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Changes in Amino Acid Metabolism During Activation of Mouse Macrophage-like Cell Lines

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Abstract. *Background: Recent reports have suggested the applicability of salivary amino acids as markers for various diseases. In order to understand the role of macrophages in the age-related changes of salivary amino acid composition, we compared the amino acid production and consumption between control and activated macrophages. Materials and Methods: Mouse macrophage-like cells (RAW264.7, J774.1) were activated by Escherichia coli lipopolysaccharide (LPS). Concentrations of nitric oxide (NO) and amino acids in the culture medium during culture were determined by Griess method and amino acid analyzer, respectively, to delineate their metabolic rates. Results: LPS activated these macrophages, as judged by the enhanced production of NO and citrulline. The activated macrophages produced greater amounts of glycine, glutamic acid, alanine and histidine as compared with control cells, and consumed serine and glutamine at the highest rates. Conclusion: The present study suggests the possible role of activated macrophages in age-related changes in salivary amino acid composition.*

It has been recently reported that salivary peptides and amino acids can be utilized in the diagnosis of various diseases (1-3). We found that during the university examination period, concentrations of glycine, alanine, threonine and histidine in the saliva of undergraduate students were slightly elevated, whereas those of glutamic acid and lysine were reduced (4), and that glycine, the most abundant amino acid in the saliva, increased significantly with aging, regardless of gender difference (5). In order to understand the role of activated macrophages in the age-related changes in salivary amino acid composition, we investigated amino acid metabolism in macrophages before and after activation, using two popular mouse macrophage-like cell lines RAW264.7 (6) and J774.1 (7).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), phenol red-free DMEM (GIBCO BRL, Gland Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), LPS from *Escherichia coli* (Serotype 0111:B4) (Sigma Chem Co., St Louis, MO, USA); trichloroacetic acid (TCA) (Wako Pure Chem Co., Tokyo, Japan).

Cell culture. RAW264.7 and J774.1 cells were cultured at 37°C in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. These cells were inoculated at 1.5×10^6/ml (1 ml) in a 24-well plate (Becton Dickinson, Labware, NJ, USA) and incubated for 1-2 hours to achieve complete adherence. The attached cells were treated for 24 hours without or with LPS (100 ng/ml). The cells were replenished with 0.5 ml of fresh phenol-red free DMEM supplemented with 10% heat-inactivated FBS, glutamine and sodium pyruvate, and incubated for a further 4 hours. The number of attached viable cells was determined by hemocytometer after staining with trypan blue solution. The amino acids and nitric oxide (NO) released into the culture medium were then determined as described below.

Assay for NO. NO was determined by Griess reagent, using the standard curve of NO_2^- (8).

Determination of free amino acids. Culture medium (0.1 ml) was mixed with 0.1 ml of 10% TCA. After centrifugation for 5 minutes at 21,000 ×*g* at 4°C, the deproteinized supernatant was collected and stored at –30°C. The supernatants (20 μl) were subjected to a JLC-500/V amino acid analyzer (JEOL, Tokyo, Japan) and amino acids were detected by the ninhydrin reaction (9). The production or consumption (Δ)(nmol/10^6 cells/h) was determined by the following equation, when the cell number attached to the plate is $n\times10^6$ cells: Δ={[amino acid concentration (nmol/ml) after

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4 hours] – [initial amino acid concentration (nmol/ml)]} ×(1/4)×(1/n). Since glutamine was degraded at the rate of 14.9 nmol/ml/h at 37°C, glutamine consumption was corrected for its spontaneous degradation.

Statistical analysis. The mean values and standard deviations were calculated. The average values were compared by paired *t*-test. The value of statistical significance was set at the 0.05 level.

Results

Enhanced production of NO. RAW264.7 and J774.1 cells were activated by incubating them with LPS (100 ng/ml) for 24 hours. These activated cells produced significantly greater amounts of proinflammatory mediator NO into the culture medium (18- and 5.3-fold over control cells, respectively), during the subsequent 4 hours (Figure 1).

Amino acid production. Using the same cells treated with or without LPS, we measured the amino acid production and consumption over 4 hours. LPS treatment of RAW264.7 cells resulted in the production of histidine at the highest rate (32 nmol/10^6 cells/h, a 2.5-fold increase over control cells), followed by glycine (30 nmol, 3.2-fold) > alanine (22.8 nmol, 1.5-fold) > glutamic acid (20.1 nmol, 6.7-fold) > citrulline (10.6 nmol, 5.6-fold) (left column in Table I). The enhanced production of citrulline further confirmed the activation of RAW264.7 cells by LPS, since citrulline and NO are generated from arginine at a 1:1 molar ratio by inducible NO synthase (10).

