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Effects of L-amino Acids on Human Peripheral Neutrophil Granulocyte Activation

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Abstract: *Objective:* The objective was to investigate the early (20 minutes) effects of 21 L-amino acids on the activation of human neutrophils and to determine in healthy individuals the effects of a meal on the 1) number and relative luminescence unit (RLU) of peripheral neutrophils, 2) serum glutamate and glucose levels and 3) mTOR signaling network.

Methods: The RLU of neutrophils stimulated by Ca^{2+} ionophor (CaI) and phorbol myristate acetate (PMA) following amino acid supplementation (3×10^{-4} M) or after consuming a meal was determined. L-glutamate was measured by HPLC.

Results: All amino acids resulted in significant inhibitions of neutrophil RLU, except for arginine, which stimulated neutrophils. The ratios of amino acid induced inhibition were significantly higher in the cells stimulated by PMA than by CaI. The consumption of a meal resulted in a significant serum glutamate elevation compared to baseline (2.3 *versus* 0.9 $x10^{-4}$ M) 90 minutes after ingestion of the meal. It was independent of the body mass index and returned near fasting levels after 150 minutes. The number of neutrophils was significantly elevated 90 minutes after the meal but the PMA induced RLU was significantly decreased.

Conclusion: Our *ex vivo* and *in vivo* results suggest that the L-amino acids, independent of their metabolic significance, may continuosly and quickly modify the activity of human peripheral neutrophils, and also the outcome of various immunologic reactions. The activation of the mTORC1 complex likely involves a transient impairment in the function of mTORC2 complex in these processes.

Keywords: L-amino acids, L-glutamate, mTOR, neutrophils, RLU.

INTRODUCTION

Plasma free amino acid concentrations have been measured for various purposes, such as to evaluate the individual's protein nutritional status, to determine amino acid requirements and circadian variations and to gain an understanding of the various metabolic aspects of amino acids [1]. Other than being components of proteins, some amino acids have individual biological or biochemical effects. Leucine [2], arginine [3], glutamine [4], and glutamate [5] have been extensively studied as signaling agents. In addition to its important role as a neurotransmitter, glutamate signaling has been described in peripherial tissues, e.g. in bone, pancreas, skin [6]. Furthermore, ionotropic glutamate receptors have been characterized in human lymphocytes [7].

Although there are some studies reporting on the effects of some amino acids on imunne modulation, there are no comprehensiuve studies. Therefore the current study investigated the effects of 21 individual amino acids on neutrophil activation. The effects of eating a simple meal on serum glutamate and glucose concentrations in healthy subjects, along with parallel measurements of the number and RLU, namely, chemiluminescence (CL) of peripheral neutrophil granulocytes, were determined. Furthermore, we measured and compared the effects of $3x10^{-4}$ M of 21 amino acids, 20 minutes into the reaction, on the RLU of peripheral neutrophil granulocytes, stimulated and not stimulated by CaI and PMA *ex vivo,* in relation to the mTOR (mechanistic target of rapamycin) signaling network; thereby testing the dose dependent effect of rapamycin (R). Aminoacids have been shown to not only activate the mTOR1 complex but are also required for activation by other stimuli [8].

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SUBJECTS, MATERIALS AND METHODS

Donors

A total of 34 healthy individuals (23 women, 18-51 years of age and 11 men, 17-60 years of age) free from disease and not consuming drugs or medications were included in the study. Fifteen of them took part in both the *in vivo* (two times) and *ex vivo* studies (five times). The protocol was approved by the Institutional Review Board, and informed consent was obtained from all participants. The body mass indexes (BMI) were determined from height and weight.

Breakfast

After a 12 hour fast, the volunteers of the study were asked to eat 28 g of commercial cheese and 88 g of salt rolls at 8 am. The meal contained 16.8 g of protein, 59.4 g of carbohydrate and 10.5 g of fat. Water was consumed *ad libitum.*

Blood testing

Prior to consumption of breakfast and 90 minutes following breakfast, serum samples were prepared from each subject for testing of glucose and glutamate levels. Additionally, in 3 subjects the levels of glutamate were measured at 150 and 210 minutes after the meal.

