Endocrine, vol. 4, no. 3, 199–205, June 1996 0969-711X/96/4:199–205/$6.75

Desensitization of the Adipocyte A_1 Adenosine Receptor During Untreated Experimental Diabetes Mellitus

William W. Barrington, Michelle Crum, Curtis Forst, Mark Scheetz, and Lamont G. Weide

The Department of Internal Medicine, Sections of Cardiology and Diabetes, Endocrinology and Metabolism, The University of Nebraska Medical Center, Omaha, NE

We examined the changes that occur in the adenosine receptor system during diabetes mellitus. Experimental diabetes mellitus was induced in male Lewis rats with streptozocin (65 mg/kg), and A_1 adenosine receptor binding was characterized with [^{125}I]N^6-2-(4-aminophenyl) ethyladenosine. In adipocytes, high-affinity A_1 adenosine receptor binding decreased from 1466 ± 228 of protein to 312 ± 123 fmol/mg of protein ($p < 0.01$) following 14 d of untreated diabetes mellitus. Neither the dissociation constant (K_d = 1.3 ± 0.2 n*M*) nor the basal level of adenylate cyclase activity (2.8 ± 1.1 pmol cAMP/mg of protein/min) was altered by diabetes mellitus. The dose–response curve for the inhibition of adenylate cyclase by N^6-R-phenylisopropyladenosine (R-PIA), however, did show a rightward shift, indicating that diabetic adipocyte membranes were less sensitive to the effects of adenosine than nondiabetic adipocyte membranes. In contrast, the A_1 adenosine receptor-binding characteristics and adenylate cyclase dose–response curve for cerebral cortical tissue were unchanged by diabetes. These findings suggest that diabetes has tissue-specific effects on the A_1 adenosine receptor system. Furthermore, the decreased sensitivity to adenosine potentially worsens the hyperlipidemia associated with diabetes mellitus. Such alterations in the adenosine receptor system may play a previously undescribed role in the pathophysiology of diabetes mellitus and may help explain why some organs are severely affected by diabetes, but others are relatively spared. Understanding these alterations in adenosine receptor function may lead to novel therapies of this common metabolic disease.

Key Words: Adenosine receptor; diabetes mellitus; adipocytes; adenylate cyclase.

Received December 4, 1995; Revised January 4, 1996; Accepted January 30, 1996.

Author to whom all correspondence and reprint requests should be addressed: William W. Barrington, Department of Internal Medicine, Section of Cardiology, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, NE 68198–2265.

Introduction

Adenosine is a widely distributed nucleoside that modulates a variety of physiologic processes through specific membrane-bound receptors. Pharmacologic intervention with agonist adenosine analogs desensitize the receptor (Parsons and Stiles, 1987), and treatment with antagonist analogs sensitizes the same receptor system (Green and Stiles, 1986) to the effects of adenosine. Although altered sensitivity following exposure to pharmacologic doses of agonist or antagonist analogs is common, several investigators have observed altered sensitivity to adenosine in the setting of diabetes mellitus (Downing, 1985; Gasser et al., 1993; Pflueger and Osswald, 1994), a disorder that does not obviously result in pharmacologic alterations in the level of endogenous adenosine. Changes in adenosine receptor number or affinity have been suggested as possible explanations for the altered diabetic sensitivity, but very little has been done to characterize the diabetic adenosine receptor system rigorously.

One important site of adenosine receptor activity is adipose tissue, where adenosine acts (through the A_1 adenosine receptor) as a potent inhibitor of lipolysis (Schwabe et al., 1973). This inhibition should counteract some of the increased lipolysis associated with insulin-deficient diabetes mellitus. If diabetes does alter the sensitivity of the adenosine receptor system, a likely location is adipose tissue, where adenosine receptor activation and insulin deficiency have opposite effects on the lipolytic process.

This project was designed to characterize the A_1 adenosine receptor system in adipocytes from diabetic rats as a first step in understanding adenosine's role in the pathophysiology of diabetes mellitus.

Results

Induction of Experimental Diabetes Mellitus

Diabetes was induced in male Lewis rats (225–250 g) with a 65 mg/kg iv injection of streptozocin (STZ). The mean (± SD) serum glucose concentration for the nondiabetic (control) Lewis rats was 6.8 ± 0.6 m*M* (122 ± 10 mg/dL),

which rose to 23.0 ± 2.2 m*M* (415 ± 39 mg/dL, $p < 0.01$) within 24 h of the STZ injection and remained elevated for the duration of the study. The percentage of glycosylated hemoglobin also rose from 5.1% in the nondiabetic animals to 11.4% ($p < 0.01$) after 2 wk of experimental diabetes. This elevation persisted throughout the remainder of the study.

