Stimulation of Glucose Transport in Rat Thymocytes by Human Albumin Preparations*

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Attempts to disclose effects of fatty acids on glucose transport in rat thymocytes led us to observe that a fatty acid-free human albumin preparation (0.2%) stimulated glucose transport dramatically and quickly. Of the next several albumin preparations tested, a fatty acid-free (acid/charcoal extracted) human albumin preparation stimulated strongly, an unextracted human albumin preparation did not stimulate, a charcoal-extracted bovine albumin preparation did not stimulate, and an unextracted bovine albumin preparation did not stimulate glucose transport. We suspected that acid charcoal extraction unmasked a stimulatory factor peculiar to human albumin. However, we have since found unextracted human albumin preparations with stimulatory activities comparable to those of extracted preparations. Lack of stimulation is the exception. Addition of fatty acids to fatty-acid free albumin did not reduce stimulatory activity, and charcoal extraction of an unstimulatory preparation of human albumin did not evoke stimulatory activity. The stimulatory activity was partially destroyed by trypsin treatment. We suspected contamination by insulin-like activity. We were unable to mimic the stimulatory effect with insulin, sulfation factor, multiplication-stimulating activity, T lymphocyte growth factor, or epidermal growth factor. In gel filtration and DEAE-sephadex chromatography, the stimulatory activity eluted with the albumin peak. Treatment with excess N-ethylmaleimide, 4,4'-dithiodipyridine, or benzoquinone did not obliterate activity. Treatment with 1 molecule of mercaptoethanol/10 mol of albumin did not stimulate activity. Treatment with 1 molecule of dithiothreitol/50 molecules of albumin did obliterate activity. From this we conclude that the stimulatory agent contains a highly reactive critical disulfide, is a minor component of the preparation, and is active at concentrations less than 3 × 10^-7 M. The cellular response was not demonstrably Ca++-dependent and appeared to be slightly antagonized by catalase.

In studies intended to test for possible effects of fatty acids on glucose transport in rat thymocytes, we found that a fatty acid-free albumin preparation stimulates glucose transport dramatically and quickly. We were curious about the effects of fatty acids on glucose transport in rat thymocytes-as seen in control experiments of Fig. 1 and all other figures, [14C]methylglucose exit as [14C]methylglucose after incubation with N-[14C]ethylmaleimide as a confirmatory method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. (13).

RESULTS

Human Albumin Preparations Stimulate Glucose Transport in Rat Thymocytes—As seen in control experiments of Fig. 1 and all other figures, [14C]methylglucose exits the thymocytes in two phases. The fast phase has a half-time of about 1-3 min (10, 14) and is essentially finished by 10 min. The slow phase has a half-time of 30-50 min. We (15) and others (16) have shown that the two phases reflect transport of two kinds of cells, active and quiescent. About ½ of the methylglucose space is in active cells, and ½ in quiescent cells. In these experiments, where cells were loaded with [14C]methylglucose, synalbumin insulin antagonist (7), or a thymosin (8) or related agent (9).

EXPERIMENTAL PROCEDURES

Preparation of Cells—Rat thymocyte suspensions were prepared as described earlier (10). Thymus glands were minced, transferred to a loose fitting Dounce homogenizer in 25 ml of medium, and crushed with one slow but firm stroke to free the thymocytes. The suspension was passed through nylon net and deposited over three layers of Percoll-containing medium (p = 1.05, 1.08, 1.10). After centrifugation for 7 min at 5000 × g, erythrocytes were on the bottom and thymocytes were between the upper two Percoll layers. They were harvested, labeled with [3H]insulin, centrifuged, and resuspended to medium at a cytocrit of about 10%. The cell medium was the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered balanced salt solution, containing β-hydroxybutyrate and lactate, described earlier (11).

Transport Tests—Cell suspensions (50 μl) were incubated with 1 μCi/ml of 3-O-[14C]methyl-D-glucose with 0.1 mM unlabeled methylglucose for 1 h, then diluted with 5 ml of medium to initiate methylglucose exit. Samples (0.75 ml) were taken at 0.2 min, 10 min, and at equal intervals thereafter to 30 or 34 min. The samples were assayed for cellular radioactivity as described earlier (11). Data are expressed as [14C]methylglucose in cells relative to that in the first sample of control cells taken 0.2 min after dilution. These were plotted against time after dilution.

