Pinocytosis and Intracellular Degradation of Exogenous Protein: Modulation by Amino Acids

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ABSTRACT Intracellular degradation of exogenous (serum) proteins provides a source of amino acids for cellular protein synthesis. Pinocytosis serves as the mechanism for delivering exogenous protein to the lysosomes, the major site of intracellular degradation of exogenous protein. To determine whether the availability of extracellular free amino acids altered pinocytic function, we incubated monolayers of pulmonary alveolar macrophages with the fluid-phase marker, [14C]sucrose, and we dissected the pinocytic process by kinetic analysis. Additionally, intracellular degradation of endogenous and exogenous protein was monitored by measuring phenylalanine released from the cell monolayers in the presence of cycloheximide. Results revealed that in response to a subphysiological level of essential amino acids or to amino acid deprivation, (a) the rate of fluid-phase pinocytosis increased in such a manner as to preferentially increase both delivery to and size of an intracellular compartment believed to be the lysosomes, (b) the degradation of exogenously supplied albumin increased, and (c) the fraction of phenylalanine derived from degradation of exogenous albumin and reutilized for de novo protein synthesis increased. Thus, modulation of the pinosome-lysosome pathway may represent a homeostatic mechanism sensitive to the availability of extracellular free amino acids.

There are four sources of essential amino acids available for cellular protein synthesis: intracellular free amino acids, amino acids derived from degradation of cellular protein, extracellular free amino acids, and amino acids derived from degradation of extracellular protein. Work in our laboratories (1, 18, 27, 29) and those of others (see references 28 and 34 for review) has demonstrated the intracellular compartmentation of amino acids from these sources. Historically, degradation of serum proteins has not been considered a significant source of amino acids for de novo protein synthesis, based almost solely on the work of Eagle and Piez (11). However, new, though indirect, experimental evidence strongly suggests that alveolar macrophages can use exogenous (serum) proteins, specifically albumin, as a source of amino acids for protein synthesis in the presence of physiological (27) and subphysiological (17) levels of extracellular free amino acids. It is also known that serum proteins are internalized by both fluid-phase and adsorptive pinocytosis (12, 13, 32) and are degraded in the lysosomes (10, 12, 14, 25). Therefore, since pinocytosed serum proteins might serve as an alternative source of amino acids (to free extracellular amino acids), we hypothesized that: (a) the rate of pinocytosis might be regulated by the availability of extracellular free amino acids, and (b) under conditions of amino acid deprivation, intracellular degradation of exogenous protein would increase. We tested these hypotheses using monolayers of alveolar macrophages and employing a kinetic analysis that we have described recently (4).

MATERIALS AND METHODS

Cells: Pulmonary alveolar macrophages were lavaged from lungs of male rabbits (or guinea pigs) with calcium- and magnesium-free phosphate-buffered saline (PBS) as described previously (22). After being washed and counted, the cells were suspended in Dulbecco's PBS supplemented with 5.6 mM glucose, pH 7.4 (PBSg), plated at 2-4 × 10⁶ macrophages per 35-mm Falcon tissue culture dish (Falcon Labware, Oxnard, CA), and allowed to form adherent monolayers for 1 h at 37°C before experiments were begun. The medium used for all experiments was Dulbecco's PBS supplemented with amino acids and vitamins, as specified.

HeLa cells were grown in Eagle's minimal essential medium (MEM) supple-
mented with 10% donor calf serum, 0.075% NaHCO₃, and 20 μg kanamycin/ml. Cultures were incubated at 37°C as a closed vessel system.

Pinocytosis, Accumulation, and Exocytosis of [14C]Sucrose: Accumulation of [14C]sucrose into alveolar macrophage monolayers was determined as previously described (4). Briefly, cell monolayers were preincubated with PBSB, supplemented with various concentrations of L-amino acids and vitamins for 1 h at 37°C. Experiments were initiated by adding [14C]sucrose (negligible volume) and concluded by removing media, washing the monolayers with ice-cold PBSB, and solubilizing the cell layer with 1% SDS. Controls for accumulating radioactivity at zero time and at 4°C were performed routinely.