LPS treatment of J774.1 cells resulted in the production of alanine at the highest rate (27.9 nmol/10^6 cell/h, 1.8-fold increase over control cells), followed by histidine (23.9 nmol, 2.6-fold) > glutamic acid (20.0 nmol, 5.6-fold) > glycine (8.3 nmol, 10.4-fold) > citrulline (5.3 nmol, 4.8-fold) (right column in Table I). LPS treatment enhanced citrulline production in J774.1 cells to an extent comparable to that observed in RAW264.7 cells.

Amino acid consumption. LPS treatment of RAW264.7 cells resulted in the consumption of serine (34.9 nmol/10^6 cells/h, 3.0-fold over control cells) and glutamine (>22.9 nmol, >2.4-fold) at the highest rates, followed by isoleucine (9.4 nmol, 1.3-fold) > leucine (9.0 nmol, 1.4-fold) > cysteine (8.6 nmol, 3.9-fold) > threonine (5.9 nmol, 2.2-fold) > valine (4.9 nmol, 1.5-fold) > arginine (4.9 nmol, 1.5-fold) > methionine (2.3 nmol, 2.9-fold) (left column in Table I).

LPS treatment of J774.1 cells resulted in the consumption of serine at the highest rate (22.7 nmol/10^6 cell/h, 2.1-fold increase over control cells), followed by glutamine (20.1 nmol, 1.7-fold) > leucine (9.9 nmol, 1.5-fold) > isoleucine (9.1 nmol, 1.3-fold) > cysteine (7.1 nmol, 4.2-fold) > valine (5.0 nmol, 1.3-fold) and arginine (5.0 nmol, 1.6-fold) (right column in Table I).

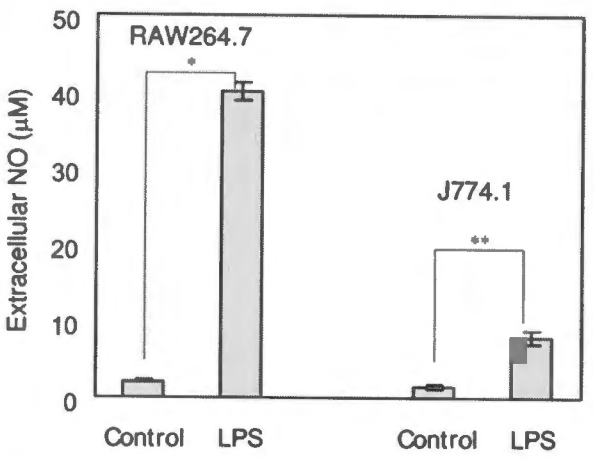


Figure 1. *Enhanced production of NO during the activation of mouse macrophage-like cells. RAW264.7 or J774.1 cells were treated for 24 hours without or with 100 ng/ml LPS in DMEM supplemented with 10% FBS. After removal of the medium, the cells were cultured for a further 4 hours in phenol red-free medium, and the viable cell number, and the extracellular NO and amino acid concentrations were determined. Viable cell number and amino acid concentrations were used to determine the metabolic rate as shown in Table I. Each value represents the mean±S.D. of triplicate assays. *p=0.00000058 and **p=0.00030.*

The extracellular concentrations of tyrosine, phenylalalnine and tryptophan changed very little during 4 hours' incubation.

Discussion

We first confirmed that LPS actually activated both RAW264.7 and J774.1 cells, as judged by stimulated production of NO and citrulline (Figure 1, Table I), as well as tumor necrosis factor (TNF)-α production (11). We found that the production of NO in the LPS-activated RAW264.7 and J774.1 cells (40 and 7.9 μM, respectively) was less than half that of citrulline (85 and 38 μM, respectively). This indicates that more than half of the NO produced decomposed during culture or the NO determination. NO, frequently used as a marker of macrophage activation, is too unstable for accurate determination (12). The simultaneous determination of citrulline is recommended for the accurate determination of NO.

The present study demonstrated that the activated macrophages consumed nine amino acids (serine, glutamine, isoleucine, leucine, cysteine, threonine, valine, arginine, methionine) at 2.2 or 1.7-fold higher rates on the average, as compared with control cells [total consumption: 46.6 (control) *vs.* 103.0 (+LPS) nmol/ml for RAW264.7 cells; 48.5 (control) *vs.* 81.4 (+LPS) nmol/ml for J774.1 cells] (Table I). Among them, serine was consumed at the highest rate, further confirming that serine is required for the growth and survival of macrophage-lineage cells (13-16).

