Amino Acids (2004) 26: 133–138
DOI 10.1007/s00726-003-0052-6

Amino Acids
Printed in Austria

Mediated exodus of L-dopa from human epidermal Langerhans cells

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Received May 30, 2003
Accepted September 18, 2003
Published online December 15, 2003;

Summary. L-3,4-dihydroxyphenylalanine (L-dopa) is not metabolized within human epidermal Langerhans cells (LC); yet they can take up substantial amounts of this amino acid which subsequently can be released into the extracellular space. We recently reported that human epidermal energy metabolism is predominantly anaerobic and that the influx mechanism is a unidirectional L-dopa/proton counter-transport system and now we describe conditions for the mediated transport of L-dopa out of the LC. It is demonstrated that certain amino acids and one dipeptide can effectively trigger the efflux of L-dopa taken up by the LC.

Thus, α-methyl-dopa (α-m-dopa), D-dopa and the dipeptide, met–ala at the outside of the plasma membrane stimulated the efflux of L-dopa from L-dopa loaded LC. Similar effects were achieved by a variety of other amino acids in the extracellular fluid while some other amino acids were inactive. The time required for 50% D-methionine-induced exodus of L-dopa from L-dopa loaded LC was in the range of 5–7 min and a complete exodus of L-dopa was attained at about 20 min of incubation. This dislocation of L-dopa to the extracellular fluid is interpreted as an expression of *trans*-stimulation. In the case of α-m-dopa, D-dopa and met–ala, which admittedly were not able to penetrate the plasma membrane of LC, the concept of *trans*-stimulation was given a new purport, since none of them were able to participate in an exchange reaction. Finally, it could be concluded that L-dopa escaped by a route different from the one responsible for L-dopa uptake in LC.

Thus, while the influx of L-dopa supports extrusion of protons deriving from anaerobic glycolysis in the LC, L-dopa efflux can provide the cells with useful amino acids in an energy-saving way, altogether a remarkable biological process. From this follows that L-dopa has a biological function of its own, besides being a precursor in the catecholamine and pigment syntheses.

Keywords: Amino acid transport – Epidermis – Langerhans cells – L-dopa – Trans-stimulation

Abbreviations: α-m-dopa, α-methyl-dopa; AnB, amino n-butyric acid; AnBMe, α-methyl-n-aminobuturyl methyl ester; MeAIB, N-methyl-α-aminoisobutyric acid; DOPS, 3,4 dihydroxyphenylserine; KRB, Krebs-Ringer-bicarbonate buffer; KRP, Krebs-Ringer-phosphate buffer; LC, Langerhans cells; L-dopa, L-dihydroxyphenylalanine; ala–ala, alanine–alanine; met–ala, methionine–alanine; val–ala, valine–alanine

Introduction

We have reported the existence of a pronounced production of lactate in the human epidermis and a deficient mitochondrial apparatus in the keratinocytes (Ronquist et al., 2003). This anaerobic energy metabolism is due to the distance from the capillary system combined with a low oxygen tension in the intercellular fluid. The epidermal Langerhans cells (LC) are, in contrast to the keratinocytes, highly energy-demanding cells; they are immunocompetent, and, *i.a.*, capable of rapidly moving around among the keratinocytes (Warfvinge et al., 1989), and of performing intense endocytosis manifesting itself in the formation of plasma membrane-derived Birbeck granules (Bartosik, 1992). Followingly, the LC must be provided with efficient pumps for extruding protons. One such pump, probably set in motion only in activated LC has been disclosed. It has long been known that LC can take up large amounts of L-dopa (*cf.* Falck et al., 1993) and this uptake mechanism was shown to be different to any other described amino acid transport systems; we found the influx of L-dopa to be highly concentrative, largely pH-independent and not dependent upon the Na^+-gradient, glucose and oxygen for its energization. Instead, the L-dopa transport is most likely fuelled by a proton gradient generated by anaerobic lactate formation in human LC giving rise to a unidirectional L-dopa/proton counter-transport mechanism (Falck et al., 2003). L-dopa is not metabolized by LC (Falck et al.,

2003) but may be rapidly dislocated to the intercellular space by certain extracellular amino acids.