The kinetics of [14C]sucrose pinocytosis and subsequent exocytosis were determined as previously described (4), including control experiments and methods of data analysis. A brief summary of these procedures is provided in text and in the legend to Table I.

Degradation of Exogenous and Endogenous Protein and Reutilization of Amino Acids: Investigating protein turnover is simplified when the amino acid(s) used to monitor the rates of protein synthesis and degradation is essential and not otherwise metabolized by the cell under study. Previous studies by Woodside and Massaro (33) and Hammer and Ranzoel (17) have established that phenylalanine is suitable for monitoring protein turnover in pulmonary alveolar macrophages. In the absence of cycloheximide, changes in the concentration of extracellular free phenylalanine reflect the balance between protein synthesis and degradation and shall be referred to as net phenylalanine release. In the presence of cycloheximide (20 μM), changes in the concentration of extracellular free phenylalanine reflect protein degradation alone and shall be referred to as total phenylalanine release. We used this approach to calculate (a) the rate of intracellular degradation of both endogenous macrophage proteins and exogenously supplied proteins (i.e., bovine serum albumin [BSA]) and (b) the fraction of amino acids (i.e., phenylalanine) derived from degradation of endogenous and exogenous proteins and reutilized for de novo protein synthesis.

Macrophage monolayers were washed free of nonadherent cells and preincubated for a 1 h period with media containing 2.2 μM phenylalanine, 20 μM cycloheximide, and either 150 μM physiological levels (see below) or no additional amino acids (amino acid deprivation). Following the preincubation period the media were removed and replaced with fresh media of identical composition (except with or without 2% BSA) and incubated at 37°C for 3 h. It was during this 3-h interval that phenylalanine release into the media was monitored: the media were removed from the monolayers and acidified (pH 7.3–7.5) with 0.5 ml of 0.1 M perchloric acid and 50 μl of 1 M leucyl-alanine. Precipitate was removed by centrifugation at 2,500 rpm for 10 min at 4°C in a clinical centrifuge. The phenylalanine content of the supernatant was determined by ion-exchange chromatography as described previously (31), using the leucyl-alanine as an internal standard. Phenylalanine release was measured against a background of 2.2 μM phenylalanine, this concentration of phenylalanine is the lowest known to provide sufficient substrate for protein synthesis in alveolar macrophages (15).

Radioassay: Radioassay for 14C was performed with the scintillant, Aquasol-2, on a Packard Tri-Carb liquid scintillation spectrometer (model 3320, Packard Instrument Co., Inc., Downers Grove, IL). Quench was monitored by external standard.

Protein Determination: Protein content of solubilized cell monolayers and filtrates were determined according to the method of Lowry et al. (23) using BSA as a standard.

Assay for [14C]Sucrose Binding to BSA: To determine whether [14C]sucrose was bound to BSA under culture conditions, we centrifuged 0.5-ml aliquots of media containing [14C]sucrose, 0 or 2% BSA, and either a complete complement of amino acids or no amino acids at 800 rpm for 10 min at room temperature through conical filters designed to retain macromolecules with molecular weights greater than 25,000 (Amicon Centriflo membrane cones, type CF25, Amicon Corp., Scientific Systems Div., Danvers, MA). Protein determinations on the filtrates indicated that 95-99% of the BSA was removed by the filters. Radioassay indicated that identical amounts of [14C]sucrose were retained on the filters regardless of whether or not 2% BSA was present. Thus, 2% BSA did not bind [14C]sucrose in excess of the 5% precision of this assay.

Assay for Cathepsin D: Cathepsin D activity in cell lysates was assayed by modifying method III described by Barrett (2). Adherent cell layers were removed from culture vessels and solubilized in 0.2% Triton X-100, so as to determine total cathepsin D activity. Lysates were assayed at pH 3.0 for 30 min at 37°C. Enzymatic activities were normalized per milligram of cell lysate protein. Inhibition of activity by pepstatin A (final concentration 71 μg/ml) was used as an index of the specificity of the assay for cathepsin D (2, 8). Samples and blanks were assayed in triplicate. All reagents were kindly provided by Dr. Balvin H. Chua, Hershey Medical Center, Hershey, PA.

