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EFFECT OF *N*-ETHYLMALEIMIDE ON LEUCINE TRANSPORT IN CHANG LIVER CELLS

I. STIMULATORY EFFECT ON NA^+-INDEPENDENT TRANSPORT AT LOW CONCENTRATIONS OF LEUCINE

TSUNEO TAKADERA and TETSURO MOHRI

Department of Physiological Chemistry, Hokuriku University, School of Pharmacy, Kanazawa 920-11 (Japan)

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Pretreatment of Chang liver cells with *N*-ethylmaleimide (0.5 or 1 mM) stimulated Na^+-independent uptake of leucine at low concentrations ($\leq$1 mM). The stimulatory effect of *N*-ethylmaleimide on the uptake of leucine measured in Na^+-replete medium was completely blocked by the addition of *b*-2-aminobicyclo[2,2,1]heptane-2-carboxylate (5 mM), which shows that the L system participates in the stimulation. The Na^+-dependent uptake of glycine was depressed by *N*-ethylmaleimide pretreatment. The stimulation of the Na^+-independent component of leucine uptake continued for at least 30 min after *N*-ethylmaleimide treatment, while the inhibition of glycine uptake was progressive with time and the Na^+-dependent uptake of leucine became depressed later, after the treatment. It has been demonstrated that treatment of cells with *N*-ethylmaleimide is capable of increasing the Na^+-independent influx of leucine and at the same time slightly decreasing the efflux of it. These results suggest that *N*-ethylmaleimide attacks the Na^+-independent system of amino acid transport at the reactive SH groups(s) of relevant protein(s) in favor of specific activation of that system in this cell.

Introduction

N-Ethylmaleimide, a sulfhydryl blocker, has frequently been used to study the necessity of the SH group in the functional activity of proteins and also of active transport systems. It has been shown that treatment of various tissues and cells with *N*-ethylmaleimide decreases the active transport of amino acids [1–6] and sugars [2,3,5,7,8]. Several other SH reagents such as *p*-chloromercuribenzoate (PCMB), *p*-chloromercuriphenylsulfonate (PCMBS) and iodoacetate also inhibit amino acid uptake by cells [4–6,9,10]. These findings strongly suggest that SH groups are directly involved in the mechanism of amino acid transport. On the other hand, authors have reported that certain kinds of amino acid and glucose have their rate of transport elevated to significantly high levels after treatment of cells with *N*-ethylmaleimide[1,11], PCMBS[12], idoacetate[11] or H_2O_2[13,14].

We have found in the present work that pretreatment of Chang liver cells with *N*-ethylmaleimide can stimulate their Na^+-independent leucine uptake, especially at low concentrations of the amino acid in the incubation medium. Na^+-dependent glycine uptake was inhibited by the same treatment. Therefore it has been suggested

Abbreviations: PCMB, *p*-chloromercuribenzoate; PCMBS, *p*-chloromercuriphenylsulfonate; *b*-BCH, *b*-2-aminobicyclo[2,2,1]heptane-2-carboxylic acid; Me-AIB, *N*-methyl-2-aminoisobutyric acid; AIB, 2-aminoisobutyric acid.

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that functionally different sites on the proteins pertaining to the two amino acid transport systems are attacked by *N*-ethylmaleimide. A preliminary account of part of this work was presented in an abstract[15].

Materials and Methods

Chemicals. ^{14}C-Labelled amino acids were purchased from the Radiochemical Centre, Amersham, U.K. *b*-2-Aminobicyclo[2,2,1]-heptane-2-carboxylic acid(*b*-BCH) was generously given by Dr. H.N. Christensen of the University of Michigan. *N*-Methyl-2-aminoisobutyric acid(Me-AIB) was prepared in the Research Laboratory of Kyowa Hakko Kogyo Co., Tokyo, and provided by courtesy of Dr. M. Naruse. *N*-Ethylmaleimide and sodium *p*-chloromercuriphenylsulfonate (PMCBS) were purchased from Sigma. Other reagents used here were of special grade from Wako Pure Chemicals Co., Tokyo.