Materials

Calcium ionophore (ionomycin) (CaI), phorbol 12-myristate 13-acetate (PMA), rapamycin (R), luminol (5-amino-2, 3-dihydro-1, 4-phtalazinedione), and the 21 L-amino acids were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Meaurement of Glucose and L-glutamate

The measurement of glucose was carried out by an automated hexokinase/glucose 6 phosphate method (Roche) and glutamate was determined by RP- HPLC. The analytical methods for the determination of free amino acids in serum are well known [9]. The deproteinization of samples by methanol was followed by derivatization with phenyliso-thiocyanate (PIRC) to form stable phenylthiocarbamylamino acid (PTCA) derivatives. The derivatives were separated by RP-HPLC and the glutamate peaks were evaluated using a D-6000 interface and a D-7000 system-maneger softwere (Merck-Hitachi, Burladingen, Germany).

Measurement of RLU of Human Neutrophils Stimulated by CaI or PMA ex vivo

The erythtocyte-neutrophil rich pellets of Ficoll-Uromiro gradient centrifugation were sediment by dextrane at 37 ^{o}C for 45 minutes. The erythrocytes contaminating the suspensions of neutrophils were lysed by hypotonic NH_4Cl. The final suspensions of neutrophils were adjusted to 10^7/ml in (methyl-red free, colourless) Hank's culture medium.

The purity and viability of cells were higher than 95 %. Neutrophils (5×10^6/ml) were stimulated by 10^{-7}M of calcium ionophore (CaI) or 10^{-9}M of PMA in the presence of 10^{-7}M of luminol. The RLU of cells was measured by a luminometer (Bertold, Germany) and its value was expressed in relative luminescence unit (RLU). The RLU values measured at the 20th minute time periods were compared in the stimulated and not stimulated cell suspensions in the absence or presence of 3×10^{-4}M of each amino acid, dissolved in Hank's solution.

For a stock solution, R was dissolved in ethanol from which further dilutions were made using Hank's solution. The final concentrations of R were as follows: 10^{-8}M (R1), 10^{-7}M (R2), 10^{-6}M (R3).

Testing of the Viability of Neutrophils

The viability of neutrophils was tested after 20 minutes using the Trypan-blue exclusion test and evaluated micro-scopically (never falling below 90%) or the measurement of lactate dehydrogenase release by an automated laboratory equipment (Roche) (never exceeding the value of 10 U/l).

Statistical Analysis

The results represent the mean $\pm$ SD values of at least three repeated experiments. Normal distribution of data was tested using the Kolmogorov-Smirnov test. Kruskal-Wallis test was used to compare the groups of data in the *in vivo* experiments. Data of *ex vivo* investigations were analysed for statistically significant differences using paired *t* and Pearson correlation tests. The difference between the various groups was tested by the analysis of variance (ANOVA) with Tukey *post hoc* test. The correlation coefficients were calculated using the Spearman rank test. The statistical analysis was performed by the SPSS 15.0 statistical software (SPSS Inc. Chicago, IL). The statistical significance was defined as $p \leq 0.05$.

RESULTS

Changes in the Serum Levels of L-glutamate and Glucose Measured Before and After Breakfast

Thirty one healthy subjects were tested *in vivo* after a 12 hours fast and 90 minutes after eating breakfast for changes in serum glutamate and glucose concentrations. The levels of glutamate increased significantly from baseline after the meal (0.94 ± 0.41 *versus* 2.28 ± 1.76 x 10^{-4}M. p<0.001), as well as the serum glucose concentration 4.36 ± 0.54 to 5.06 ± 0.99 x 10^{-3}M (p<0.001).

The increase in the serum glutamate levels was independent of the BMI (correlation coefficient = 0.20, n.s.); whereas the glucose elevation was dependent on BMI (correlation coefficient = 0.53, p< 0.003).