Further evidence of clinical diabetes was the loss of epididymal fat. Gross visual examination of the epididymal fat pads showed the amount of fat decreased as the duration of experimental diabetes increased. A graph of the mean (± SD) normalized fat pad mass (normalized mass = g epididymal fat/animal body wt in kg) vs the duration of diabetes (Fig. 1) demonstrated a 65% loss of epididymal fat over the 28-d course of this study. An analysis of variance (ANOVA) showed the mean fat pad mass of all the diabetic time-points was significantly different from the normalized mass of the nondiabetic controls ($p < 0.01$). Each of the diabetic time-points was statistically different from the adjacent point as well ($p < 0.025$), with the exception of the 7- and 14-d points, which were statistically indistinguishable (p = NS).

In addition to marked hyperglycemia, elevated levels of glycosylated hemoglobin, and loss of epididymal fat, the STZ-treated animals lost over 10% of their body weight, and experienced polyuria and polydipsia, all of which constituted further evidence of clinical diabetes mellitus.

Although diabetes was induced with iv STZ in the majority of animals, the results from a cohort of animals (data not shown) that were treated with alloxan to induce diabetes were statistically indistinguishable from those treated with STZ. Consequently, all the animals in this article were treated with STZ.

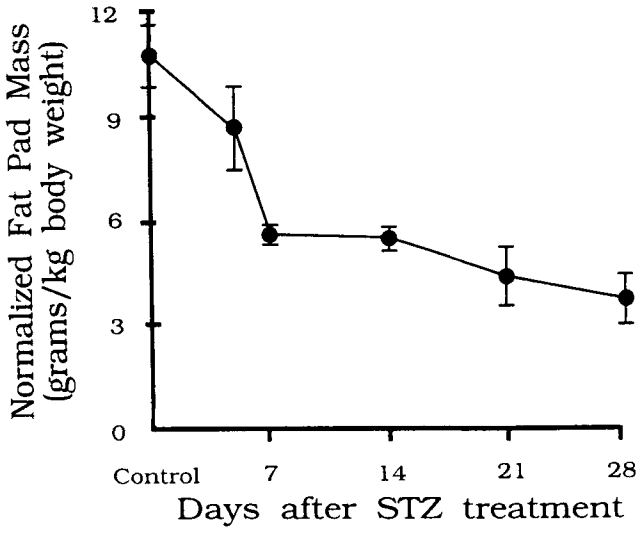

Fig. 1. Normalized epididymal fat pad mass. The normalized mass is based on the mass of the animal (in kg) on the day of sacrifice and is equal to the mass of epididymal fat (g)/mass of the animal (kg). The mean normalized fat pad mass (± SD) is shown at day 0 (nondiabetic control) and at 5, 7, 14, 21 and 28 d after STZ injection. Each time-point represents the average results of between three and five experiments. The mean fat pad mass at each time-point is statistically different from the adjacent points ($p \leq 0.025$), except for the 7- and 14-d values, which are statistically identical.

Adipocyte Adenosine Receptor Characterization

Anywhere from three to six separate saturation binding experiments were performed at each of the experimental time-points. The A_1 adenosine receptor was present at a mean (± SD) density (B_{max}) of 1466 ± 228 fmol/mg of protein in nondiabetic adipocyte membranes. In the setting of diabetes, this value decreased by 61% after 5 d (mean B_{max} = 562 ± 279 fmol/mg of protein) and reached a nadir of 312 ± 123 fmol/mg of protein on day 14 (Fig. 2A). Statistical analysis showed that the mean B_{max} at all of the diabetic time-points was significantly different from the nondiabetic control value ($p < 0.01$), and that all of the diabetic B_{max} values (days 5–28) were statistically indistinguishable (p = NS).

In contrast, the mean adipocyte dissociation constant (K_d) from these experiments was unaffected by experimental diabetes (Fig. 2B). None of the diabetic K_d values were significantly different from the nondiabetic control value of 1.3 ± 0.2 n*M* (p = NS).

Competitive binding studies (data not shown) using [^{125}I]N^6-2-(4-aminophenyl)ethyladenosine ([^{125}I]APNEA) and varying concentrations of N^6-R-phenylisopropyladenosine (R-PIA), N^6-*S*-phenylisopropyladenosine (S-PIA), and 5'-*N*-ethylcarboxamidoadenosine (NECA) revealed a potency order of R-PIA > NECA > S-PIA, which is unique for the rat A_1 adenosine receptor (Stiles et al., 1985, 1986), confirming that [^{125}I]APNEA labeled the appropriate adenosine receptor subtype.

Adipocyte Adenylate Cyclase Activity

Since the modulation of lipolysis was postulated as one site of interaction between diabetes mellitus and the adenosine receptor system, adenylate cyclase activity was examined. Three separate adenylate cyclase inhibition experiments were performed. The basal adipocyte adenylate cyclase activity was unaffected by diabetes. Nondiabetic adipocyte membranes produced 2.8 ± 1.1 pmol cAMP/mg of protein/min (mean ± SD), whereas the animals sacrificed after 4 wk of diabetes produced 3.0 ± 1.6 pmol cAMP/mg of protein/min (p = NS). The presence of 10^{-5} *M* R-PIA produced the maximal inhibition of adipocyte adenylate cyclase activity (35–40% inhibition) in both the diabetic and control animals. The dose–response curve for the inhibition of adenylate cyclase activity, however, was shifted to the right in the diabetic animals (Fig. 3). The mean IC_{50} value (± SD) for inhibition of cAMP production was $3.2 \pm 2.2 \times 10^{-9}$ mol/L in control animals and $3.5 \pm 4.4 \times 10^{-7}$ mol/L in diabetic animals ($p < 0.05$ compared to control).