Cell. Chang liver cells were cultured as monolayers in polystyrol petri dishes (35 mm), each with 2 ml of Eagle's minimum essential medium [16] supplemented with 45% (v/v) Hanks' salt solution containing 0.4% (w/v) lactalbumin hydrolysate and 10% (v/v) bovine serum, under 5% CO_2 in air at 37°C.

Measurement of amino acid uptake. Incubation for measurement of amino acid uptake was carried out with cells attached to the surface of dishes as confluent sheets for 2 min, unless otherwise mentioned, either in Krebs-Ringer phosphate buffer supplemented with 0.1% glucose or in a substitute in which NaCl and sodium phosphate of Krebs-Ringer phosphate buffer were replaced by equimolar concentrations of choline chloride and potassium phosphate, respectively, as specified in the Results. Krebs-Ringer phosphate buffer thus supplemented is hereafter referred to as Na^+ medium and the sodium-depleted medium as choline medium. The incubation medium contained a given labelled and nonlabelled amino acid of desired concentration expressed in the Results in total. All experiments were preceded by 30 min preincubation of cells at 37°C in the medium, similar to that in the subsequent incubations without addition of amino acid and *N*-ethylmaleimide, to deplete endogenous amino acids. Then pretreatment of cells with *N*-ethylmaleimide was performed with either of the two media mentioned above for 10 min at 37°C. *N*-Ethylmaleimide was also added to the incubation medium for measurement of amino acid uptake. Control cells were preincubated and incubated in parallel in corresponding media without addition of *N*-ethylmaleimide. To stop the assay incubation the medium was removed rapidly by aspiration and both the inside surface of the dishes and the cell layers were washed thoroughly three times, each with 2 ml of ice cold Krebs-Ringer phosphate buffer, within 15 s. From the data of efflux determination of accumulated labelled leucine, the loss of intracellular leucine in that term of washing is assumed to be some 10% of the initial. The cell sheets of each dish were scraped off with a rubber policeman, collected in 0.8 ml of 70% ethanol in a centrifuge tube, and stored at 4°C overnight. Each tube was centrifuged to obtain a clear supernatant fraction, an aliquot of which was dried on a glass filter (Whatman GF/B 24 mm) and counted for radioactivity using conventional toluene scintillation fluid. Radioactivity was converted to moles of amino acids using their specific activities in the medium, and counting efficiencies. It was affirmed experimentally using ^{14}C-labelled inulin in medium that contamination of radioactivity or unwashed extracellular space can generally be estimated to be less than 3% of the radioactivity measured as the uptake.

Measurement of amino acid uptake in extended incubation after N-ethylmaleimide treatment of cells. After cells were treated with *N*-ethylmaleimide (0.5 mM) for 10 min they were rinsed twice with Krebs-Ringer phosphate buffer, maintained in Na^+ medium containing only 2-mercaptoethanol (2 mM) up to 30 min at 37°C and checked on the 2-min uptakes of labelled leucine, glycine and Me-AIB (each 0.17 mM) in Na^+ or choline medium at the intervals indicated in the results. The uptakes were compared with those in the cells (control) incubated with amino acids in Na^+ or choline medium in parallel after preincubation for 10 min and additional incubation up to 30 min in Na^+ medium without 2-mercaptoethanol. That concentration of 2-mercaptoethanol had no effect on the transport of amino acid in normal cells for at least 15 min of treatment.

Protein determination. Cellular protein in the sediment obtained by the centrifugation mentioned above determined by the method of Lowry et al. [17] using bovine serum albumin as standard.