Thus, the biological importance of how L-dopa accumulates in LC and subsequently escapes into the extracellular fluid has been accentuated. Conceivably, L-dopa can present a different specificity pattern for entry and for exodus in human LC, which presupposes the coexistence of two apparently distinct transport systems for L-dopa. The aim of the present investigation was to define the influence of certain amino acids and one dipeptide on the exodus of L-dopa from LC residing in small epidermal biopsies, *i.e.* in a practically undisturbed microenvironment.

Materials and methods

Chemicals

The amino acids and porcine trypsin were obtained from Sigma Chemical Company (St. Louis, Mo., USA). All chemicals were of the purest grade commercially available.

Biopsies

Thin skin biopsies containing the epidermis and upper dermis were obtained by punching (2 mm) without anaesthesia from the volar forearm skin of voluntary healthy adults after informed consent.

Incubation procedures

Unless otherwise stated the *in vitro* procedure was executed as follows.

1. Loading of the epidermal LC with L-dopa was carried out in 4 ml Krebs-Ringer-phosphate buffer (KRP) containing L-dopa (4 or 10 mmol/L) and glucose (10 mmol/L), generally for 60 min at +37°C and at pH 7.4.
2. The biopsies were transferred to the same volume of KRP containing the test amino acid or dipeptide at different concentrations (see Tables 3–6) and incubated for 30 or 60 min under otherwise the same conditions. In some experiments the KRP was replaced by isotonic NaCl solution. Controls were always run concomitantly and consisted of L-dopa loaded LC incubated in corresponding buffer only.
3. In addition, uptake experiments were carried out in KRP with α-methyl-dopa (α-m-dopa) (10 mmol/L, 60 min, +37°C) and D-dopa (2–10 mmol/L, 60 min, +37°C) to study possible uptake of any of these amino acids in LC with and without a preincubation with α-methyl-n-aminobuturyl methyl ester (AnBMe) (20 mmol/L for 30 min). These incubations were generally followed by a washing in isotonic NaCl solution for 15–30 min. Competitive, inhibitory uptake experiments were also performed in KRP involving coincubation with α-m-dopa in excess and L-dopa (see Table 2), sometimes preceded by a preincubation with only α-m-dopa (10 mmol/L, 30 min).

Fluorescence histochemistry

After the incubation procedure the biopsies were placed into 0.05% porcine trypsin in KRP at +4°C. The length of time for trypsin exposure, before separation of the epidermis was possible, was 25–50 min. The epidermis was gently teased off the dermis and processed according to the Falck-Hillarp histofluorescence technique which is a highly specific and sensitive method for the visualization of certain catechol and indole derivatives. The epidermal biopsies were treated essentially as thin tissue sheets are processed for the visualization of the transmittor in adrenergic nerves (Falck, 1962; Falck et al., 1982). Briefly, the epidermal sheets were placed on a slide, corneal surface downwards, dried overnight in a vacuum chamber, then exposed to formaldehyde gas for 1 h at +80°C and mounted in liquid paraffin. This method selectively visualizes, against an essentially dark background, a population of epidermal LC capable of taking up and accumulating L-dopa (Falck et al., 2003).

Biopsies were coded and viewed in the fluorescence microscope on at least two different occasions by two observers thus unaware of the type of experiment concerned. Intracellular L-dopa uptake was evaluated from the fluorescence intensity in accordance with a previous investigation (Falck et al., 2003).

