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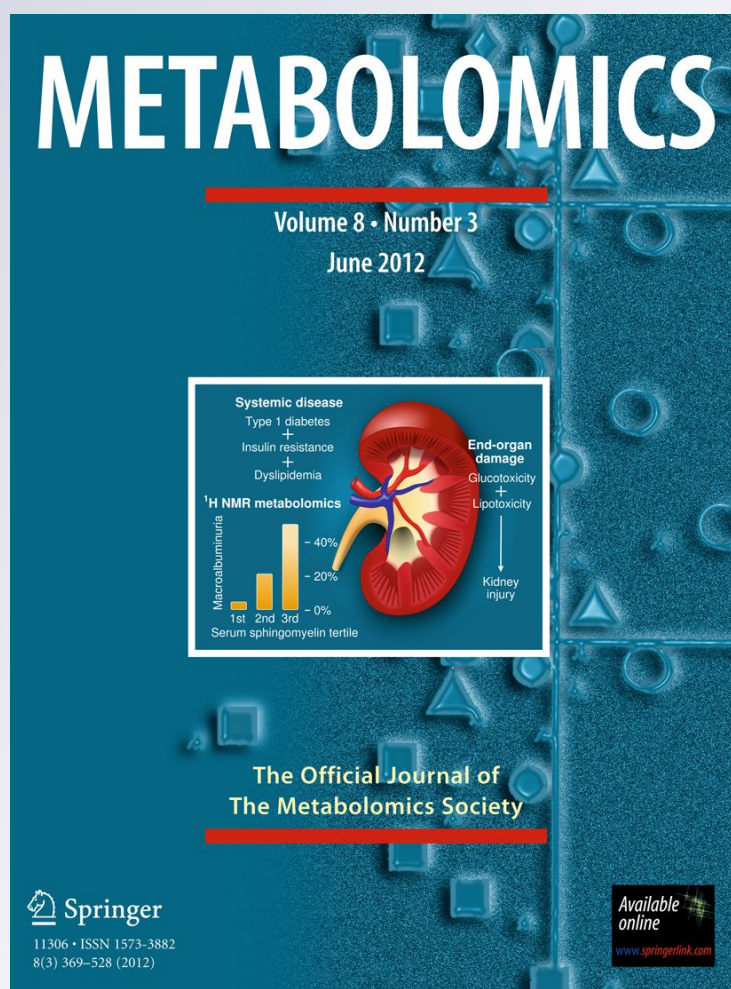
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Non-targeted metabolite profiling in activated macrophage secretion

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Abstract Periodontal diseases are inflammatory infectious diseases that affect the periodontal tissue. Macrophages play a central role in inflammatory conditions, leading to the destruction of tissues. Identifying the signaling molecules secreted by macrophages would be valuable to the study of these diseases. Here, we present non-targeted analysis using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) for the profiling of extracellular metabolites released during macrophage activation. Lipopolysaccharide (LPS)-induced activation of a mouse macrophage-like cell line RAW264.7 was used as a model system. Cells were treated without (control) or with LPS for 22 h and, after washing, were incubated for 1 h in phosphate-buffered saline. The accumulation of metabolites in the culture supernatant was monitored. LPS treatment significantly enhanced the accumulation of prostaglandins, tumor necrosis factor- α ,

nitric oxide and citrulline in the culture medium. RAW264.7 cells produced 46 metabolites and 66% of these showed significant changes ($P < 0.05$) following cell activation. In particular, the production of leucine, hypoxanthine, choline, putrescine, N_8 -acetylspermidine, succinate, itaconate, and 4-methyl-2-oxopentanoate was significantly increased by cell activation ($P < 0.001$). Significantly elevated production of lactate and glycine was also observed. Here, we present the first catalog of the up and down-regulation of the various metabolites secreted by macrophages following inflammatory activation.

Keywords Macrophage activation · Lipopolysaccharide · RAW264.7 cells · Capillary electrophoresis-mass spectrometry

1 Introduction

Periodontal disease and its milder form, gingivitis, affects 50–90% of adults worldwide, depending on the precise definition of the disease (Pihlstrom et al. 2005). The disease results in the destruction of periodontal tissues and the supporting dental structure, and is associated with systemic diseases such as diabetes, cancer, cardiovascular disease, and preterm birth (Meyer et al. 2008). Periodontal diseases are caused by complex interactions between the host tissue and bacteria at the junctional and crevicular epithelia, and is mainly mediated by the host's response to bacteria (Van Dyke and Serhan 2003).