In three healthy donors, the increased serum glutamate levels returned to near fasting levels 150 minutes after the breakfast. (These data are not presented.)

Changes in the Number and RLU of Peripheral Neutrophils, 90 Minutes after Breakfast in Healthy Subjects

In 12 healthy subjects, the number of circulating neutrophils was significantly elevated 90 minutes after breakfast (3.8 ± 1.7 *versus* 4.40 ± 2.1 G/L p< 0.05) tested *in*

Table 1.   **The *ex vivo* effects of various L-amino acids on the RLU of human neutrophils stimulated or not stimulated by CaI and PMA.**

Amino acids with non polar, aliphatic groups	Cells+Amino acids RLU % means(n=3)	Cells+Amino acids +CaI RLU % means(n=3)	Cells+Amino acids +PMA RLU % means(n=3)
Glycine	55*	46*	32**
Alanine	91	95	66
Valine	82	94	67
Leucine	92	103	57*
Isoleucine	87	104	67
Proline	57*	57*	34**
Methionine	49*	48*	14**
Amino acids with aromatic groups	**Cells+Amino acids RLU % means(n=3)**	**Cells+Aminio acids +CaI RLU % means(n=3)**	**Cells+Amino acids +PMA RLU % means(n=3)**
Thyrosine	40*	52*	6***
Tryptophan	110	82	14***
Phenylalanine	51*	44*	25**
Aminoacids with uncharged groups	**Cells+Amino acids RLU % means(n=3)**	**Cells+Amino acids +CaI RLU % means(n=3)**	**Cells+Amino acids +PMA RLU % means(n=3)**
Serine	44*	38**	15***
Threonine	47*	43*	16***
Cysteine	36**	37**	2***
Cystine	69	75	35**
Asparagine	47*	49*	15***
Glutamine	46*	42*	20**
Aminoacids with negatively charged groups	**Cells+Amino acids RLU % means**	**Cells+Amino acids +CaI RLU % means**	**Cells+Amino acids + PMA RLU % means**
Glutamate	48* (n=15)	42* (n=15)	21** (n=15)
Asparagine acid	47* (n=3)	49* (n=3)	15*** (n=3)
Aminoacids with positively charged groups	**Cells+Amino acids RLU % means(n=3)**	**Cells+Amino acids +CaI RLU % means(n=3)**	**Cells+Amino acids + PMA RLU % means(n=3)**
Lysine	39**	48*	20**
Histidine	37**	56*	22**
Mean ± SD (n=20)	*58.70 ± 21.80*	*60.20± 22.82*	*28.15 ± 20.33******
Arginine	*244 (NO)***	*191(NO)***	*93 (NO)*

RLU: relative luminescence unit (chemiluminescence); CaI: Ca^{2+} ionophore (ionomycin); PMA : phorbol myristate acetate; NO: nitric oxide.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

vivo. The RLU of the circulating, not stimulated neutrophils (standardized on the same cell count) was also significantly increased (273± 129 *versus* 342± 46 RLU, p<0.05), whereas the PMA induced RLU of these cells was significantly decreased (2200 ± 751 *versus* 1681 ± 519 RLU, p< 0.05), measured *ex vivo* in whole heparinized blood.

The *ex vivo* Effects of Various L-amino Acids on the RLU of Human Neutrophils Stimulated or Not Stimulated by CaI and PMA

The RLU data of purified neutrophils, stimulated or not stimulated for 20 minutes by CaI and PMA *ex vivo* in the presence of 21 L-amino acids, $(3 \times 10^{-4} M)$ are depicted in Table **1**. In the Table, a RLU value measured in the presence of a certain amino acid was expressed as a percent of the RLU found in the absence of the amino acid (RLU=100 %); only the statistical means of the RLU data (derived from at least three measurements) for each amino acid and the levels of significance are depicted. The following conclusions can be drawn: a.) the effects of some amino acids (alanine, valine, leucine, isoleucine, tryptophan) were not significant on the CaI stimulated and non-stimulated cells compared to other amino acids resulting in significant inhibitions in all experimental systems (except for arginine); b.) the CL of neutrophils was significantly lower (28.15 ± 20.33 *versus* 60.20 ± 22.82; RLU, $p<0.001$) in the cells stimulated by PMA than in those stimulated by CaI in the presence of 20 amino acids even at a much lower concentration of PMA ($10^{-9} M$) than that of CaI ($2 \times 10^{-7} M$); c.) arginine alone increased significantly the RLU in the non-stimulated cells and in those stimulated by CaI. These data are shown in Table **1**.