Results

Effect of N-ethylmaleimide pretreatment on leucine uptake in choline medium

It was shown in our laboratory that the uptake of leucine at low concentrations in Na^+ medium was significantly promoted after treatment of cells with 0.5 mM *N*-ethylmaleimide for 10 min (data not shown). Therefore the 2 min uptake of leucine by the cells was measured after treatment for 10 min with 0.5 mM *N*-ethylmaleimide over a concentration range from 0.17 to 2 mM in choline medium and compared with that of control cells. Increased of leucine uptake was remarkable at leucine concentrations of 0.17, 0.25, 0.33 and 0.5 mM, whereas it was small, or rather inhibited, in higher concentrations (Table I).

Effect of N-ethylmaleimide pretreatment on leucine uptake in the presence of system-specific model amino acids in Na^+ medium

It is shown in Table II that the stimulatory effect of *N*-ethylmaleimide on the uptake of leucine in lower concentrations in Na^+ medium is observed even in the presence of a large excess of Me-AIB, but completely cancelled in the presence of *b*-BCH. Therefore it would be deduced that those mechanisms of mediated transport of leucine at lower concentrations which are shared with transport of *b*-BCH are specifically activated by pretreatment of cells with *N*-ethylmaleimide.

Effect of N-ethylmaleimide pretreatment on glycine uptake in Na^+ medium

Glycine uptake for 2 min following 10 min of NEM treatment in Na^+ medium was significantly low compared to control uptake both in 0.17 and 0.25 mM substrate (Table III).

Residual effect of N-ethylmaleimide on the uptakes of leucine, glycine and Me-AIB in Na^+ and choline media in extended incubation

Changes in the 2-min uptakes of the three kinds of amino acid were followed after extended incubation of cells in Na^+ medium containing 2 mM 2-mercaptoethanol for up to 30 min following 10 min of treatment of the cells with 0.5 mM

TABLE I

EFFECT OF *N*-ETHYLMALEIMIDE PRETREATMENT ON LEUCINE UPTAKE IN CHOLINE MEDIUM

Cells were incubated for 2 min with 0.17–2 mM labelled leucine in the presence or absence of 0.5 mM *N*-ethylmaleimide (NEM) in choline medium after 10 min of preincubation in the medium without leucine. Each value represents the mean ± the standard error of mean of triplicate determinations. n.s., Not significant.

Leucine conc. (mM)	Uptake nmol/2 min/mg protein		Stimulation by NEM (%)	*P*
	−NEM	+NEM		
0.17	2.68±0.30	4.78±0.02	78	<0.01
0.25	2.82±0.24	5.28±0.24	87	<0.01
0.33	3.22±0.24	5.48±0.18	70	<0.01
0.5	4.02±0.22	5.58±0.08	39	<0.01
1.0	5.70±0.16	6.78±0.16	19	<0.01
2.0	9.12±0.46	7.96±0.28	−13	n.s.

TABLE II

DIFFERENCE IN THE EFFECT OF *N*-ETHYLMALEIMIDE ON LEUCINE UPTAKE IN THE PRESENCE OF TWO COMPETITIVE AMINO ACIDS IN Na^+ MEDIUM

Cells were incubated in the simultaneous presence of labelled leucine at concentrations indicated in the table and either Me-AIB or *b*-BCH in Na^+ medium with or without addition of 0.5 mM *N*-ethylmaleimide (NEM) for 2 min after 10 min of preincubation in the medium without amino acids. Each value represents the mean ± the standard error of mean of triplicate determinations.

Addition 5 mM	Leucine concn. (mM)	Uptake nmol/2 min/mg protein		Stimulation by NEM (%)
		−NEM	+NEM	
Me-AIB	0.17	4.48±0.12	6.26±0.18	40
	0.33	5.68±0.38	7.30±0.54	29
b-BCH	0.25	0.90±0.06	0.80±0.06	−11
	0.33	1.40±0.10	1.24±0.06	−11

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE PRETREATMENT ON GLYCINE UPTAKE

Cells were incubated for 2 min with labelled glycine in the presence or absence of 0.5 mM *N*-ethylmaleimide (NEM) in Na^+ medium after 10 min of preincubation in the medium without glycine. Each value represents the mean ± the standard error of mean of triplicate determinations.