Assay of L-dopa efflux

L-dopa loaded LC were examined regarding remaining fluorescence intensity (Falck et al., 2003) after the incubation procedure. This fluorescence

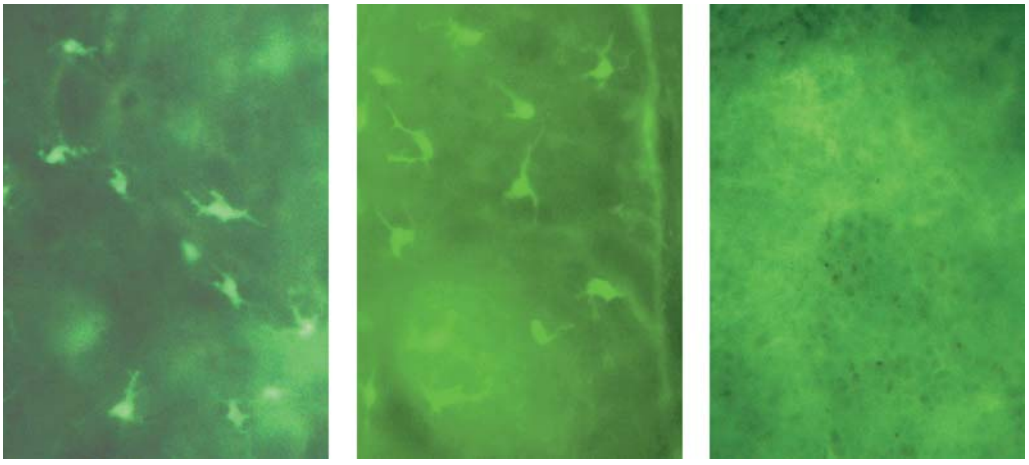

Fig. 1. L-dopa dislocation in Langerhans cells estimated by fluorescence intensity after incubation with L-dopa (see Methods) using an arbitrary stepwise scale from 0 to 3. **a** Maximal fluorescence intensity = no exodus; **b** intermediate, and **c** very low fluorescence intensity = almost total dislocation of the amino acid

intensity was evaluated according to an arbitrary scale containing 3 steps including half-steps from no exodus at all, *i.e.* 100% remaining fluorescence, to total exodus, *i.e.* 0% remaining fluorescence (Fig. 1a–c).

Results

α-Methyl-dopa is a fluorogenic compound similar to L-dopa, although the relative fluorescence intensity is only about $1/3$ of that of L-dopa (Jonsson, 1967). Hence, an uptake of α-m-dopa in LC would be possible to detect in the same fashion as for L-dopa. However, as is given in Table 1, no uptake of α-m-dopa occurred in LC, not even after preincubation with α-methyl-n-aminobuturyl methyl ester (AnBMe), a procedure resulting in an intracellular release of protons and enhanced uptake of L-dopa in LC (Falck et al., 1993). Based on this finding, competitive uptake experiments in LC were possible between L-dopa and α-m-dopa. As seen in Table 2, α-m-dopa was inefficient as an inhibitor of the L-dopa uptake in LC even when it was given in 10-fold excess over L-dopa. Albeit the incompetence of α-m-dopa being a substrate for concentrative uptake in LC and its shortcoming as an inhibitor of L-dopa uptake, it worked effectively in the facilitated efflux of L-dopa from L-dopa loaded LC and, accordingly, no L-dopa was remaining inside LC after exposure to α-m-dopa (Table 5). In the experiments where α-m-dopa was replaced by D-dopa the outcome was similar. Hence, D-dopa was not taken up to any observable extent which was confirmatory of a previous investigation (Falck et al., 2003), and did not work as a competitive inhibitor of L-dopa uptake. Still, the effect on L-dopa exodus was unambiguous and the enhancing effect was apparent already at 2 mmol/L (Table 3).

L-valine, being a physiological amino acid opposite to α-m-dopa and D-dopa, also exhibited a stimulatory effect on the efflux of L-dopa from L-dopa loaded LC already at low concentrations (Table 4). The D-methionine-induced exodus of L-dopa was observed during a 30 min period (Fig. 2). The volume of the medium was at least 1000 times the estimated cellular water of LC, and thus, a re-uptake of L-dopa was ruled out. The time required for 50% loss of the preloaded L-dopa in LC was in the range of 5–7 min, whereas a complete loss of L-dopa was achieved at about 20 min of incubation.