The *ex vivo* Effects of Rapamycin on the RLU of Human Neutrophils Stimulated by CaI and PMA in the Presence and Abscence of L-glutamate

Table **2**. shows the changes in RLU of neutrophils, with or without glutamate, stimulated by CaI and by PMA in the absence or presence of three different doses of rapamycin (R), a blocking agent of mammalian mTORC1 complex 1 [8]. The final concentrations were as follows: $R1:10^{-6}$, $R2:10^{-7}$, $R3:10^{-8} M$, L-glutamate: $3 \times 10^{-4} M$. The three concentrations of R decreased the RLU of neutrophils stimulated by CaI. This inhibition, however, was only significant at $10^{-7} M$ (RLU: 59%). In the non-stimulated cells, R caused a minimal non significant elevation. The inhibitions caused by the combination of R and glutamate were additive but not significant.

Testing the changes in RLU of neutrophils stimulated by PMA in the presence and abscence of three different doses of R and with or without L-glutamate, R resulted in a weak, non significant, non dose dependent increase in the RLU of both stimulated and not stimulated neutrophils. In addition, the presence of R did not alter the inhibition caused by glutamate in the PMA stimulated cells. Whereas, R slightly increased the RLU of PMA free neutrophils even in the presence of glutamate.

DISCUSSION

Knox van Dyke summarized the main processes of neutrophil RLU as based upon the activation of NADPH oxidase, producing superoxide anions, followed by the participation of dismutases and myeloperoxidase [10]. In both CaI or PMA induced RLU reactions of neutrophils, the mobilization of intracellular Ca^{2+}, the production of diacyl glycerol (DAG) by phopholipase C, furthermore, the production of arachidonic acid by phospholipase A_2 enzymes triggering PKC enzymes have been shown to play crucial roles [11-14]. NADPH oxidase can be activated by two types of protein kinase C isoenzymes 1.) requiring both Ca^{2+} and DAG, 2.) requiring only DAG [12-14].

L-glutamate is the most widely investigated amino acid as a signaling molecule [6], having receptors on human lymphocytes [7]. The current *in vivo* study showed that glutamate concentration increased significantly 90 minutes after breakfast, it reached the values of $2.28 \pm 1.76 \times 10^{-4}$ M but it returned near the fasting value at 150 minutes. For this reason a nearly similar concentration of 3.0×10^{-4} M was used for the other amino acids in the *ex vivo* studies. Similar *in vivo* investigations were carried out also in Taiwan in 1999 [1]. In this study, the circadian variations in the plasma levels of glutamate, glutamine and alanine were measured in 10 healthy men fed ordinary Taiwanese meals (included breakfast, lunch and dinner). The mean age of subjects was 24 years, the mean body weight was 63.8 kg, and the mean height was 170 cm. In our 34 heathy donors, the number of men was 11, the number of women was 23, with a mean age of 40.9 years, mean weight of 73.7 kg and mean height of

Table 2.   **The *ex vivo* effects of rapamycin on the RLU of human neutrophils stimulated by CaI and PMA in the presence and abscence of L-glutamate.**

