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CHARACTERIZATION OF HUMAN ADIPOCYTE ADENOSINE RECEPTORS

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^{125}I-Hydroxyphenylisopropyl adenosine (^{125}I-HPIA) was used to characterize adenosine receptors in human adipocyte plasma membranes. Steady state binding was achieved after 6 h at 37°. Scatchard plots were linear, with a K_D of approx. 2.5 nM, and B_{max} of 360-1800 fmol/mg protein. (-)N^6-phenylisopropyl adenosine (PIA) was a more potent inhibitor of binding than N-ethyl carboxamido adenosine, and (+)PIA was more than 10-fold less potent than (-)PIA, consistent with A_1 adenosine receptor binding. Theophylline was a potent inhibitor of binding (IC_{50} approx. 10 μM). Photoaffinity cross-linking studies demonstrated that the receptor is a single subunit, M_r approx. 43 kDa. The findings demonstrate that the human adipocyte adenosine receptor is similar to the A_1 adenosine receptor of rat adipocytes, although its molecular weight is higher, and its affinity for HPIA is lower than that of the rat.

Adenosine has a wide range of biological actions (1). In adipose tissue, this purine nucleoside has been demonstrated to inhibit lipolysis and increase the sensitivity of both lipolysis and glucose metabolism to the actions of insulin (2-4). Adenosine is thought to act by binding to cell surface receptors (5,6). These receptors for adenosine are coupled to either inhibition or stimulation of adenylate cyclase. Distinct receptor populations have been identified as responsible for these effects on cyclase, and have been termed A_1 and A_2 adenosine receptors, respectively (5,6). Rat adipocytes possess primarily adenosine receptors of the A_1 class (7,8). Human adipocytes are also thought to possess A_1 receptors, based on the findings that adenosine, and specific A_1 receptor agonists (such as N^6-phenylisopropyl adenosine) inhibit lipolysis in these cells, presumably via inhibition of adenylate cyclase (9-11). Furthermore it has been suggested that under basal conditions, lipolysis is normally under tonic inhibition by adenosine in human adipose tissue (11).

The A_1 receptor of rat adipocytes has been characterized by binding studies, utilizing various radioactive adenosine analogs (7,12), and by photoaffinity cross-linking and photoaffinity labeling (13-15). However, no such studies have been reported for the human adipocyte adenosine receptor. In this paper we report biochemical characteriza-

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The abbreviations used are: PIA, N^6-phenylisopropyl adenosine; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HPIA, (-)-N^6-p-hydroxyphenylisopropyl adenosine, NECA, 5'-N-ethylcarboxamido adenosine, ANB-NOS, N-5-azido-2-nitrobenzoyloxysuccinimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IC_{50}, concentration causing half-maximal inhibition.

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tion of the human adipocyte adenosine receptor, using an adenosine receptor agonist that binds preferentially to the A_1 receptor, ^{125}I-hydroxyphenylisopropyl adenosine.

METHODS

Discarded human subcutaneous adipose tissue was from severely burned patients undergoing therapeutic debridement. Before use, any burned tissue was carefully dissected and removed. The study protocol was approved by the Institutional Review Board, University of Texas Medical Branch, Galveston. Rat adipose tissue was from epididymal fat pads of male Sprague-Dawley rats. Adipocytes were isolated from human and rat adipose tissue as described by Rodbell (16), with minor modifications (3).

Isolation of adipocyte membranes

Adipocytes were washed three times in a buffer consisting of 137 mM NaCl, 5 mM KCl, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 5 mM glucose, 20 mM Hepes, pH 7.4 plus bovine serum albumin (10 mg/ml). The cells were then washed once in homogenizing buffer (250 mM sucrose, 1 mM EDTA, 0.1 mM PMSF, 20 mM Hepes, pH 7.4) and homogenized in a volume of buffer equal to twice the packed cell volume, using 8 strokes of a Potter-Elvehjem homogenizer fitted with a teflon pestle. The homogenate was centrifuged for 5 min at 1,000 g, and the supernatant was centrifuged for 15 min at 16,000 g. The resulting pellet was resuspended and layered onto a "cushion" of 35% (w/w) sucrose, and centrifuged at 108,000 g for 1 h in a Sorvall SW-28 rotor. The band of membranes at the interface of the 35% sucrose was collected, diluted with homogenizing buffer and centrifuged at 16,000 g for 30 min. The resulting pellet was resuspended in 154 mM NaCl, 10 mM $MgCl_2$, 50 mM Hepes, pH 7.6. The protein concentration was measured by the method of Bradford (17) using bovine γ-globulin as a standard, and adjusted to 1-2 mg/ml.