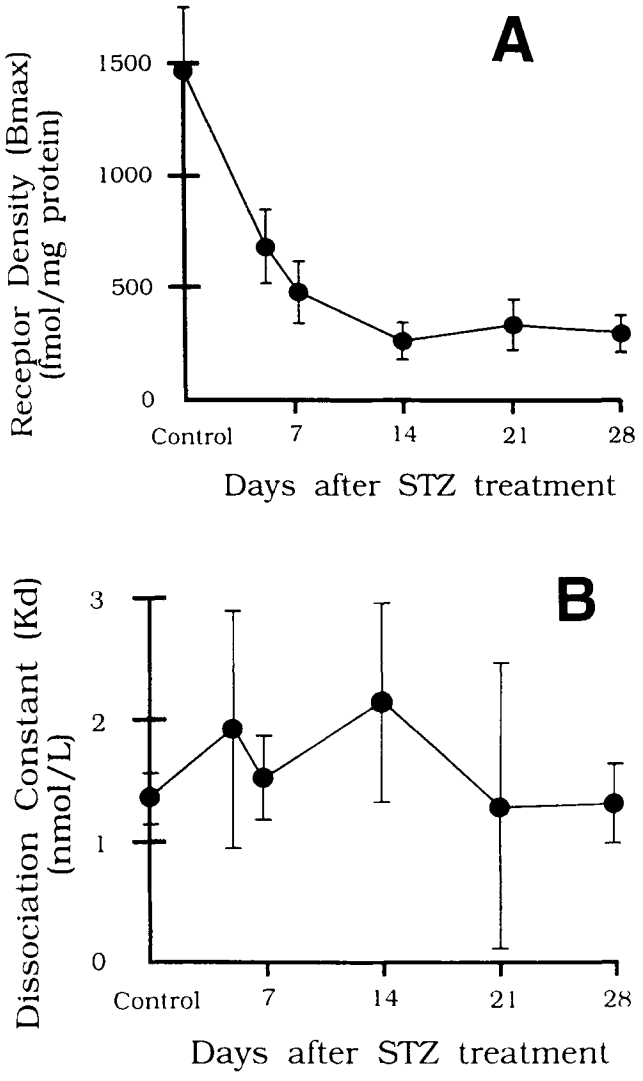

Fig. 2. Adipocyte A_1 adenosine receptor characterization. The mean receptor density (B_{max}) during the course of experimental diabetes mellitus is shown in **A**. This is displayed as the mean B_{max} (± SD) at day 0 (nondiabetic control) and at 5, 7, 14, 21, and 28 d after STZ injection. The nondiabetic B_{max} is significantly different from the B_{max} at the 5-, 7-, 14-, 21-, and 28-d time-points ($p < 0.01$ in all cases), but the 5-, 7-, 14-, 21-, and 28-d values are not significantly different (p = NS) from each other. The mean adipocyte dissociation constant (K_d) during the course of experimental diabetes is shown in **B**. The mean K_d (± SD) is again displayed at day 0 (nondiabetic control) and at 5, 7, 14, 21, and 28 d after STZ injection. None of the mean K_d values are significantly different (p = NS) from the control value. Each time-point (in both A and B) represents the average results of between three and six experiments.

Cerebral Cortex Adenosine Receptor Characterization and Adenylate Cyclase Activity

Anywhere from three to five separate experiments were performed with the rat cerebral cortical membranes. A_1 adenosine receptor binding in the cerebral cortex was unaffected by experimental diabetes. The mean (± SD) A_1 adenosine receptor density (B_{max}) was 390 ± 95 fmol/mg of protein in nondiabetic control animals, 445 ± 36 fmol/mg of protein following 2 wk, and 387 ± 73 fmol/mg of protein after 5 wk of uncontrolled diabetes (p = NS compared to control). The dissociation constant was unchanged as well with a mean (± SD) K_d value of 2.2–0.4 nM in the nondiabetic animals, 2.2 ± 0.6 nM after 2 wk of diabetes, and 2.2 ± 0.3 nM following 5 wk of untreated diabetes (p = NS).