Other Procedures—Thiol content of albumin was measured by the method of Grassetti and Murray (12) using 4-PDS.1 We also measured acid-precipitable 14C after incubation with N-[14C]ethylmaleimide as a confirmatory method. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Fairbanks et al. (13). Materials—Percoll and chromatographic gels were obtained from Pharmacia. 1-[4,5-3H]Isoleucine (1000 mCi/mg) and 3-O-[14C]methylglucose (0.25 μCi/μg) were obtained from New England Nuclear. Albumin, insulin, trypsin, soybean trypsin inhibitor, and catalase were obtained from Sigma; albumin was also obtained from United States Biochemical Corp. and ICN Nutritional Biochemicals. Rat T lymphocyte growth factor was obtained from Bethesda Research Laboratories. CR-multiplication-stimulating activity was obtained from Collaborative Research, Inc. Mouse epidermal growth factor was a kind gift of Dr. Stanley Cohen (Department of Biochemistry, Vanderbilt University), and rat sulfation factor was a kind gift of Dr. William D. Salmon, Jr. (Department of Medicine, Vanderbilt University).

The abbreviation used is: 4-PDS, 4,4'-dithiodipyridine.

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methylglucose for 1 h, about half of the sugar is in active cells and half in quiescent cells.

As seen in Fig. 1, the presence of fatty acid-free albumin in the efflux medium caused a larger fraction of fetal bovine methylglucose to exit in the fast phase, showing that many of the quiescent cells quickly exhibited transport activity comparable to that of active cells. It can be seen in Fig. 1 and other figures that about 10–20% of methylglucose exited slowly after exposure to fatty acid-free albumin, indicating that 60–80% of the quiescent cells were stimulated. Most of the remaining 20–40% were not stimulated with doses as high as 3% (data not shown). Fig. 1 also shows that an unextracted preparation of albumin did not stimulate. We have also failed to observe stimulation with fatty acid-free or unextracted bovine albumin from Sigma and Collaborative Research, Inc.

We were deceived by results of the kind shown in Fig. 1 into thinking that stimulation and nonstimulation are characteristics of extracted and unextracted human albumin, respectively. In fact, there is a great deal of lot-to-lot variability. We have not found another charcoal-extracted preparation as active as 70F-9310, and we have found only two other unextracted preparations (ICN 103704, lot 11276; Sigma A1653, lot 81F-9389) which may be as inactive as 30F-02271. Of six unextracted human albumins tested, one from Sigma (A2386, lot 121F-0277) was strongly stimulatory. Perhaps, strong stimulation will be found more frequently among charcoal-extracted preparations and lack of stimulation will be found more frequently among unextracted preparations, as our experience would suggest, but we must conclude that human albumin preparations typically stimulate glucose transport in thymocytes.

Adding back oleic acid and linoleic acid to charcoal-extracted human albumin in various fatty acid/albumin ratios did not reduce the stimulatory activity, so we could not attribute the stimulatory action to fatty acid poverty per se.

The fatty acid removal process (17) involved acidification with HCl in the presence of charcoal, centrifugal removal of charcoal, and neutralization with NaOH. We have not been able to elicit the stimulatory activity by acidifying (pH 3) and neutralizing human fraction V (Sigma A2386, lot 30F-02271), by alkalizing (pH 11) and neutralizing human fraction V, or by subjecting human fraction V to the entire fatty acid-removal procedure (using Darco charcoal or Norit-A and Milli-pore filtration to remove traces of charcoal remaining after centrifugation).

**Dose and Time Dependences of Glucose Transport Stimulation by Fatty Acid-free Human Albumin**—Fig. 2 shows the effects of various concentrations of albumin included in the medium with which the suspension was diluted to initiate exit. Submaximal doses stimulated a smaller number of cells. In this experiment, 0.1% albumin resulted in an obvious stimulation, and 0.05% resulted in a questionable stimulation.

Fig. 3 shows the results of several experiments in which 0.6% albumin was added after the fast exit phase was complete (active cells equilibrated by efflux). Efflux from quiescent cells was stimulated fully without any visible delay, judging from the linear extrapolation of the stimulated time course back to the control time course at the point of albumin addition.