Glycine concn. (mM)	Uptake nmol/2 min/mg protein		Change due to NEM (%)
	−NEM	+NEM	
0.17	11.0±0.24	7.34±0.30	−33
0.25	16.1±0.24	9.92±0.76	−39

N-ethylmaleimide. Leucine uptake measured in Na^+ medium was much lower in *N*-ethylmaleimide-pretreated cells than in control cells after 15 and 30 min of extended incubation, although it was higher in the pretreated cells shortly after treatment (Fig. 1). However, when the uptake was measured in choline medium, the increase of leucine uptake due to *N*-ethylmaleimide pretreatment was apparently larger than that observed in Na^+ medium and lasted for at least 30 min. The inhibitory effect of *N*-ethylmaleimide on the uptake of glycine was elicited just after the treatment, in agreement with the result shown in Table III, and continued during the extended incubation, progressive with time after treatment (Fig. 2). Time-dependent depression of the uptake of Me-AIB after *N*-ethylmaleimide treatment was very comparable to that of glycine uptake (data not shown). It has been recognized here that the effect of *N*-ethylmaleimide on the Na^+-independent component of leucine transport is irreversible and so is the inhibitory effect on Na^+-dependent transport of amino acid; it is even progressive after removing *N*-ethylmaleimide.

Time-courses of leucine uptake by control and N-ethylmaleimide-pretreated cells

Uptake of leucine (0.17 mM) and the effect of 1 mM *N*-ethylmaleimide pretreatment on it were examined after 0.5, 1, 2 and 5 min of incubation in choline medium (Fig. 3). A promoted leucine uptake in the treated cells, 2 to 3 times as high as control uptake, was shown over all the incubation times.

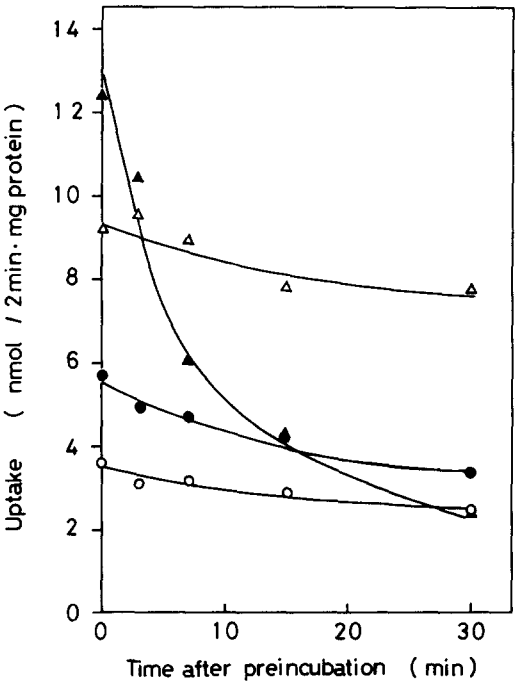

Fig. 1. Changes of leucine uptake measured in Na^+ and choline mediums with time after *N*-ethylmaleimide treatment of cells. Cells were incubated to measure the uptake of labelled leucine (0.17 mM) in Na^+ medium (▲, △) or choline medium (●, ○) for 2 min immediately (time zero in the abscissa) after 10 min of preincubation in Na^+ medium with (▲, ●) or without (△, ○) addition of *N*-ethylmaleimide or after various periods of further incubation in Na^+ medium with (▲, ●) or without (△, ○) addition of 2 mM 2-mercaptoethanol as indicated in the figure. Each point represents the mean of triplicate determinations.