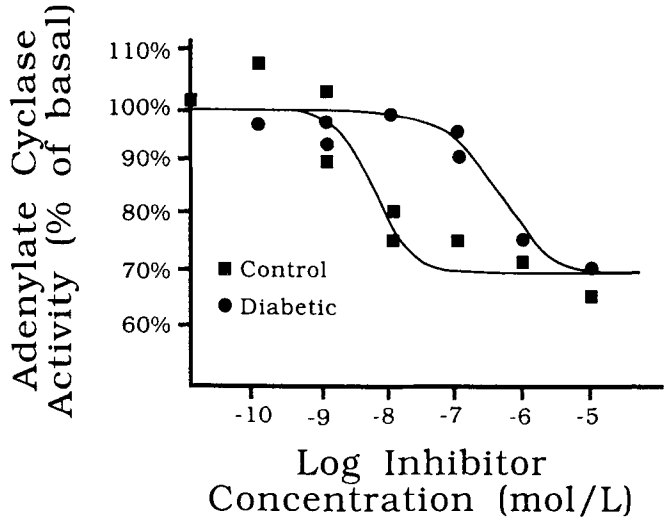

Fig. 3. Dose–response curve for inhibition of adenylate cyclase activity. Adenylate cyclase activity is plotted as a percentage of basal activity for a typical experiment. The left-hand curve reflects the dose response to increasing concentrations of R-PIA for the nondiabetic control animals, whereas the right-hand curve reflects the diabetic group. The mean IC_{50} (± SD) for inhibition of cAMP production was 3.2 ± 2.2 × 10^{-9} mol/L in control animals and 3.5 ± 4.4 × 10^{-7} mol/L in diabetic animals (p <0.05, n = 3).

Basal adenylate cyclase activity in the cerebral cortex did not change with diabetes and showed a 35–40% inhibition in the prepense of $10^{-5}M$ R-PIA without a shift in the dose–response curve.

Photoaffinity Labeling of the A_1 Adenosine Receptor

Direct photoaffinity labeling of the A_1 adenosine receptor-binding subunit showed the expected specific band on SDS-PAGE (Fig. 4) with an apparent mol wt of 36–38 kDa. This apparent molecular weight did not change in either the diabetic adipocyte or cerebral cortical membrane preparations.

Discussion

The results of this study are consistent with the metabolic derangements that characterize clinical diabetes, but they also suggest that changes in the adenosine receptor system play a previously undescribed role in the pathophysiology of diabetes mellitus.

The decrease in high-affinity adipocyte A_1 adenosine receptor binding makes the diabetic adipocytes less sensitive to a given level of endogenous adenosine. The functional significance of this finding is the rightward shift of the adenylate cyclase inhibition curve (Fig. 3), which shows

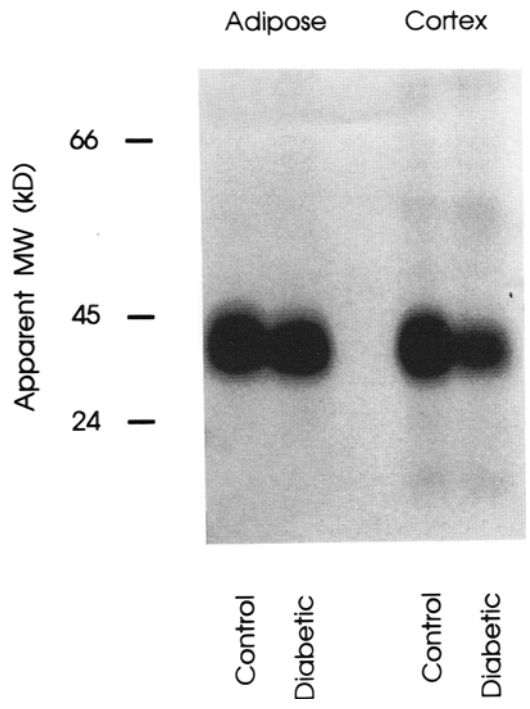

Fig. 4. Autoradiograph of the rat adipocyte (left) and cerebral cortical (right) A_1 adenosine receptor-binding subunit. The labeling was performed using [^{125}I]AZPNEA with the nondiabetic (control) labeled binding subunit in the left lane of each pair and the diabetic labeled binding subunit in the right lane of each pair. The adipocyte diabetic lane is loaded with approximately five times as much protein as the adjacent nondiabetic adipocyte lane to provide a similar intensity in the specifically labeled bands.

the diabetic adipocytes require nearly 100 times more adenosine to achieve the same degree of adenylate cyclase inhibition as the nondiabetic adipocytes. Since the inhibition of adenylate cyclase activity is the mechanism by which the A_1 adenosine receptor system inhibits lipolysis, this finding may be clinically relevant. Hyperlipidemia is an important complication of diabetes and has been attributed to the metabolic derangements associated with insulin deficiency (Nathan, 1993). This study, however, suggests that changes in the sensitivity of the adipocyte A_1 adenosine receptor system could contribute to hyperlipidemia by diminishing the inhibitory influence of endogenous adenosine.

The decrease in B_{max} suggests a downregulation of the diabetic adipocyte A_1 adenosine receptor, but the concentrations of [^{125}I]APNEA used in this study (up to ≈2 n*M*) only recognizes one of the two affinity states reported in rat adipocyte membranes (Lohse et al., 1984). Although we cannot conclude that true downregulation has occurred, since the loss of high affinity binding may be owing to a shift from the high- to low-affinity binding state, our conclusion that the diabetic adipocyte is less sensitive to the influence of adenosine is still consistent with our findings. Gasser et al. (1993), in fact, found such a shift from high- to low-affinity binding in the A_2 adenosine receptor system of human diabetic platelet membranes. Interestingly, they also observed a rightward shift in the dose–response curve for A_2 adenosine receptor-mediated platelet aggregation, which they believed indicated decreased sensitivity of the A_2 adenosine receptor system.