A_1 adenosine receptor binding assay

Adenosine receptors were investigated by binding of ^{125}I-HPIA, an A_1 adenosine receptor agonist (12,18), to the membrane preparations as follows. Membranes (approx. 20 μg protein) were incubated in 154 mM NaCl, 10 mM $MgCl_2$, 50 mM Hepes, pH 7.6, adenosine deaminase (10 μg/ml) and 0.2 nM ^{125}I-HPIA, plus unlabeled HPIA (0-100 nM) in a total volume of 70 μl. After incubation for various periods at 37° the membranes were precipitated as follows. Bovine γ-globulin solution (20 μl of 12.5 mg/ml in 50 mM Hepes, pH 7.5) was added, followed by 500 μl of ice cold 15% (w/v) polyethylene glycol. Duplicate 200 μl samples were transferred to plastic 300 μl microtubes (W. Sarstedt & Co.) containing 50 μl of 15% polyethylene glycol, and centrifuged in a Beckman Microfuge for 3 min. The tips of the microtubes, containing the precipitate, were cut and counted in a γ-counter. Non-specific binding was determined in the presence of 10 μM HPIA. All data have been corrected for non-specific binding. Preliminary experiments (not shown) revealed that these conditions for measurement of A_1 adenosine receptors were optimum with respect to incubation conditions and stopping procedure.

Photochemical cross-linking of adenosine receptors

^{125}I-HPIA was covalently coupled to human A_1 adenosine receptors by a slight modification of our published technique (13), as follows. Membranes (80 μg protein) were incubated with 1 nM ^{125}I-HPIA for 6 h at 37°. ^{125}I-HPIA was then covalently cross-linked to the receptors by use of the photoactive heterobifunctional compound, ANB-NOS. The labeled membranes were analyzed by SDS-PAGE (10% gels) and autoradiography as previously described (13).

RESULTS

Figure 1 shows a time course of binding of ^{125}I-HPIA to human fat cell membranes. The time course of association was fairly slow, reaching equilibrium after approximately 6 h at 37°. After 8-10 h, binding decreased somewhat, probably as a result of receptor denaturation. This rate of association was much slower than in membranes from rat adipocytes, in which steady state was reached after only 2.5 h at the same temperature. Furthermore, the non-saturable component of ^{125}I-HPIA binding (i.e. non-specific binding) forms approximately 20% of total binding, in contrast to only about 5% in rat membranes (not shown).

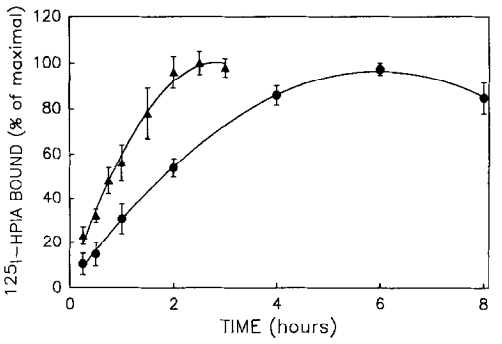


Figure 1. Time course of binding of ^{125}I-HPIA.
Human (●) and rat (▲) adipocyte membranes were incubated with 0.2 nM ^{125}I-HPIA. Binding was determined after the indicated times as described in Methods. The data are means (±SEM) of four experiments, representing four different rats, and four different human subjects, each assayed in duplicate.

Figure 2A is a competition curve, showing the effect of unlabeled HPIA on binding of ^{125}I-HPIA to human adipocyte membranes. In Figure 2B the data are presented in the form of a Scatchard plot. This analysis demonstrates a linear plot, indicative of a single affinity form of binding sites. In the example shown, the K_D, calculated from the slope of