Efflux of leucine from cells in the presence of N-ethylmaleimide in the medium

Dishes of cells previously loaded with labelled leucine were added to Na^+ medium for 5 min and then incubated in choline medium with or without addition of 1 mM *N*-ethylmaleimide for 0.5, 1 or 2 min at 37°C. Accumulation of radioactive leucine within the cells (time zero) and retention of it after the subsequent incubation were determined at the intervals. Semi-lorgarithmic plots of the data (Fig. 4) show that the retention curve apparently follows first-order kinetics in either group and

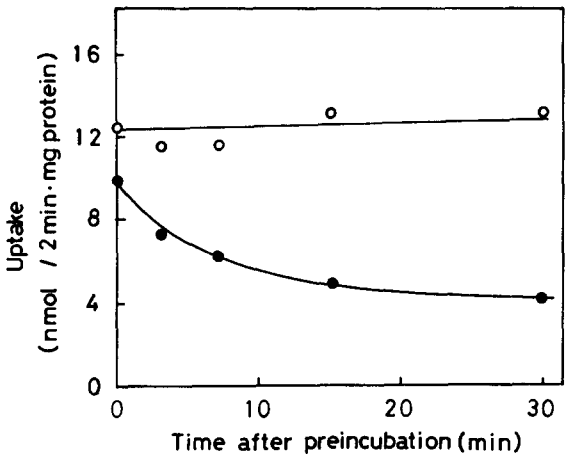

Fig. 2. Change of glycine uptake with time after *N*-ethylmaleimide treatment of cells. Experimental conditions were similar to those of Fig. 1, except that labelled glycine (0.17 mM) was used in place of leucine in the incubation after preincubation with (●) or without (○) addition of *N*-ethylmaleimide and further incubation with (●) or without (○) addition of 2-mercaptoethanol. Only Na^+ medium was employed in incubation throughout the experiment. Each point shows the mean of triplicate determinations.

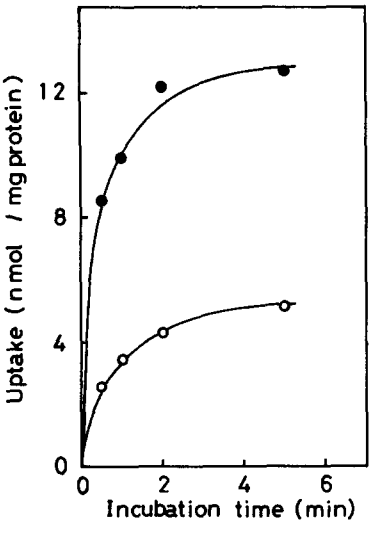

Fig. 3. Effect of *N*-ethylmaleimide pretreatment on the time-course of leucine uptake in choline medium. Cells were incubated for 0.5, 1, 2 or 5 min with labelled leucine (0.17 mM) in the presence (●) or absence (○) of 1 mM *N*-ethylmaleimide in choline medium after 10 min of preincubation in the similar medium without leucine. Each point represents the mean of triplicate determinations.

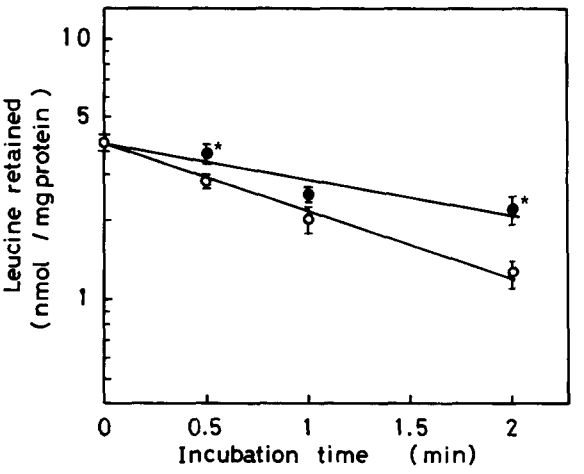

Fig. 4. Effect of *N*-ethylmaleimide on leucine efflux in choline medium. Dishes of cells were incubated previously with labelled leucine (0.17 mM) in Na^+ medium for 5 min, rapidly rinsed twice with choline medium and then additionally incubated for the various periods (0–2 min) indicated in the figure at 37°C in choline medium (2 ml per dish) with (●) or without (○) 1 mM *N*-ethylmaleimide. Accumulation (time zero) and retention of leucine within the cells after periods of incubation were determined by the same procedures as for measurement of uptake and plotted on semi-logarithmic scales. Each point represents the mean ± 1 S.E. of triplicate determinations. * The difference from control was significant ($P < 0.05$).

affords efflux values of $t\frac{1}{2} = 0.98$ min and 1.9 min, for control and treated samples, respectively.