One other published study has examined the effect of diabetes on the A_1 adenosine receptor. In that report, Green and Johnson (1991) reported a functional abnormality in the coupling of G_i (the inhibitory guanine nucleotide-binding protein) to the diabetic adipocyte A_1 adenosine receptor, but they failed to find any change in the number of functional A_1 adenosine receptors. Those findings clearly contradict the results of this article, but this discrepancy may be explained by several significant differences in experimental technique. Specifically, in the former study, animals were diabetic for only 7 d before they were sacrificed, and they were supplemented with exogenous insulin for the entire 7 d. We demonstrated that the untreated diabetic animals needed at least 5–7 d to achieve a steady-state receptor density. It is possible that the supplemental insulin employed by Green and Johnson altered the time-course of the B_{max} changes to such a degree that significant changes in the number of functional A_1 adenosine receptors were not present after only 7 d. Additionally, the former study utilized a polyethylene glycol precipitation technique to quantify specific receptor binding at a single radiolabeled ligand concentration. In contrast, we used a filtration technique to quantify receptor binding over a range of radiolabeled ligand concentrations and rigorously characterized receptor binding in terms of a B_{max} and K_d using an exact physical-chemical model of receptor ligand binding (Munson and Rodbard, 1980; Munson, 1992).

Differences in experimental technique can account for the discrepancy sited above, but the similar contours of Fig. 1 and Fig. 2A raised a concern that the loss of epididymal fat pad mass somehow resulted in an artifactual decrease in the adipocyte B_{max} and not true receptor desensitization. We believed this was not the case for three reasons.

First, although the shape of the two curves appears similar, the statistical analysis of variance showed that they are not the same. The fat pad mass (Fig. 1) at all of the diabetic time-points (except for 7 and 14 d) was statistically different from the adjacent points ($p < 0.025$), whereas the diabetic B_{max} values shown in Fig. 2A were statistically indistinguishable. This can be interpreted as showing the B_{max} (Fig. 2A) reaching a steady minimum plateau on days 5–7, whereas the fat pad mass shown in Fig. 1 decreased over the entire 28-d course of the study. This disparity in the contour of the curves along with the difference in the curves at the end of the study (65% decrease for the fat pads vs 80% decrease for the B_{max}) suggest that the loss of high-affinity binding cannot be explained by the loss of epididymal fat alone.

Second, the observation that basal adenylate cyclase activity does not change during diabetes also supports the

conclusion that the decrease in high-affinity binding is not owing to the loss of epididymal fat. If the decrease in high-affinity binding was owing to a decrease in the number of adipocytes or some generalized loss of adipocyte cellular components, we would expect the catalytic unit of adenylate cyclase (and subsequent activity) to be affected as well. This is because both the catalytic unit of adenylate cyclase and the A_1 adenosine receptor-binding subunit are proteins that reside on the cell membrane, so if the A_1 binding subunit was decreased by 80% as a consequence of the loss of epididymal fat, we should observe some decrease in basal adenylate cyclase activity well. Since the values for the adipoctye B_{max} and adenylate cyclase activity show different responses to diabetes-associated loss of epididymal fat (even though both were measured on a per-mg-of-protein basis), it is unlikely that the drop in high-affinity adipocyte binding is an artifact of the epididymal fat loss.

Finally, our rigorous approach to receptor characterization, the correlation between the loss in high-affinity binding, and the shift in the adenylate cyclase inhibition curves as well as the similarity of our results to findings in the A_2 adenosine receptor system all demonstrate that diabetes alters the adipocyte A_1 adenosine receptor system in a manner that makes diabetic adipocytes less sensitive to the effects of endogenous adenosine.

In contrast to the dramatic changes seen in diabetic adipocytes, the A_1 adenosine receptor system in the cerebral cortex appears unaffected by diabetes. This discrepancy isconsistent with the clinical observation that some tissues are severely affected by diabetes, whereas others are relatively spared, but the explanation for this difference remains to be seen.

What then is the mechanism(s) responsible for the desensitization of the adipocyte A_1 adenosine receptor? It is very unlikely that diabetes leads to significantly elevated levels of adenosine. In vivo, endogenous adenosine deaminase (ADA) reduces adenosine's circulating half-life to 1 or 2 s. Continuous iv infusions can maintain elevated adenosine levels, but most investigators have resorted to adenosine analogs that are resistant to adenosine deaminase to study sensitization in this receptor system (Green and Stiles, 1986; Parsons and Stiles, 1987). Part of the explanation may lie in the different tissue requirements for insulin. In adipose, skeletal, and cardiac muscle, insulin greatly enhances the transport of glucose into the cell where it can be metabolized. In central nervous system tissues, however, insulin has very little effect on glucose transport. The greater importance of insulin for glucose transport may correlate with the loss of high-affinity binding and suggests that these two processes may be related. Finally, it is possible some circulating factor leads to the loss of adipocyte high-affinity binding, but since this substance cannot cross the blood–brain barrier, it has no effect on the cerebral cortex. In any event, further work is needed to understand the mechanism surrounding these findings.