Materials: The following materials were obtained from the indicated sources: male guinea pigs, Canadian Breeders Laboratory, St. Constant, Quebec, Canada; New Zealand white rabbits, Charles River Breeding Laboratories, Wilmington, MA; Eagle's basal medium (BME) amino acid solution (50 x) without glutamine, MEM amino acid solution (50 x) without glutamine, MEM vitamin solution (100 x), Gibco Laboratories, Grand Island, NY; fraction five BSA (Pentex), Miles Laboratories Inc., Research Products Div., Elkart, IN; [14C]sucrose, specific radioactivity 477 mCi/mmol, Amersham Corp., Arlington Heights, IL. All other chemicals were reagent grade.

RESULTS

Effect of Amino Acids on [14C]-Sucrose Accumulation

Two approaches were used to determine whether the availability of extracellular essential amino acids altered pinocytic function. First, the time course of macrophage accumulation of [14C]sucrose was determined in three different media, one free of amino acids and two containing essential amino acids at levels frequently employed in cell culture. Fig. I illustrates that, whereas there was little difference in the response to the two amino acid mixes, omitting amino acids increased net accumulation of [14C]sucrose ~40% without noticeably changing the time course of accumulation. These results may explain why fluid-phase pinocytic rates reported by others for macro-
of amino acids, the process of fluid-phase pinocytosis and of the intracellular compartments involved and of the flux of corresponding to lysosomes) (4). It was within the context of previously established that in the presence of a physiological level between endocytosis and exocytosis (4). However, a kinetic observation that pinocytic rates reported for macrophages cultured in complete media could be attributed to the presence of physiological levels of essential amino acids, it was found that adding a complete vitamin mix to amino acid-free medium did not result in the lower pinocytic rate observed in the presence of essential amino acids (Fig. 1). That nonlinear accumulation was observed in all cases was consistent with our previous findings (4) and those of others (3) for fluid-phase markers in macrophages, an observation we have analyzed in detail recently (4).

**Effect of Amino Acids on Kinetics of [14C]-Sucrose Exocytosis**

The approach described above for quantitation of fluid-phase pinocytosis provided information concerning only the net accumulation of [14C]sucrose, which reflects the balance between endocytosis and exocytosis (4). However, a kinetic analysis of exocytosis of accumulated pinocytic marker ([4]; summarized in the legend to Table I) allowed for a more detailed examination of the pinocytic process through analysis of the intracellular compartments involved and of the flux of pinocytic marker through these compartments. We have previously established that in the presence of a physiological level of amino acids, the process of fluid-phase pinocytosis and subsequent exocytosis requires at least two intracellular compartments in series, one small compartment turning over rapidly (probably corresponding to pinosomes), and the other, apparently larger compartment turning over slowly (probably corresponding to lysosomes) (4). It was within the context of this two-compartment model (Fig. 2) that the effect of extracellular amino acid depletion on pinocytic function was analyzed.

Macrophage monolayers were allowed to pinocytose [14C]-sucrose for 20 min and the exocytosis of this marker back into isotope-free medium was expressed as the percentage of [14C]sucrose remaining inside the cell as a function of reincubation time. The resultant curves were dissected into their exponential components by "curve peeling"; the slope of each component process was taken as a measure of that component's half-life. The y-intercepts obtained by back extrapolation to time zero were taken as a measure of the distribution of the [14C]sucrose among the component processes (4). Results indicated that pinocytosis and subsequent exocytosis involved at least two intracellular compartments (processes) under all three media conditions tested (Table I). These results were consistent with previous findings (4) and with the working model shown in Fig. 2. However, the sizes of these two compartments and the associated intercompartmental fluxes were clearly affected in a prescribed way (Table I). Specifically, the size of compartment 1 appeared essentially unaffected by varying the extracellular amino acid concentration, whereas the size of compartment 2 increased 30-37% in the absence of a physiological level of essential amino acids. Moreover, although the overall uptake rate (K₁) increased only slightly (7-12%), this increase was transmitted in toto to K₂, effectively increasing the flux from compartment 1 to compartment 2 by 31-38%. Hence, the increase in fluid-phase pinocytosis in response to reducing the cellular amino acid depletion was a consequence of an increase in the size of compartment 2.