Various other physiological and non-physiological amino acids were tested regarding their ability to induce an enhanced efflux of L-dopa out of LC. Table 5 gives a variegated pattern in this matter and any attempt of systematization is obstructed. *E.g*. stereospecificity was not always obligatory, since both L- and D-forms of methionine were effective contrasting to L-valine being effective (Table 4), but not D-valine. On the other hand, D-leucine was superior to L-leucine. Although structurally similar (both contain an OH-group) serine was inefficient, very much in contrast to threonine. Differences also existed between kindred compounds, amino n-butyric acid (AnB) (good) versus N-methyl-α-aminoisobutyric acid (MeAIB) (poor) (Table 5). A similar discrepant behaviour was apparent also among the dipeptides. Ala–ala and val–ala were invalid contrary to met–ala (Table 6).

Table 1. Failure of α-methyl-dopa (10 mmol/L) uptake in epidermal LC with (+) or without (−) preincubation with 20 mmol/L of AnBMe

	Preincubation with AnBMe	Washing in isotonic NaCl, min	Uptake in LC	n
1	+	30	0	3
2	−	−	0	12
3	−	15	0	9
4	−	30	0	6

n, number of experiments (carried out on different occasions)

Discussion

The L-dopa transport system into epidermal LC is complex, and not yet fully elucidated. Still, accumulated evidence from our previous studies (Falck et al., 2003; Ronquist et al., 2003) points to the proton gradient as motive force across the plasma membrane of LC regulating

Table 2. Deficient competitive inhibition of α-methyl-dopa on L-dopa uptake in LC with (+) or without (−) preincubation with 10 mmol/L of α-methyl-dopa

	Preincubation with α-methyl-dopa	Incubation, α-methyl-dopa: L-dopa ratio (mmol/L)	Washing in isotonic NaCl, min	Inhibitory effect	n
1	+	0:10	−	0	3
2	+	40:4	−	0	3
3	−	40:4	30	0	10

n, number of experiments (carried out on different occasions)

Table 3. Effect of D-dopa at different concentrations and incubation times on L-dopa efflux

mmol/L	Incubation time, min	Remaining L-dopa (%)	n
0*	30&60	100	6
1*	60	15–100	5
2*	30	65–100	8
2*	60	0–100	9
5*	30	15	3
5	30	15	6
5	60	0	10

* Loading of LC using 4 mmol/L L-dopa
n, number of experiments (carried out on different occasions)

Table 4. Effect of L-valine at different concentrations and incubation times on L-dopa efflux

mmol/L	Incubation time, min	Remaining L-dopa (%)	n
0.1	60	100	3
0.3	60	15–100	6
0.5	60	0–15	3
1	30	65–100	9
1	60	0–15	6
10	30	0	4

n, number of experiments (carried out on different occasions)

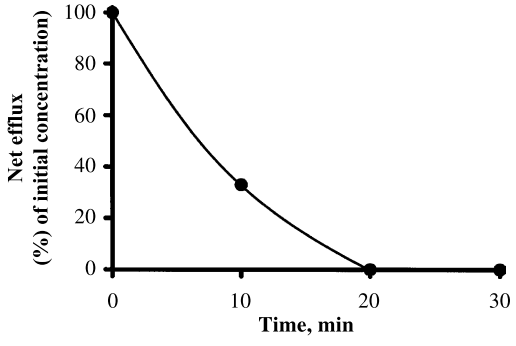

Fig. 2. Time course of D-methionine-induced net efflux of L-dopa. Each point represents results of 3 experiments carried out on different occasions with identical outcome

Table 5. Effect of various amino acids at different concentrations on L-dopa efflux

Amino acid, mmol/L	Remaining L-dopa (%)	n
α-Methyl-dopa, 10–30	0	15
MeAIB, 10–30	100	24
L-alanine, 20	65	3
D-alanine, 10–20	100	5
AnB, 10	0	14
L-cysteine, 5–10	0	12
D-cysteine, 10	65–100	6
Arginine, 10	100	3
DOPS, 7*	15	3
Glycine, 10–30	100	6
Histidine, 10	0–15	8
L-isoleucine, 10	0–15	9
D-isoleucine, 10	100	7
L-leucine, 10	65–100	21
D-leucine, 10	0	9
L-methionine, 10	0–15	3
D-methionine, 10	0	10
Norbornane**, 20	100	6
L-norleucine, 10	0	6
D-norleucine, 10	0	3
Proline, 10	100	5
Sarcosine, 10	100	5
L-serine, 10	100	5
D-serine, 10	100	3
L-threonine, 10	0–15	6
L-tyrosine, 2	0–15	11
DL-o-tyrosine, 10	0–15	6
D-valine, 10	100	8