In conclusion, this study demonstrates that diabetic adipocytes are approx 100 times less sensitive to the effects of adenosine than nondiabetic adipocytes owing to an 80% loss in high-affinity A_1 adenosine receptor binding. The significance of this result is to decrease the inhibitory effect of endogenous adenosine on the activated lipolysis that occurs in insulin-deficient diabetes. This alteration in the lipolytic process potentially contributes to the hyperlipidemia that increases the risk of cardiovascular disease in the diabetic population. Although the best way to treat diabetic hyperlipidemia would be restoration of normal insulin levels with intensive insulin therapy (The Diabetes Control and Complications Trial Research Group, 1993), this goal is difficult to achieve in all but the most motivated insulin-dependent diabetic patients and may not be appropriate for the 90% of diabetic adults that have noninsulin-dependent diabetes. The recognition that abnormalities in adenosine receptor system function may contribute to the pathophysiology of diabetes mellitus suggests new approaches to treatment in the diabetic population. Techniques to restore normal adenosine receptor system function could potentially serve as novel tools for the treatment of the hyperlipidemia that is associated with this common metabolic disease.

Materials and Methods

Materials

ADA was obtained from Boehringer Mannheim (Indianapolis, IN). $Na^{125}I$ (carrier-free) was obtained from the Amersham Corp. (Arlington Heights, IL). R-PIA, S-PIA, NECA, phenylmethanesulfonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor, 3-[3-(cholamido-propyl)-dimethylamino]-1-propanesulfonate (CHAPS), crude collagenase (Type II), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). STZ was obtained by one of the investigators (L. G. W.) from Eli Lilly Co. (Indianapolis, IN). N^6-2-(4-aminophenyl)ethyl-adenosine (APNEA) was generously provided by Ray Olsson (University of South Florida, Tampa, FL). Electrophoretic supplies were obtained from Bio-Rad (Hercules, CA), and the autoradiography film (XAR-5) was obtained from Kodak (Rochester, NY). Male Lewis rats (225–250 g) were obtained from Harlan-Sprague-Dawley (Indianapolis, IN), and all other materials were of the highest available grade obtained from standard sources.

Animal Utilization

The methods related to animals used in this study were based on previously described techniques performed in accordance with protocols approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). Pairs of animals were housed in separate cages, and allowed free access to chow and water.

Induction of Diabetes

Experimental diabetes was induced by a single 65 mg/kg iv injection of STZ (Green and Johnson, 1991). The success of these injections was confirmed by random tail vein blood glucose measurements ≥ 22 m*M* (400 mg/dL). Less than 5% of the animals failed to attain this blood glucose level within the 48 h following injection. Animals that failed to reach this blood glucose level were reinjected.

A second cohort of experimental diabetic animals was created with an iv injection of alloxan (50 mg/kg) (Lee et al., 1988). The same criteria for establishing the diagnosis of diabetes was employed in both the alloxan and STZ groups.

Adipocyte Membranes

Adipocyte membranes were prepared in the manner previously described (Stiles et al., 1985). Epididymal fat pads were harvested from freshly killed rats and placed in ice-cold Kreb's phosphate buffer (pH 7.4) containing 128 m*M* NaCl, 10 m*M* Na_2HPO_4, 5.3 m*M* KCl, 1.4 m*M* $MgCl_2$, 3% (w/v) BSA, and 1.5 mg/mL collagenase. The pads were minced and incubated at 37°C for 45 min with constant agitation, and the dissaggregated adipocytes were aspirated and washed twice (by floatation) in the Kreb's buffer without collagenase. After the final wash, the adipocytes were placed into 10 vol of homogenization buffer composed of 10m*M* Na_2HPO_4, 5 m*M* EDTA, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, and 10^{-4} *M* PMSF adjusted to pH 7.4. The cells were disrupted with 10 strokes of a motor-driven Teflon™ homogenizer and then centrifuged at 15,000*g* for 10 min at 4°C. The fat adhering to the sides of the centrifuge tube was removed, and the pellet was resuspended in homogenization buffer and centrifuged again at 15,000*g*. This final pellet was suspended in buffer composed of 50 m*M* Tris, 10 m*M* $MgCl_2$, and 1 m*M* EDTA (hereafter referred to as 50/10/1 buffer) adjusted to pH 8.26 at 5°C. ADA was added (0.5 U/mL), and the membrane suspension was incubated with constant agitation at 37°C for 10 min. These membranes were used immediately for binding studies.