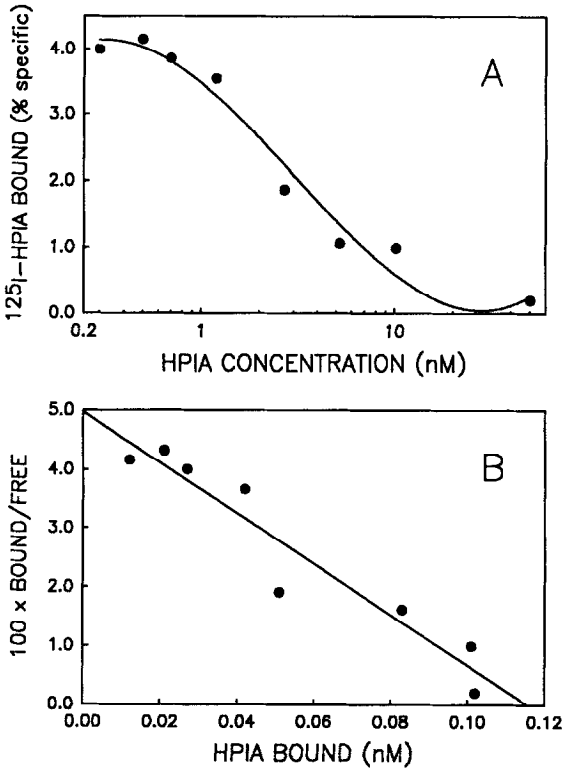


Figure 2. Equilibrium binding of HPIA to human adipocyte membranes.
Human adipocyte membranes were incubated with 0.2 nM ^{125}I-HPIA plus 0-10 nM unlabeled HPIA for 6 h. Binding was determined as described in the methods section. Panel A is a competition curve. In panel B, the data are expressed in the form of a Scatchard plot. The figure shows a representative experiment.

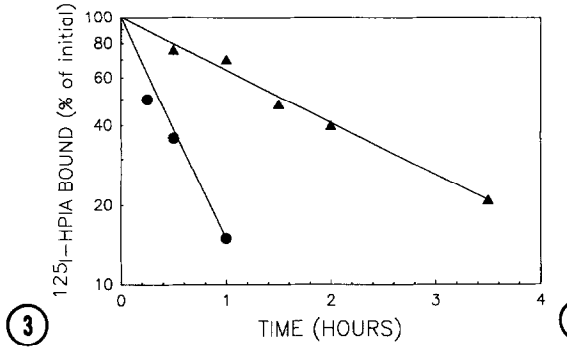
the Scatchard plot, is approximately 2.3 nM, and the B_{max} is 1600 fmol/mg protein. In the four subjects studied, the K_D ranged from 2.3 to 3.3 nM, and the B_{max} ranged from 360 to 1800 fmol/mg. These B_{max} values are similar to those reported for rat adipocytes, but the K_D is higher (in the rat it is about 0.5-0.9 nM (18,19)). This indicates that human adipocytes have similar numbers of adenosine receptors to rat adipocytes, but their affinity for HPIA is lower.

The rate of dissociation of ^{125}I-HPIA from human and rat adipocyte membranes is illustrated in figure 3. Dissociation is approximately first order in both human and rat, but the rate of dissociation is much faster in the human. The half-time for dissociation is approximately 1.5 h in the rat, but only about 15 min in the human.

Adenosine receptors were initially classified pharmacologically, based on the order of potency of various agonists (6). (-)PIA is more potent than NECA at A_1 receptors, while the reverse is true for A_2 receptors. As shown in figure 4, both (-) and (+)PIA, and NECA, inhibit binding of ^{125}I-HPIA to human adipocyte membranes. The most potent is (-)PIA, with an IC_{50} of about 0.55 nM. NECA is a less potent inhibitor (IC_{50} approx. 1 nM). Furthermore, (+)PIA (IC_{50} approx. 9 nM) is more than 10-fold less potent than (-)PIA, which is again typical of A_1 adenosine receptors, in contrast to A_2 receptors, where the two stereoisomers are almost equipotent (6).

Methyl xanthines inhibit adenosine receptor binding in all systems that have been reported (20,21). As demonstrated in figure 5, theophylline inhibited binding of ^{125}I-HPIA binding in the human adipocyte membranes. The IC_{50} was approximately 10 μM, similar to that in the rat membranes.

We have reported that ^{125}I-HPIA can be covalently coupled to rat adipocyte A_1 adenosine receptors by use of the heterobifunctional, photoactive cross-linker, ANB-NOS (13). SDS-PAGE and autoradiography demonstrate that this technique labels a 38



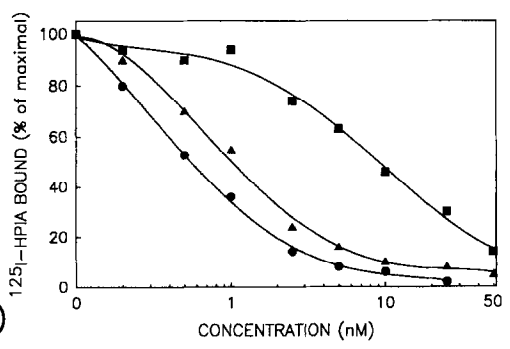
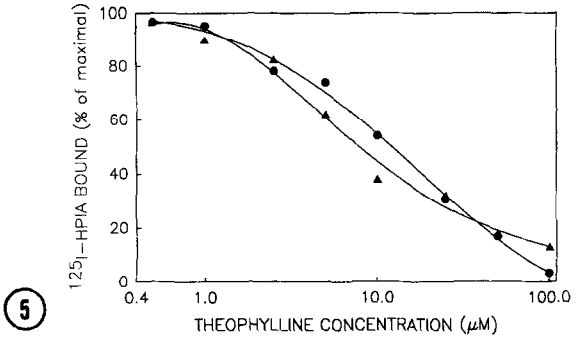