* Loading of LC using 4 mmol/L L-dopa; DOPS, 3,4 dihydroxyphenylserine
** 2-Amino-2-norbonane-carboxylic acid
n, number of experiments (carried out on different occasions)

Table 6. Effect of 3 different dipeptides on L-dopa efflux

Dipeptide, mmol/L	Remaining L-dopa (%)	n
Ala–ala, 10–20	100	6
Met–ala, 10–20	35	5
Val–ala, 20	100	3

n, number of experiments (carried out on different occasions)

the uptake of L-dopa in these cells. A proton gradient has previously been shown to drive transport of other organic ions through plasma membranes (Crane, 1977). As shown here, stimulation of L-dopa exodus from L-dopa loaded LC still being in their intercellular context was possible by certain amino acids and one dipeptide, present in the extracellular medium.

We observed no uptake of α-m-dopa in LC, *i.e.* the cells remained non-fluorescent after exposure to this fluorogenic amino acid. Nor was it capable of inhibiting L-dopa uptake in LC. Still, α-m-dopa exerted a remarkable effect on LC in that it worked as an excellent enhancer of L-dopa efflux from L-dopa loaded LC. Hence, since α-m-dopa admittedly was not able to penetrate the plasma membrane of LC and to interact with the receptor site of L-dopa transport thus being a competitive inhibitor, it might have an allosteric-like action at the external surface of the L-dopa carrier system of the plasma membrane. By such an interac-

tion the carrier would have undergone a conformational change and therewith been brought into a permissive state of releasing intracellular L-dopa. A similar effect was most probably exerted by D-dopa. However, the registration sensitivity of uptake of D-dopa in LC is somewhat compromised by the fact that D-dopa interacts with extracellular matrix proteins in a hitherto unknown fashion leading to a general background fluorescence. Nevertheless, D-dopa, if taken up at all, was not accumulated in LC to any observable extent. Accordingly, for very good reasons we conclude that the efficient enhancing effect of D-dopa on L-dopa efflux was not the result of an exchange reaction, but rather of an interaction between D-dopa and the external surface of LC, analogous to the behaviour of α-m-dopa.

This assumed working principle for α-m-dopa and D-dopa would also be applicable for at least some of the other amino acids perhaps especially the D-isomers and one of the dipeptides supposed not to be able to penetrate the plasma membrane. However, it should be pointed out that this working principle was not a general one for amino acids, since nearly half of the tested amino acids were ineffective. Also, two of the three dipeptides were without effect. As regards the other active amino acids (other than α-m-dopa and D-dopa) we cannot exclude the possibility that perhaps most of them (D-amino acids might be excepted) could indeed be transported into LC and therefore behave differently and in line with the *trans*-stimulatory concept (see below) as this is commonly defined (Christensen, 1964). L-valine, expected to be a substrate of an L-system carrier-mediated transport into LC, was an efficient enhancer of L-dopa efflux, while D-valine, not expected to be a corresponding substrate, was completely inefficient. In this context, it is also worth mentioning that *trans*-stimulation of L-system amino acid transport has indeed been reported (Segel et al., 1988). We also noticed that arginine, as a representative of the cationic amino acids and as such with an extra charge at neutral pH and therefore expected to be transported by the y+ (formerly Ly+) system, was completely inactive. This is in line with our previous verification that the y+ system is not involved in L-dopa transport into LC (Falck et al., 2003).