Neutrophils and macrophages play important roles in the innate inflammatory response during the progression of periodontal disease, with sequential events being triggered by lipopolysaccharide (LPS) from gram-negative bacteria at the tooth root surface. Polymorphonuclear leukocytes are

M. Sugimoto and H. Sakagami contributed equally to this work.

M. Sugimoto (✉) · H. Onuma · M. Kaneko · M. Mori · Y. Sakaguchi · T. Soga · M. Tomita
Institute for Advanced Biosciences, Keio University,
Tsuruoka, Yamagata 997-0017, Japan
e-mail: msugi@sfc.keio.ac.jp

M. Sugimoto · T. Soga · M. Tomita
Systems Biology Program, Graduate School of Media and
Governance, Keio University, Fujisawa, Kanagawa 252-8520,
Japan

H. Sakagami
Divisions of Pharmacology, Meikai University School
of Dentistry, Sakado, Saitama 350-0283, Japan

Y. Yokote
Faculty of Science, Josai University, Sakado, Saitama 350-0295,
Japan

recruited to the site, and monocytes and activated macrophages respond to endotoxin by releasing various cytokines and interleukin-1 β , which stimulates further tissue destruction (Giannobile et al. 2009). Therefore, understanding the biochemical cascades induced by macrophages is important for the diagnosis and treatment of periodontal diseases.

Macrophages perform a multitude of functions essential for tissue remodeling and the immune response, and produce a wide array of pro-inflammatory cytokines, growth factors, lysozymes, proteases, complement components, coagulation factors and prostaglandins upon stimulation with LPS or hypoxic stress (Bingle et al. 2002). A mouse macrophage-like cell line (RAW264.7) (Ralph and Nakoinsz 1977) has been used by many investigators in experiments designed to elucidate the signal transduction events during macrophage activation. Using this cell line, we have previously profiled amino acid release and found that the extracellular levels of glycine were significantly elevated during LPS-induced activation (Nishiyama et al. 2010). Glycine is the most abundant amino acid found in saliva (Nakamura et al. 2010) and salivary glycine levels are significantly elevated in elderly persons regardless of gender (Tanaka et al. 2010). This suggests that activated macrophages may be involved in age-related changes to salivary composition. Elevated levels of glycine in the saliva of elderly persons may aggravate periodontitis by stimulating the production of prostaglandin E₂ and cyclooxygenase-2 (COX-2) protein by interleukin-1 β -stimulated human gingival fibroblast cells (Rausch-Fan et al. 2005). However, the importance of changes in glycine levels relative to other intracellular metabolite changes induced in activated macrophages remains unclear.

Metabolomics, the simultaneous quantification of all intercellular or intracellular metabolites, has become a powerful new tool that can provide insight into cellular functions (Soga et al. 2003). Several metabolites have been identified as endogenous chemical mediators that orchestrate the host's response in periodontal diseases. These metabolites include lipid-derived mediators, key inflammation signaling molecules (Van Dyke and Serhan 2003), and adenosine and inosine (Cronstein et al. 1999a, b), which are upregulated by cells in response to stress. Although the metabolite profiles of individual pathways, including lipid (Van Dyke and Serhan 2003), amino acid (Li et al. 2007) and carbohydrate (Rodriguez-Prados et al. 2010) metabolites, have been revealed, a broad understanding of these metabolomic profiles is necessary to determine the pathogenic mechanisms involved in innate macrophage inflammation in periodontal diseases.

In this study, we present the first comprehensive metabolomic analysis of the culture supernatants of unstimulated and stimulated macrophage cells to identify the metabolites secreted from macrophages at inflammatory

sites such as periodontal tissues during periodontal disease. To profile a wide range of metabolites, non-targeted analyses of the extracellular environment of RAW264.7 cells treated without and with LPS were conducted using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) (Soga et al. 2006).