Figure 3. Dissociation of ^{125}I-HPIA from adipocyte membranes.
Human (●) and rat (▲) adipocyte membranes were incubated with 0.2 nM ^{125}I-HPIA until steady state was reached (2.5 h for the rat, 6 h for the human membranes). At time zero on the figure, 10 μM unlabeled HPIA was added, and binding was determined at the indicated times.

Figure 4. Effect of NECA and (-) and (+)PIA on ^{125}I-HPIA binding.
Human adipocyte membranes were incubated with 0.2 nM ^{125}I-HPIA and the various concentrations of (-)PIA (●), (+)PIA (■) or NECA (▲),for 6 h at 37°.



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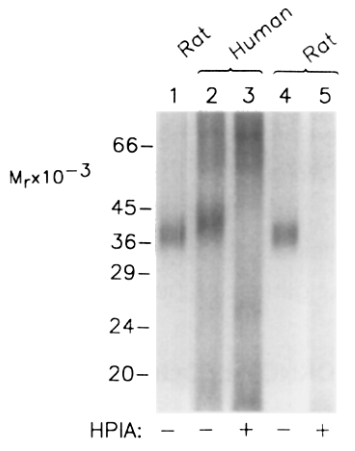

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Figure 5. Effect of theophylline on adenosine receptor binding.
Human (●) or rat (▲) adipocyte membranes were incubated with ^{125}I-HPIA plus the indicated concentrations of theophylline, for 6 h (human) or 2.5 h (rat).

Figure 6. Photoaffinity cross-linking of adenosine receptors.
Rat (lanes 1, 4 and 5) and human (lanes 2 and 3) adipocyte membranes were incubated with 1 nM ^{125}I-HPIA, and then treated with ANB-NOS and light, as described in methods. In lanes 3 and 5, excess unlabeled HPIA (10 μM) was included to detect non-specific labeling. The membranes were separated by SDS-PAGE and autoradiography. The positions and molecular weights of standards, detected by Coomassie blue staining, are indicated to the left of the autoradiogram.

kDa band. Similarly, photoaffinity labeling studies have suggested that the rat A_1 adenosine receptor has a molecular mass of approximately 36-38 kDa (14,15). When we used our technique to label human adipocyte membranes, the results shown in Figure 6 were obtained. As can be seen, a single band is labeled specifically in the human membranes, but it is slightly larger than in the rat. The calculated apparent molecular weight for the human A_1 receptor is about 43 kDa, i.e., about 5 kDa larger than the rat A_1 receptor.

DISCUSSION

We have demonstrated that human fat cell plasma membranes contain high affinity binding sites for ^{125}I-HPIA, a well characterized A_1 adenosine receptor ligand. The characteristics of binding were similar, but not identical to, the A_1 adenosine receptor of rat adipocyte membranes. Most striking among the differences were first, that the affinity of the human receptor for HPIA is much lower than that of the rat receptor. The rate of association of ^{125}I-HPIA was slower in the human membranes. Furthermore, the rate of dissociation was faster than in the rat. This combination is the probable explanation for the higher K_D in the human membranes.

A second difference between the human and rat adipocyte adenosine receptor is the somewhat higher molecular weight of the binding subunit, as determined by photoaffinity cross-linking. However, the human adipocyte adenosine receptor is very similar to the rat in terms of the effects of agonists (NECA, PIA) and antagonists (theophylline).

Together with the reports that adenosine and its analogs inhibit lipolysis in human adipose tissue (9,11), it therefore seems that human fat cells possess fairly typical A_1 adenosine receptors. Furthermore, (-)PIA is a more potent inhibitor than NECA, and the K_D is in the nanomolar range. This is in contrast to typical A_2 receptors, where NECA is more effective than PIA, and K_D's are in the micromolar range (6).

In summary, we have found that human adipocytes have adenosine receptors. Their affinity for HPIA is lower than in rat adipocytes, and their molecular weight is higher, but otherwise they seem to be typical A_1 adenosine receptors.

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