Although a single nondiabetic animal provided sufficient epididymal fat to perform a complete binding experiment, the diabetic animals experienced sufficient loss of epididymal fat that the pads from two or three animals at the identical time-point were pooled to perform one experiment. This step was necessary because it was difficult to obtain consistent membrane preparations with the small amounts of tissue found in single diabetic animals.

Cerebral Cortex Membranes

Cerebral cortical membranes were also prepared as previously described (Stiles et al., 1985). Cortex was dissected free of the remaining brain tissue and placed in ice-cold buffer composed of 50 m*M* Tris, 5 m*M* EDTA, 5 μg/mL leupeptin, 5 μg/mL soybean trypsin inhibitor, and 10^{-4}*M* PMSF adjusted to pH 7.4 at 25°C. The cortex was disrupted with 10 strokes of a motor-driven Teflon™ homogenizer and then centrifuged at 600*g* for 5 min at 4°C. The supernatant was retained, transferred to a clean tube, and centrifuged at 40,000*g* for an additional 10 min. This pellet was washed by resuspension in the same buffer and centrifuged at 40,000*g* for 10 min. The final pellet was suspended in 50/10/1 buffer containing 0.3 U/mL of ADA and incubated with constant agitation at 37°C for 15 min. These membranes were used immediately. The cerebral cortex from a single animal provided sufficient material for a given experimental time-point in both the diabetic and nondiabetic groups.

Radioligand Binding

The same procedure was employed for radioligand binding in both adipocyte and cerebral cortex membrane preparations. Membrane binding utilized a total reaction volume of 250 μL, consisting of 150 μL of membranes in 50/10/1 buffer, 50 μL of the appropriate concentration (sequential dilutions between ~0.05 and ~2.0 n*M*) of [^{125}I]APNEA (Stiles et al., 1985), and 50 μL of either water or the competing ligand. After a 60-min agitated incubation at 37°C, the bound ligand was separated from the free ligand by rapid vacuum filtration over glass fiber filters (No. 31 Schleicher and Schuell) that had been pretreated for 60 min with 0.3% (w/v) polyethylenimine. The filters were washed with three 3-mL aliquots of ice-cold 50/10/1 containing 0.01% CHAPS (w/v), and then placed in polypropylene tubes and counted in a γ-counter. The nonspecific binding (defined with 10^{-5}*M* R-PIA) was typically <10% of the total binding in adipocyte membranes and <15% of the total in cortical membranes.

Adenylate Cyclase Activity

Activity of membrane-bound adenylate cyclase activity was determined in both adipocyte (Parsons and Stiles, 1987) and cerebral cortical (Green and Stiles, 1986) membranes using [α-^{32}P]ATP as the tracer by previously published techniques. The assays were performed in triplicate using a total reaction volume of 100 μL, consisting of 50 μL of membranes, 50 μL of ATP mix, and 20 μL of effector or water. With the addition of the ATP mix, the final reaction contained 1 μCi [α-^{32}P]ATP, 25 U creatinine phosphokinase, 5 m*M* creatine phosphate, 1.35×10^{-4}*M* dATP, 10^{-6}*M* cAMP, 5×10^{-6}*M* GTP, 30 m*M* Tris (pH 7.4 at 30°C), 5 m*M* $MgCl_2$, 80 m*M* NaCl, 1 m*M* DTT, 2 U/mL ADA, and 0.1 m*M* papaverine. The reaction proceeded for 30 min at 30°C before being terminated by placing the reaction tubes in an ice bath and adding 1 mL of ice-cold stop solution (0.4 m*M* ATP, 0.3 m*M* cAMP, and 20,000–30,000 cpms of [^{3}H]cAMP). The cAMP was separated from the other reaction products using the dowex/alumina chromatography method outlined by Solomon (1974).

Photoaffinity Labeling

Photoaffinity labeling of the A_1 adenosine receptor-binding subunit was performed with the azide derivative of [^{125}I]APNEA, [^{125}I]N^6-2-(4-azido-3-iodophenyl) ethyl-

adenosine ([^{125}I]AZPNEA) (Stiles et al., 1986), in both adipose and cerebral cortical membranes. Briefly, adipose and cerebral cortical membranes were prepared as above and 1–2 mL aliquots are incubated with ~1.0 n*M* [^{125}I]-AZPNEA in foil-wrapped tubes, at 37°C for 1 h with constant agitation. At the end of this time, under subdued lighting, the labeled membranes were washed with a 10-mL aliquot of ice-cold 50/10/1 buffer containing 0.02% (w/v) CHAPS and centrifuged at 40,000*g* for 5 min. This pellet was resuspended in an additional 10 mL of 50/10/1 buffer with 0.02% CHAPS and pelleted by centrifugation at 40,000*g* for 5 min. This final pellet was then resuspended in 1–2 mL of 50/10/1 buffer without CHAPS, poured into a Petri dish resting on a bed of ice, and exposed to UV light from a UVGC-25 mineral light at a distance of 2 cm for 5 min. The photoincorporated membranes were washed once in 50/10/1 buffer, centrifuged at 40,000*g* for 5 min, and the remaining pellet was solubilized for electrophoresis as outlined below.