**Partial Destruction of Stimulatory Activity by Trypsin**—Suspecting that contaminating insulin-like activity might be responsible for the stimulation, we treated the albumin with trypsin according to a procedure (18) reported to destroy insulin-like activity in albumin preparations. In fact, trypsin treatment reduced the stimulatory activity in a time-dependent and dose-dependent manner (Fig. 4).

Gel electrophoresis revealed that the main contaminant was a protein considerably larger than albumin. It was completely destroyed by trypsin treatments which affected only a minor fraction of the stimulatory activity, so we believe that this major contaminant is not the stimulant. It will be seen below that the agent is, in fact, a minor component of the albumin preparation.

**Lack of Stimulation by Known Anabolic Hormones**—Since glucose transport stimulation by serum fractions is usually attributed to insulin-like activity, we tested insulin and several anabolic factors for ability to stimulate methylglucose efflux. None of these agents stimulated when present in the medium with which the suspensions were diluted to initiate efflux. The dose ranges tested were bovine insulin, 1–100 nM; rat T lymphocyte growth factor, 0.2–2 units/ml; mouse epidermal growth factor, 1–600 ng/ml; multiplication-stimulating activity, 10–3000 ng/ml; and rat sulfation factor, 0.02–0.2 units/ml.

**Attempts to Separate Stimulatory Activity from Albumin**—

![Fig. 1. Effects of various lots of human albumin on methylglucose efflux.](image)

![Fig. 2. Effects of various concentrations of fatty acid-free human albumin on methylglucose (MeGc) efflux.](image)
We attempted to separate the stimulatory activity from albumin by gel filtration on a variety of bed types with a variety of methods. These included Sephadex G-50 with incubation medium, Sephadex G-50 with 30 mM acetic acid, Sephadex G-50 with 900 mM acetic acid and 150 mM NaCl, Sephadex G-50 with 155 mM acetic acid and 155 mM NaCl, Sephadex G-100 with 155 mM acetic acid and 155 mM NaCl, Sephadex G-200 with 155 mM acetic acid and 155 mM NaCl, and Sephadex G-200 with incubation medium. In each case, the stimulatory activity eluted in the albumin peak. Stimulatory activity coincided with the albumin peak also on DEAE-Sephadex chromatography (A-50-120; 1.5 × 12 cm; gradient elution with 75 ml of 15 mM Tris-Cl (pH 7.2) and 75 ml of 15 mM Tris, 15 mM acetic acid, 20 mM NaCl brought to pH 3 with HCl). These methods failed to distinguish the main stimulatory agent from albumin.

Effects of Thiol Reagent on Stimulatory Activity—Using the method of Grassetti and Murray (12), one can titrate about 1 thiol group/2 or 3 albumin molecules with 4-PDS. A 1% albumin solution, then, contains about 0.1 mM reactive thiol groups. Such thiol compounds as cysteine, glutathione, and dithiothreitol at concentrations less than 0.1 mM stimulate glucose transport dramatically and quickly by a mechanism which involves oxidation by molecular oxygen and is mediated by the resulting H2O2 (10). Although it seemed unlikely that such a reactivity would be exhibited only by the thiol in albumin (present at less than 1 group/molecule) and that such a reactivity, if present, could survive chromatography in oxygen-containing media, we decided to test the possibility by blocking the sulphydryl groups of albumin. Blocking agents would, of course, react with sulphydryl groups of any contaminating protein, so the broader question examined was whether reactive thiols are essential to the stimulatory agent.

Preliminary studies showed that fatty acid-free and untreated human albumin contained the same amount of 4-PDS-reactive thiols, 0.3 group/molecule. However, the fatty acid-free albumin reacted somewhat faster, and the reaction went to completion with a slightly shorter half-time. Fatty acid-free albumin samples were then treated with excess thiol reagents (4-PDS, benzoquinone, and N-ethylmaleimide) and then passed through Sephadex G-50 columns equilibrated with incubated medium to remove the excess reagents. 4-PDS reduced stimulatory activity slightly both times it was tested. Benzoquinone reacted quickly with the albumin, producing a pink product characteristic of thio-substituted benzoquinone (19). This did not reduce stimulatory activity. Likewise, al-

Fig. 3. Time course of glucose transport stimulation. Fatty acid-free human albumin (0.6%) was added at 10.3 min (arrow). This experiment was repeated four times with the same result. Lot 70F-9310 was used. MeGlc, methylglucose.

