast-twitch red (type IIa) or fast-twitch white (type IIb) fibers. This finding led them to conclude that the effect of blocking adenosine receptors on glucose uptake in contracting muscles perfused with insulin is limited to slow-twitch red fibers. They also reported, however, that hindquarter glucose uptake under those conditions was reduced ~25%. It can be calculated from the glucose transport rates reported for the different fiber types by Vergauwen et al. (15) and the evidence that slow-twitch red fibers make up only ~5% of the total muscle mass in rat hindlimbs (27) that the decrease in insulin plus contraction-stimulated glucose uptake caused by CPDPX or caffeine would have been just ~4% if the effect was, in fact, only on type I fibers.

Contractions and insulin stimulate muscle glucose transport via separate pathways. This is evidenced by the findings that 1) the maximal effects of insulin and contractions on both glucose transport (7) and the increase in GLUT4 at the cell surface (29,30) are additive, and 2) it is possible to completely inhibit insulin-stimulated glucose transport without affecting contraction-stimulated glucose transport (30–32). Our results show that adenosine acts on both contraction-stimulated and insulin-stimulated glucose transport. This finding provides evidence that the potentiating effect of adenosine on stimulated glucose transport in muscle occurs at a common step beyond the contraction and insulin signaling pathways, thus pointing the direction for future studies on the mechanism of adenosine action.

In conclusion, the results of this study show that removing adenosine with adenosine deaminase or blocking its action with CPDPX markedly reduces the responsiveness of muscle glucose transport to stimulation by insulin, by contractions, and by insulin and contractions together. The decrease in insulin-stimulated glucose transport is mediated by a smaller increase in GLUT4 at the cell surface. The decrease in stimulated glucose transport induced by removing adenosine or blocking its action occurs in both fast-twitch and slow-twitch muscle. These findings raise the possibility that decreased adenosine production or action could play a role in the development of insulin resistance.

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