Discussion

One of the authors and his collaborators previously reported that the plasma membrane of Chang liver cells has at least two distinct transport systems for neutral amino acids which are characterized by preferential affinities, one for glycine and the other for leucine [18]. The specificities and properties of these systems are very similar to those of the transport systems, A (preferring alanine and glycine, accepting Me-AIB as a specific substrate) and L (preferring leucine and valine, accepting *b*-BCH specifically), described earlier for the Ehrlich ascites cell [19]. Activity of system A is sensitive to sodium ion concentration, while system L is independent of it. As a matter of fact it has also been found that leucine transport is partly Na^+ dependent in the Chang liver cell. In the

present work it has been revealed that the Na^+-independent component of leucine transport is able to be specifically stimulated by pretreatment of the cells with no more than 1 mM *N*-ethylmaleimide. On the other hand, glycine transport was markedly depressed by the *N*-ethylmaleimide pretreatment both in 0.17 and 0.25 mM of substrate.

On the basis of the following experimental results, it is likely that the Na^+-dependent component of leucine transport and the transport of glycine and Me-AIB in Na^+-replete medium behave very similarly in response to *N*-ethylmaleimide; (1) glycine uptake, which was previously shown not to be affected by the presence of an excess of *b*-BCH [18], and leucine uptake in the presence of *b*-BCH in Na^+ medium were inhibited or had a tendency to decrease after *N*-ethylmaleimide pretreatment of cells (Tables II and III); and (2) the uptakes of glycine, Me-AIB and leucine measured in Na^+ medium were all directed to decrease progressively with time after *N*-ethylmaleimide treatment of cells in spite of the continuing maintenance of the Na^+-independent component of leucine uptake at stimulated levels for at least 30 min under the same conditions (Figs. 1 and 2). These results suggest that *N*-ethylmaleimide irreversibly attacks the Na^+-independent system (probably the L system) for leucine transport at the reactive SH group(s) of relevant protein(s), which results in a specific increase of the uptake of leucine at low concentrations, whereas it directly damages the Na^+-dependent system (presumably the A system). It is of interest that pretreatment of cells with PCMBS (0.1 mM) markedly reduced leucine uptake at any concentration within a range from 0.17 to 2 mM in choline medium and also glycine uptake in Na^+-replete medium (data not shown).

Feng and Riggs [11] have also shown in the rat uterus in vitro that uptakes of amino acids such as phenylalanine, cycloleucine and valine, which commonly utilize the L system as a major route of membrane transport, can be stimulated by the addition of a proper concentration range of *N*-ethylmaleimide to the incubation (2 h) or preincubation (10 min) medium.

Kwock et al. [12] reported that PCMBS and 5,5′-dithiobis(2-nitrobenzoate) can stimulate the uptake of AIB in rat thymocytes to various extents depending on the levels of the reagents. They propose that there are in those cells several categories of SH groups distinguished by their reactivities with 5,5′-dithiobis(2-nitrobenzoate), possibly embedded in the cell surface, and that titration with SH blockers of the most rapidly reacting category results in stimulation of amino acid uptake. Some important postulations have been made implying intramolecular hydrogen bonds involving SH groups and interaction between lipid double bonds and SH groups of membrane proteins [20]. From our data on the time course of the uptake of leucine (Fig. 3) it can be postulated that a large increase in the initial rate of uptake is substantially responsible for the promotion of leucine uptake due to *N*-ethylmaleimide pretreatment. The identification of the entity in the membrane that is reactive with *N*-ethylmaleimide and capable of promoting leucine transport on alkylation to help elucidate the mechanism of the *N*-ethylmaleimide effect is now under investigation in our laboratory.

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