Fig. 4. Effects of trypsin treatment on stimulatory action of fatty acid-free human albumin. Albumin (Alb) (Lot 70F-9310) was dissolved at 5% in 155 mM NaCl, and pH was adjusted to 7.4. Aliquots (1 ml) were then incubated at 37°C with 0.1 mg/ml trypsin for various intervals, after which 0.1 mg/ml of soybean trypsin inhibitor was added at 0 min (●), 10 min (▲), 20 min (■), 40 min (□), and 60 min (△). In one case (△), the trypsin and inhibitor concentrations were 0.2 mg/ml. The samples were then made to 6 ml with appropriate salts to constitute the efflux medium. Neither trypsin, trypsin inhibitor, nor the combination affected basal transport (not shown). MeGlc, methylglucose.

Fig. 5. Obliteration of stimulatory activity by mercaptoethanol treatment. Albumin (Alb) (Lot 70F-9310) was dissolved at 3% (0.45 mg/ml) in 10 mM Tris-Cl buffer (pH 7.4) and pH adjusted to 7.4. Mercaptoethanol was added to 1 ml portions in various molar ratios, and the mixtures were incubated 1 h at 37°C and then passed through Sephadex G-50 columns (1.5 × 18 cm) equilibrated with incubation medium. The three 2-ml fractions from each column with greatest 280-nm absorbance were combined and diluted with medium to 0.25% albumin. Of this, 5 ml was used as the dilution medium to initiate efflux. The residue was examined in N-[2-(3-ethylthiophenyl)]maleimide binding and 4-PDS reactivity. By these criteria, the lowest doses of mercaptoethanol (Mercpt) (▲ which obliterated stimulatory activity) increased protein sulfhydryl groups slightly, whereas larger doses increased protein sulfhydryl groups in a proportional manner. MeGlc, methylglucose.
kylation of most of the sulfhydryl groups with excess N-ethylmaleimide failed to diminish stimulatory activity. The slight effect of 4-PDS may have been mediated by thiolpyridine released in the reaction with protein thiols (see below).

By contrast to thiol-reactive reagents, thiol compounds did obliterate stimulatory activity. Mercaptoethanol obliterated activity when added at low molar ratios (Fig. 5). The results suggested that the active agent contained an essential disulfide and was present in a molar ratio to albumin less than 0.1. In order to get a better upper limit estimate of the ratio of critical disulfides to albumin molecules, we treated the albumin preparations with dithiothreitol (DTT) shown in the figures (A, △) increased protein sulfhydryl groups very slightly, whereas larger doses increased sulfhydryl groups in a stoichiometric manner. These results are representative of eight such treatments of albumin (Alb) (70P-8310) with dithiothreitol. MeGlc, methylglucose.

Fig. 6. Obliteration of stimulatory activity by dithiothreitol treatment. The experiment was carried out as described in the legend to Fig. 5. Again, the doses of dithiothreitol (DTT) shown in the figures (A, △) increased protein sulfhydryl groups very slightly, whereas larger doses increased sulfhydryl groups in a stoichiometric manner. These results are representative of eight such treatments of albumin (Alb) (70P-8310) with dithiothreitol. MeGlc, methylglucose.

Fig. 7. Persistence of albumin responsiveness in Ca²⁺-depleted cells. Half of the cell preparation (O, △, □) was suspended in 10 ml of Ca²⁺-free medium, centrifuged, and resuspended in 0.4 ml of Ca²⁺-free medium containing EDTA equivalent to ½ of the Mg²⁺ and 1 µg/ml of A23187. It was loaded 1 h with methylglucose (MeGlc) in this medium and diluted 100 times with a medium differing only in that the A23187 concentration was 0.5 µg/ml. The other half (O, Δ, □) was similarly handled in normal medium. Human fatty acid-free albumin (Alb) (70P-9310) was present in the dilution medium as indicated.

Fig. 8. Slight sensitivity of albumin stimulation to catalase. The agents were all added to the diluting medium about 16 min prior to their addition to the methylglucose (MeGlc) loaded cells to initiate efflux. At the doses used, albumin (Alb) (△) and dithiothreitol (DTT) (○) stimulated comparably. Both doses of catalase virtually obliterated dithiothreitol stimulation (O, □) but reduced albumin stimulation only slightly (A, △).