Samples	Cells	Cells +CaI	Cells+R1	Cells +R1 +CaI	Cells+R2	Cells +R2 +CaI	Cells+R3	Cells +R3 +CaI
Cells without glutamate RLU% means (n=3)	100	100	105	78	110	*59**	115	76
Cells with glutamate RLU% means (n=3)	48*	42*	39**	37**	28**	35**	49*	31**
Samples	**Cells**	**Cells +PMA**	**Cells+R1**	**Cells +R1 +PMA**	**Cells+R2**	**Cells +R2 +PMA**	**Cells+R3**	**Cells +R3 +PMA**
Cells without glutamate RLU% means (n=3)	100	100	105	118	110	115	115	105
Cells with glutamate RLU% means (n=3)	48*	21**	35**	20**	53*	23**	68*	19***

RLU: relative luminescence unit (chemiluminescence); CaI: Ca^{2+} ionophore (ionomycin); PMA: phorbol myristate acetate; R: rapamycin; *RLU% means (4x);
** p< 0.01; *** p< 0.001

168.9 cm. However, there were stiking differences in the levels of glutamate measured in the two groups. In the Taiwanese group, both the fasting and the maximum concentrations of glutamate after breakfast were approximately half of the concentrations found in the present Hungarian cohort while consuming similar amounts of protein. These differences may be due to different mean ages (24 *versus* 40.9 years), different distributions of the genders and other components of the diets [15].

The testing of the neutrophils in the healthy donors, 90 minutes after ingesting a meal resulted in the following: 1.) The number of neutrophils and their RLU increased significantly in the blood. We speculate that the elevation in the number of circulating neutrophils is the result of cortisol, produced and released after the meal and thereby stimulating vessel wall adhered neutrophils to enter the circulation [16, 17]. The increased RLU observed might be due to the increase in serum glucose levels providing for an oxidative burst by the neutrophils [18], The activation of neutrophils by PMA in the blood, 90 minutes after the meal was significantly reduced compared to the pre-meal baseline. These findings suggest that following a high protein meal, the appearance of a transient immune-compromised state can occur. Of concern may be the impact of high protein intakes in body builders or other conditions where high protein intakes are recommended.

In order to test whether the decreased RLU of neutrophils could be related to the increased serum level of glutamate or that of other amino acids, the effects of all 21 L-aminoacids on the RLU of neutrophils stimulated and non stimulated by Ca^{2+} ionophore (CaI) and PMA, were tested *ex vivo*. Most of the amino acids used at this concentration caused significant decreases in the RLU of stimulated and non stimulated cells at the 20 minute measurement. The RLU values were significantly lower in the cells stimulated by PMA than that by CaI evaluated as means of 20 amino acids, although, the inhibitions caused by alanine, valine, leucine, isoleucine and tryptophan were minimal in the CaI stimulated and non stimulated cells. On the other hand, arginine induced a rather intensive RLU in the non stimulated and CaI stimulated cells, likely through the production of NO reacting with luminol. For the structural classification of aminoacids the table from the handbook "Lehninger Principles of Biochemistry" was used [19].

The mechanistic target of rapamycin on signaling pathways, that sense and integrate a variety of environmental cues to regulate organismal growth and homeostasis, is via the mTOR pathway. mTOR is an atypical serine/threonine proteine kinase that belongs to the phosphoinositide 3 kinase (PI3K) related kinase family and interacts with several proteins to form two distinct complexes named, mTOR complex1 (mTORC1) and 2 (mTORC2). mTORC1 regulates cell growth and cell cycle progression; amino acids activate, rapamycin inhibits its activity. mTORC 2 is related to cytoskeletal organization, cells survival and to $PKC\alpha$. In the linkages between mTORC1 and mTORC2 complexes the various protein kinase (A,G,C), phospholipase (A_2 and D) isoenzymes and the actual intracellular Ca^{2+} levels play determinate roles [14, 20-23].