SDS–PAGE

Electrophoresis was performed according to the method of Laemmli (1970) in homogeneous slabs of 12% polyacrylamide gel. Samples were solubilized for 45 min in sample buffer containing 10% (w/v) SDS, 10% (v/v) glycerol, 25 m*M* Tris–HCl, and 5% (v/v) 2-mercaptoethanol, all adjusted to pH 6.8 at 25°C. After electrophoresis, the gels were dried and exposed to Kodak XAR-5 film with dual intensifying screens for 48–72 h.

Protein Content

The protein content of the membrane suspension was determined by a modification of the biuret reaction (Lowry et al., 1951) using the Pierce (Rockford, IL) BCA protein assay reagents.

Blood Glucose Measurements

Random serum glucose levels were determined from samples of tail vein blood with a Beckman (Fullerton, CA) Glucose Analyzer 2 using glucose oxidation via the oxygen rate method (Alpert, 1973).

Glycosylated Hemoglobin Measurements

The percentage of glycosylated hemoglobin was determined from 0.3-mL samples of blood drawn from selected animals. Boronate affinity chromatography columns (Endocrine Sciences, Calabassas Hills, CA) were used to separate the glycosylated hemoglobin (gHb) from the non-glycosylated hemoglobin (non-gHb), and the absorbance of each fraction was measured. The percentage of glycosylated hemoglobin

(%gHb) was then calculated using the formula:

$$\% \text{ gHb} = 100 \times (\text{absorbance gHb}) / (5 \times \text{absorbance non-gHb} + \text{absorbance gHb}) \quad (1)$$

This assay has been validated in rodents (Weide and Scheetz, manuscript in preparation).

Data Analysis

Between three and six experiments were performed at each experimental time-point. Radioligand binding data were analyzed using an exact physical-chemical model of receptor ligand binding as implemented by the LIGAND (National Institutes of Health) (Munson and Rodbard, 1980; Munson, 1992) computer program. The dose–response curves were analyzed using the ALLFIT program (National Institutes of Health) (Delean et al., 1978, 1985). The values obtained from these analysis programs were entered into a Microsoft® Excel© spreadsheet (Microsoft Corporation, Redmond, WA) and means ± SD were calculated. Differences between the mean values of these parameters was considered statistically significant at a p value < 0.05. Student's t–test was used for comparisons between two sample means, whereas ANOVA was used for comparisons between three or more sample means. These routines were contained within the Excel© program.

Acknowledgment

M. C. was supported by American Heart Association (Nebraska Affiliate) Student Research Fellowship.

References

Alpert, N. L. (1973). *Lab World* **24,** 40.

Delean, A., Munson P. J., and Rodbard, D. (1978). *Am. J. Physiol.* **235,** E97–E102.

Delean, A., Munson, P. J., Guardabasso, V., and Rodbard, D. (1985). *A user's guide to ALLFIT.* National Institutes of Health, Bethesda, MD.

The Diabetes Control and Complications Trial Research Group (1993). *NEJM* **329,** 977–86.

Downing, S. (1985). *Am. J. Physiol.* **249 (1 pt. 2),** H102–107.

Gasser, J. A., Cooper, M. B., Tan, C. B., Saggerson, E. D., and Betteridge, D. J. (1993). *Cell. Signalling* **5,** 145–153.

Green, A. and Johnson, J. (1991). *Diabetes* **40,** 88–94.

Green, R. M. and Stiles, G. L. (1986). *J. Clin. Invest.* **77,** 222–227.

Laemmli, U. K. (1970). *Nature* **227,** 680–685.

Lee, S., Scott, M., Yancey, D., Allen, J., Chang, E., Chisari, F., and Moosa, A. (1988). *Microsurgery* **9,** 217–221.

Lohse, M. J., Lenschow, V., and Schwabe, U. (1984). *Mol. Pharm.* **26,** 1–9.

Lowry, O., Rosebrough, H., Farr, A., and Randall, R. (1951). *J. Biol. Chem.* **193,** 265–275.

Munson, P. (1992). In: *A user's guide to LIGAND.* National Institutes of Health, Bethesda, MD.

Munson, P. and Rodbard, D. (1980). *Anal. Biochem.* **107,** 220–239.

Nathan, D. M. (1993). *NEJM* **328,** 1676–84.

Parsons, W. and Stiles, G. (1987). *J. Biol. Chem.* **262,** 841–847.

Pflueger, A. and Osswald, H. (1994). *Drug Dev. Res.* (abstract) **31,** 307.

Schwabe, U. R., Ebert, R., and Erbler, H. C. (1973). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **276,** 133–148.

Solomon, Y., Londos, C., and Rodbell, M. (1974). *Anal. Biochem.* **58,** 541–548.

Stiles, G., Daly, D., and Olsson, R. (1985). *J. Biol. Chem.* **260,** 10,806–10,811.

Stiles, G., Daly, D., and Olsson, R. (1986). *J. Neurochem.* **47,** 1020–1025.