**Effect of Amino Acids on the Release of Phenylalanine from both Endogenous and Exogenous Protein Sources**

To test the hypothesis that degradation of exogenous protein is stimulated under conditions of amino acid deprivation, we measured changes in the extracellular concentration of phenylalanine in the presence and absence of a physiological level of free amino acids (Table II). In the absence of albumin, total phenylalanine release (plus cycloheximide) was unaffected by amino acid deprivation (2.24 ± 0.25 at 1X vs. 2.66 ± 0.30 at 0X). Thus, endogenous protein degradation appeared insensitive to an acute depletion in the concentration of free extracellular amino acids. In addition, the fraction of phenylalanine derived from degradation of endogenous protein and utilized for protein synthesis could be calculated from Table II. In the presence of a physiological level of amino acids (1X), total phenylalanine release (degradation) occurred at a rate of 2.24
of whether the macrophages had been cultured in the absence or presence of amino acids, and (b) the total activity of cathepsin D is 6–7 times greater in macrophages than in HeLa cells. The comparatively low level of cathepsin D in HeLa cells offers one possible explanation why Eagle and Piez (11) were unable to demonstrate use of exogenous proteins as a source of amino acids for protein synthesis in HeLa cells (see Discussion).

**Effect of BSA and Cycloheximide on Kinetics of \(^{14}C\)Sucrose Exocytosis**

The above observations indicated that increased degradation of BSA occurred in the absence of extracellular amino acids, as was predicted from increased delivery of \(^{14}C\)sucrose to a compartment believed to be the lysosomes. However, 20 \(\mu M\) cycloheximide was present to measure degradation of BSA, whereas the \(^{14}C\)sucrose kinetic studies were performed in the absence of both BSA and cycloheximide. Therefore, we assessed whether the presence of 20 \(\mu M\) cycloheximide and 2% BSA could, in and of themselves, alter \(^{14}C\)sucrose kinetics in such a way to account for the increased degradation of BSA under amino acid-deprived conditions. Irrespective of the absence or presence of extracellular amino acids, (a) cycloheximide caused compartment 2 to decrease in size by \(\sim 40\%\) and to turn over slightly faster, and (b) BSA caused compartment 2 to increase in size by \(\sim 30\%\) and to turn over slightly slower. Neither cycloheximide nor BSA appeared to affect the size or rate of turnover of compartment I. The effects of cycloheximide and BSA together were additive, resulting in little or no net effect on the steady state kinetics of \(^{14}C\)sucrose pinocytosis and subsequent exocytosis. In addition, \(^{14}C\)sucrose did not bind to BSA (see Materials and Methods). Thus, in the presence of both cycloheximide and BSA, amino acids modulated pinocytic pathway traffic.

**DISCUSSION**

Correct interpretation of pinocytic studies requires understanding the relationship between the rate of intracellular accumulation of a pinocytic marker and the actual rate of pinocytosis (4). Using the rate of intracellular accumulation of \(^{14}C\)sucrose as an index of pinocytic activity, we observed a 40–45% increase in accumulation in the absence of extracellular amino acids (Fig. 1), which is consistent with our hypothesis that amino acid deprivation would stimulate fluid-phase pinocytosis. How-
ever, because accumulation of [14C]sucrose reflects the balance between pinocytosis and exocytosis in alveolar macrophages (4), the cellular response to amino acid deprivation was examined more closely. The increase in intracellular sucrose accumulation was due to a redistribution in intracellular pathway traffic: a small increase in the pinocytic rate, Kᵢ, was observed in the absence of amino acids might result. The present findings are not consistent with this possibility. Alternatively, freshly isolated macrophages cultured for a few hours in the absence of amino acids may have increased levels of lysosomal proteases. However, Table III indicates that, at least for cathepsin D, total activity was identical for macrophages incubated in the presence or absence of extracellular amino acids.