Tests for Possible Roles of Ca²⁺ and H₂O₂—When the cells were prepared in Ca²⁺-free medium, loaded with [¹⁴C]methylglucose in Ca²⁺-free medium containing EDTA equivalent to ¼ of the Mg²⁺ and 1 µg/ml of A23187, and then diluted to initiate efflux in a similar medium, the stimulatory effect of fatty acid-free human albumin was as potent as usual (Fig. 7). These nearly lytic doses of A23187 in a Ca²⁺-free medium should have greatly upset the physiological control of Ca²⁺ compartmentation. We, therefore, think it unlikely that Ca²⁺ redistribution is a signal mediating the response.

In other experiments, fatty acid-free human albumin was compared with dithiothreitol as regards the effects of catalase on stimulation (Fig. 8). In these experiments, 20 µM dithiothreitol stimulated as did 0.2% (30 µM) albumin. Catalase present in the diluting medium blocked stimulation by dithiothreitol but antagonized stimulation by albumin only slightly. In another experiment with a series of lower catalase concentrations, 16 µg/ml of catalase blocked stimulation by dithiothreitol and had no effect on stimulation by albumin. If H₂O₂ is involved in albumin stimulation, it is not generated in the bulk medium.

**DISCUSSION**

The present studies show that many commercial human albumin preparations contain a factor(s) which stimulates glucose transport in rat thymocytes. Thus far, the most stimulatory preparation we have encountered was charcoal extracted for fatty acid removal, and the least active (inert) preparations were not so treated. However, a wide range of activities was found among treated and untreated prepara-
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Stimulatory human albumin preparations. Insulin, sulfation factor, nor any purified anabolic agent which were significant relative to pre-existing protein thiols). The dithiothreitol treatment (with dithiothreitol doses which we do not know whether the one(s) in lot 70F-9310 is typical.

That the agent might be altered albumin is suggested by our inability to separate it from albumin on gel filtration and ion exchange columns. That the agent might be a hormone-like peptide or protein rather than altered albumin is suggested by the fact that so few molecules bear the activity. It seems that a treatment (storage in whole blood, fractionation, extraction) to which all molecules are subjected might affect a larger fraction of them similarly. The upper limit estimate of 1 active molecule/50 albumin molecules may be a gross overestimate. Although the disulfides of the stimulatory agent are particularly susceptible to reduction by mercaptoethanol and dithiothreitol, a substantial fraction of reducing agent must have been consumed by disulfides in unstimulatory and dithiothreitol, a fraction of reducing agent must have been consumed by disulfides in unstimulatory albumin molecules. That albumin disulfides react readily was seen by the “stoichiometric” production of protein thiols in the dithiothreitol treatment (with dithiothreitol doses which were significant relative to pre-existing protein thiols).

The thymocyte response appears to be a rather specific assay for certain unidentified stimulatory agents, for neither insulin, sulfation factor, nor any purified arbovic agent which we have so far been able to test would mimic the effect of stimulatory human albumin preparations.

Stimulation by albumin is unlike that of plant lectins and A2s187 (11) and like that of H2O2 and N-ethylmaleimide (10) in that it does not appear to involve Ca2+ translocation. Catalase concentrations which block stimulation by dithiothreitol hardly affect stimulation by albumin, whereas much higher catalase concentrations appeared to interfere somewhat. This suggests a role of H2O2 generated at the plasma membrane, from which diffusion to its site of action would be favored over collision with extracellular catalase (by comparison with H2O2 generated from dithiothreitol in the bulk extracellular fluid).

From the beginning of our studies of glucose transport in thymocytes, we have used 0.2% bovine fraction V in our medium with the thought that this would reduce the likelihood of mechanical injury of the cells. We have been through numerous lots without seeing evidence of stimulation. After finding the stimulatory effect of fatty acid-free human albumin, we also tested charcoal-extracted (two lots) and unextracted bovine albumin at higher concentrations (0.4-3%) with negative results. We, therefore, believe stimulatory activity to be much more prevalent among human albumin preparations than bovine albumin preparations.

Since glucose transport stimulation is a common action of growth factors, the stimulatory agent in human albumin might behave as a growth factor in some circumstances. However, the transport stimulant which we assay is not essential to the growth-promoting action of albumin in lymphocyte tissue culture (20-23). The latter is attributable to albumin per se, and it is very consistent among albumin preparations within a species and among species.

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