The involvement of mTOR receptors in the reactions tested in our experiments may occur via the following mechanisms: 1) by changing the available intracellular Ca^{2+} levels required for NADPH oxidase; 2) by the linkage of mTORC2 to the phosphorylation of PKC isoenzymes needed for the activation of NADPH oxidase [12, 22]. Amino acids can induce conformational changes of mTORC1 and can increase the intracellular level of Ca^{2+} enhancing its interaction with Ca^{2+}/CaM (calcium/calmodulin dependent) kinase, causing a relative lack of calcium for other pathways [22]. Although amino acids might elevate the intracellular Ca^{2+} level during the activation of mTORC1, its consumption by Ca/CaM kinase could result in a relative lack of available calcium for the activation of $PKC\alpha$ related to mTORC2 pathway, causing a weaker stimulation of NADPH oxidase. The activation of the mTORC1 complex involves a transient impairment in the function of mTORC2 complex in these processes. In the greater inhibition of neutrophil RLU (expressed in per cent and not in absolute RLU values) caused by the amino acids in the system stimulated by a much smaller (10^{-9} M) dose of PMA than in that where the cells were stimulated by a much higher dose (10^{-7} M) ionomycin, different pathways could play the major roles. Namely, PMA stimulated RLU of human neutrophils may represent a more sensitive and specific pathway related to the PKC isoenzymes in which any intracellular metabolic change can result in a greater alteration than in the pathway involving the Ca^{2+} dependent ion channels stimulated by Ca^{2+} ionophore. We chose the 20th minute time period to be the standard time for comparision of the various test systems, because this was the time of peak RLU with PMA. In addition, any toxic effect of aminoacids tested *ex vivo* could be excluded still at this early time of treatment. The comparison of changes induced by the amino acids in the PMA and CaI stimulated neutrophils was in percent change from the untreated cells which represent 100 percent in each system.

Rapamycin, at 10^{-7}M concentration inhibited significantly the RLU of neutrophils stimulated by CaI, and showed some additive inhibitory effect along with glutamate. On the other hand, R was basicly ineffective on the RLU of PMA stimulated cells either in the presence or absence of glutamate, suggesting an opposite effect compared to the results of rapamycin-free systems. We suggest that these observations can be explained by the direct effects of R on calicium metabolism, on the preservation of Ca^{2+} homeostasis and prevention of Ca^{2+} overload via extrusion of calcium surplus [22, 23], and not only by its reaction with mTORC1.

It has to be stressed, however, that in the *ex vivo* amino acid experiments we tested only their early (20 minutes) effects and not their influence on the protein metabolism of these cells appearing later (after hours or days) [24]. We think that the differences in the early *in vivo* and *ex vivo* effects of the single amino acids can be related to the differences in their penetration times and concentrations across the cell membrane. For example, for cystine and glutamate special transporter molecules are expressed in the human peripheral neutrophils [25] (both amino acids caused really great inhibition), whereas other amino acids may

require a longer time or other transporters to enter these cells [26].

Confirming our results, articles various inflammation modulating effects of L-glutamate [27] and L-arginine [28] were observed *in vivo*.

CONCLUSION

In conclusion, our *in vivo* and *ex vivo* experimental data suggest that the L-amino acids, independent of their metabolic significance, may continuosly and quickly modify the activity of human peripheral neutrophil granulocytes and also the outcome of various immunologic reactions [27, 28]. Both the mTORC1 and mTORC2 axises of mTOR signaling pathway can be involved in these processes, as the activation mTORC1 complex by the amino acids involves a transient impairment in the function of mTORC2 complex in these processes. [20,29]. These findings suggest that high protein diets may have adverse effects on immune modulation.

ABBREVIATIONS

BMI	=	body mass index
Ca	=	calcium
CaI	=	Ca^{2+} ionophore (ionomycin)
CAM	=	calmodulin dependent
CL	=	chemiluminescence
DAG	=	diacyl glycerol
G/L	=	Giga/liter
M	=	molar
mTOR	=	mammalian target of rapamycin
n	=	number of samples
NADPH	=	nicotinamid adenine dinucleotide phosphate
NO	=	nitric oxide
PKC	=	protein kinase C
PMA	=	phorbol myristate acetate
R	=	rapamycin
RLU	=	relative luminescence unit
RP-HPLC	=	reversed phase high pressure liquid chromatography
SD	=	standard deviation

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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