We postulated that during amino acid deprivation, increased pinocytosis provides an increased alternate source of amino acids (derived from degradation of exogenous protein) for protein synthesis. Consistent with this postulate, Hammer and Rannels (17) reported that in the absence of extracellular amino acids protein synthesis decreased by ~30% and the presence of exogenous albumin (2%) could restore the synthetic rate to that measured in the presence of plasma levels of amino acids. On the basis of the rates of albumin degradation and protein synthesis determined by Hammer and Rannels (17), we calculate that only 36% of the phenylalanine derived from degraded albumin was required to restore the protein synthetic rate to normal. This value is in excellent agreement with our empirically determined rate of utilization for protein synthesis of phenylalanine derived from degradation of exogenous albumin (42%, see Results).

The findings reported here and those reported by Hammer and Rannels (17) are consistent in many, but not all, aspects. For example, Hammer and Rannels (17) used suspended alveolar macrophages and reported that the cells were in nitrogen balance in the presence of a physiological level of amino acids. We found monolayers of alveolar macrophages in negative nitrogen balance under similar conditions. Whether the origin of these differences results from comparing suspended with adherent cell function or whether these differences reflect variability inherent to the source of the cells (27) is not known.

The present findings, along with those of Hammer and Rannels (17), and Rannels et al. (27), provide strong experimental evidence that intracellular degradation of exogenous protein by pulmonary alveolar macrophages can provide a source of amino acids for protein synthesis. In contrast, Eagle and Piez (11) using HeLa cells concluded that degradation of exogenous protein was not a significant source of amino acids for de novo protein synthesis. There are at least three explanations for these differences. (a) The apparent rate of pinocytosis by HeLa cells is approximately 20 times lower than that of alveolar macrophages (6, 20, 21). (b) Compared with macrophages, HeLa cells have very low levels of lysosomal proteases (i.e., cathepsin D, Table III). (c) The experiments with HeLa cells were performed in culture medium containing either a complete supply of amino acids at supraphysiological concentrations or, likewise, omitting six amino acids (five essentials). We have demonstrated that macrophages cultured in medium containing physiological levels of amino acids utilize only 7% of the phenylalanine derived from degradation of exogenous protein for de novo protein synthesis. Whether omitting five essential amino acids would produce results analogous to those in the absence of all amino acids is not known.

The question remains as to whether the contribution of amino acids from degradation of exogenous protein is significant in the presence of a physiological level of exogenous
protein (0.6% compared with the 2% used in the studies presented here). In this regard, Airhart et al. (1) and Rannels et al. (27) have shown that in the presence of 10% serum (=0.6% exogenous protein) and a physiological level of free amino acids a significant fraction of the amino acid used to charge tRNA does not come from the extracellular pool. Furthermore, raising the concentration of extracellular amino acids could bring the specific activity of amino acyl-tRNA to that of the extracellular pool in the absence of serum, but not in the presence of serum (27). Thus, it appears that 10% serum may be capable of providing a significant source of amino acids for macrophage protein synthesis. Additionally, Hammer and Rannels (17) have shown that degradation of exogenously supplied BSA is linear over a range of concentrations from 0.02 to 6.0%. Calculations based on those findings, in relation to those presented here, predict that under conditions of amino acid deprivation 0.6% exogenous protein would provide 83% of the amino acids (i.e., phenylalanine) necessary to maintain a normal rate of protein synthesis by alveolar macrophages. Therefore, even at a physiological concentration of exogenous protein, it seems likely that pinocytosis coupled to intracellular hydrolysis of exogenous protein could provide a significant source of amino acids for protein synthesis, especially during periods of reduced availability of extracellular free amino acids. Whether this pathway is common to cell types other than alveolar macrophages remains to be determined.

Lastly, recent evidence from two different receptor-mediated systems indicates that amino acids may determine the intracellular pathway followed subsequent to internalization of ligand (7, 24). Thus, amino acid modulation of intracellular pathway traffic may be a characteristic of pinocytosis in general.

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