Table I. *Changes in amino acid production and consumption during macrophage activation in phenol red-free DMEM supplemented with 10% FBS.*

	Amino acid concentration (nmol/ml)							
		RAW264.7 cells				J774.1 cells		
	0 h	LPS (–) 4 h	LPS (+) 4 h	LPS (–) Δ	LPS (+) Δ	LPS (–) 4 h	LPS (+) 4 h	LPS (–) Δ	LPS (+) Δ
Production									
His	171±1	421±46	427±28	13.0	32.0	309±1	342±7	9.1	23.9
Gly	404±10	583±27	643±15	9.3	30.0	404±13	463±13	0.8	8.3
Ala	110±3	397±19	292±1	14.9	22.8	348±9	309±9	15.7	27.9
Glu	101±2	159±7	262±5	3.0	20.1	156±4	244±14	3.6	20.0
Cit	11±4	47±2	96±2	1.9	10.6	27±1	49±2	1.1	5.3
Total				42.1	115.5			30.3	85.4
Consumption									
Ser	394±9	169±6	115±21	–11.7	–34.9	229±3	232±31	–10.9	–22.7
Gln	242±8	0	0	<–9.5	<–22.9	0	39±3	<–12.1	–20.1
Ile	692±15	558±15	617±11	–7.0	–9.4	584±15	627±22	–7.1	–9.1
Leu	722±15	597±24	650±13	–6.5	–9.0	620±15	651±24	–6.7	–9.9
Cys	153±6	110±4	84±4	–2.2	–8.6	128±3	102±4	–1.7	–7.1
Thr	730±17	678±23	683±9	–2.7	–5.9	707±16	725±25	–1.5	–0.7
Val	759±16	701±22	718±11	–3.0	–5.1	693±16	723±26	–3.9	–5.0
Arg	362±3	300±7	323±5	–3.2	–4.9	314±6	326±10	–3.2	–5.0
Met	170±4	154±6	152±2	–0.8	–2.3	149±4	157±6	–1.4	–1.8
				–46.6	–103.0			–48.5	–81.4
No apparent change									
Tyr	335±7	342±12	339±5	0.4	0.5	328±7	334±11	–0.5	–0.1
Phe	362±8	370±13	363±4	0.4	0.1	353±7	357±12	–0.6	–0.7
Try	103±2	115±3	99±1	0.6	–0.5	109±2	100±3.5	0.4	–0.4

Each value represents the mean±S.D. of triplicate assays. Δ: Change in amino acid concentration (nmol/10^6 cells/h).

The present study also demonstrated that the activated macrophages produced five amino acids (histidine, glycine, alanine, glutamic acid, citrulline) at 2.7- or 2.8-fold higher rates on average, as compared with control cells [total production: 42.1 (control) *vs.* 115.5 (+LPS) nmol/ml for RAW264.7 cells; 30.3 (control) *vs.* 85.4 (+LPS) nmol/ml for J774.1 cells] (Table I). The balance between the total amino acid consumption and the total amino acid production makes ends meet well (103.0 *vs.* 115.5 nmol/ml; 81.4 *vs.* 85.4 nmol/ml) (Table I). It should be noted that the production of both stimulatory amino acid (glutamine) and inhibitory amino acid (glycine) was considerably increased in activated macrophages. In RAW264.7 cells, both glycine and histidine were the amino acids produced at the highest rates. In J774.1 cells, the production of glycine was increased 10.4-fold over the control during macrophage activation. This suggests the possibility that the elevation of salivary glycine in elderly persons may be achieved at least in part by its release from activated macrophages. It remains to be investigated whether this phenomenon is applicable to any type of macrophages.

At present, the biological significance of the age-related elevation of salivary glycine level (50-500 nmol/ml) (5) is unknown. Recent reports have suggested the possible role of glycine in inflammation. Glycine stimulated myelin phagocytosis and the production of pro-inflammatory NO and TNF-α by rat macrophages possibly *via* activation of neutral amino acid transporters (17). Glycine also stimulated the production of prostaglandin E_2 and cyclooxygenase-2 protein in interleukin-1 (IL-1) β-stimulated human gingival fibroblast, suggesting its involvement in the pathogenesis of periodontitis (18). Glycine and serine were essential for the growth of microglial cells that express phosphatase activity and generate inducible NO synthase and superoxide anion (19).

On the other hand, glycine exerts anti-inflammatory activity *via* glycine-activated chloride channels that suppress production of oxidants and pro-inflammatory cytokines (20). Glycine protected U937 macrophage cells (induced by pretreatment with phorbol 12-myristate 13-acetate) from the cytotoxicity of cadmium by its antioxidant activity, and inhibited their production of NO, TNF-α, IL-1 and IL-6 (21). Glycine prevented or minimized cyclosporine-induced kidney

damage in rats (22), and pretreatment of rats with glycine significantly reduced liver injury and improved liver function after partial hepatectomy (23). Glycine pretreatment of mice attenuated LPS-induced liver injury, possibly *via* the down-regulation of toll-like receptor 4 expression and the up-regulation of IL-10 production (24). These data suggest that glycine plays an important role in cell survival and activation.

It remains to be investigated how exogenously added glycine affects the viability and growth of RAW264.7 and J774.1 cells, and the expression of various types of cell surface receptors. This information may pave the way for the elucidation of the biological significance of elevated glycine production in activated macrophages, and of the possible link to the age-related elevation of salivary glycine concentration.

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