An alternative explanation to the enhanced effect on exodus displayed by a majority of the amino acids tested would implicate the application to the classical term ''*trans*-stimulation'', first introduced by Christensen (1964), and implying participation in exchange of certain amino acids transported by systems demonstrating substrate selectivity and asymmetry of operation (White et al., 1982). Such *trans*-stimulatory effects on amino acid exodus were observed in different cell types as human fibroblasts (Gazzola et al., 1980), Ehrlich cells (Christensen, 1964), rabbit reticulocytes (Christensen and Antonioli, 1969), kidney slices (Schwartzman et al., 1967), the hepatoma cell line HTC (Makowske and Christensen, 1982), and cultured astrocytes (Su et al., 1995). All these examples of a *trans*-stimulated exodus of amino acids from various cells presuppose the participation in exchange as mentioned above.

In the present study we face a phenomenon where some compounds, α-m-dopa, with all probability D-dopa, met–ala, and possibly more, do not participate in any exchange, but still work as excellent effectors in a *trans*-stimulatory context. Dipeptides cannot be transported by any amino acid carrier and there is no report in the literature on ecto-dipeptidase activity at the cell surface splitting dipeptides into two amino acids (and if so, a justified question would be raised why the other two dipeptides did not work). These statements are based on observations on plasma membranes obtained in unicellular systems. Another type of barrier is the one constituted by polarized, multicellular systems like epithelial cells of the intestine and the kidney. *E.g*. a peptide transport system was described in the small intestine meaning that the mucosal uptake of amino acids was faster when they were administered as dipeptides than an equivalent amount of amino acid mixture (Matthews et al., 1969; Burston et al., 1972). Accordingly, an improvement of L-dopa absorption was achieved using an intestinal epithelial model, when a dipeptide-mimetic derivative of L-dopa, L-dopa-phenylalanine was employed (Tamai et al., 1998). These absorption studies across cells constituting a multicellular barrier differ from the present study on L-dopa influx in and efflux out of human LC representing a unicellular system and, therefore, meaningful comparisons are obstructed. As a matter of fact, in several epithelia and at the level of brain capillary endothelium, L-dopa and other large neutral amino acids were transported by the L-system, which is a sodium-independent and 2-aminobicyclo(2,2,1)-heptane-1-carboxylic acid (BHC)-sensitive transport system (Soares-Da-Silva and Serrao, 2000). This L-system-dependency is in sharp contrast to L-dopa transport into human LC (Falck et al., 2003). We may therefore conclude that the enhancing effects observed on L-dopa efflux from LC by at least α-m-dopa, D-dopa, and met–ala were the result of a hitherto unknown interacting mechanism with the external surface of the plasma membrane of LC. Herewith, a conformational change of probably the carrier protein might have been established, leading to an enhanced efflux of L-dopa. We now are confronted with a new facet of the concept of *trans*-stimulation to be valid for also a ''remote'' (*i.e*. genuine

trans-) efflux-influence of one extracellular amino acid (which does not participate in any form of exchange reaction and therefore is not influential on a mass basis on the equilibrium between intra- and extracellular amino acids) on another amino acid being in its intracellular environment. We believe that this effect is exerted via an amino acid-membrane protein interaction leading to a conformational change of this particular protein which in turn brings about that the plasma membrane becomes more permissive of L-dopa permeability. The nature of this protein is unknown, it could be the L-dopa carrier but whether this carrier is only one (bidirectional), or, more probably, two (unidirectional) is not yet established. Furthermore, we cannot exclude the possibility that more than one outward transporter might be involved. The contrast between the weak or default of ability of many of the amino acids tested (and two dipeptides) to stimulate L-dopa efflux and the strong stimulation produced by others poses intriguing questions for future studies on L-dopa uptake and efflux mechanisms in LC.

In conclusion, our studies so far demonstrate that while the influx of L-dopa supports extrusion of protons deriving from anaerobic glycolysis in the LC, L-dopa efflux can provide the cells with useful amino acids and, furthermore, – provided some of the physiological amino acids participate in an exchange reaction with intracellular L-dopa – in an energy-saving way, altogether a remarkable biological process. From this follows that L-dopa has a biological function of its own, besides being a precursor in the catecholamine and pigment syntheses.

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