2 Materials and methods

2.1 Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO BRL (Grand Island, NY, USA), fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS, USA) and LPS from *E. coli*, Serotype 0111:B4 was purchased from Sigma (St. Louis, MO, USA). Ophthalmate was purchased from Bachem AG (Bubendorf, Switzerland). Acetohydroxamate and azetidine-2-carboxylate were purchased from Chem Service (West Chester, PA, USA). Glucose 1-phosphate and 5-aminovalerate were purchased from Fluka (Buchs, Switzerland). Betaine was purchased from Tokyo Chemical Industry (Tokyo, Japan). All other compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Wako (Osaka, Japan).

2.2 Cell culture

Mouse macrophage-like RAW264.7 cells (purchased from Dainippon Sumitomo Pharma, Osaka, Japan) were cultured at 37°C in DMEM supplemented with 10% FBS under a humidified 5% CO₂ atmosphere.

2.3 LPS activation

Cells (8×10^5 cells/ml, 2 ml) were inoculated on a six-well plate (Becton–Dickinson, Labware, NJ, USA) and incubated at 37°C for 4 h for the cells to completely adhere to the plate. The medium was changed with fresh medium and the cells were then incubated for 22 h without (LPS–) or with 100 ng/ml LPS (LPS+). The experiment was carried out in triplicate. The cells were washed twice with phosphate-buffered saline without calcium or magnesium (PBS–), and then incubated in 1 mL of PBS–. An aliquot (0.5 ml) was removed immediately from each well to provide samples to analyze the initial metabolite mixture, and the cells were then incubated for 60 min at 37°C with the remaining 0.5 ml of PBS– in a 5% CO₂ incubator. All samples were centrifuged (10,000 \times g, 60 min) through a 3-kDa cutoff filter (Pall Corporation, NY, USA) to remove macromolecules, and the filtrates were assayed to detect nitric oxide (NO), tumor necrosis factor- α (TNF- α), amino acids and other cellular metabolites as described below.

A short incubation time (60 min) was used to minimize cell damage during incubation in PBS⁻. After incubation, the number of viable cells, which were not stained with 0.15% trypan blue in PBS⁻, was counted using a hemocytometer under a light microscope. The production rate for each compound was determined by subtracting the initial value from the value obtained after 60 min incubation, and expressed as nmol/10⁶ cells/h.

2.4 NO assay

Since NO is a highly unstable compound, NO present in the culture medium was detected directly, without centrifugation, using the Griess method (Takahashi et al. 2008).

2.5 TNF- α assays

The culture supernatants were assayed using an ELISA kit (R&D Systems Inc, Minneapolis, MN, USA) to measure the concentration of TNF- α . The assay was performed according to the manufacturer's instructions.

2.6 Sample preparation for CE-TOFMS

To 100 μ l of the extracellular-dissolved samples, 400 μ l of methanol containing internal standards (50 μ mol/l each of methionine sulfone, 2-[*N*-morpholino]-ethanesulfonic acid, and *D*-camphor-10-sulfonic acid) was added. The homogenate was then mixed with Milli-Q water and chloroform at a volume ratio of 5:2:5, and the mixture was centrifuged at 20,400 $\times g$, for 15 min at 4°C. The aqueous layer was filtered to remove macromolecules by centrifugation through a 5-kDa cutoff filter (Millipore) at 9,100 $\times g$ for 2.5 h at 4°C. The filtrate (300 μ l) was concentrated by centrifugation and dissolved in 50 μ l of Milli-Q water containing reference compounds (200 μ mol/l each of 3-aminopyrrolidine and trimesate) immediately before CE-TOFMS analysis.

2.7 Instrumental parameters for CE-TOFMS

The instrumentation and measurement conditions used for CE-TOFMS are described elsewhere (Sugimoto et al. 2010a; Soga et al. 2006, 2009). Cation analysis was performed using an Agilent CE capillary electrophoresis system, an Agilent G6220A LC/MSD TOF system, an Agilent 1100 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). Anion analysis was performed using an Agilent CE capillary electrophoresis system, an Agilent G6210A LC/MSD TOF system, an Agilent 1200 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit and a G1607A Agilent CE-electrospray ionization (ESI) source-MS sprayer kit

(Agilent Technologies). For both the cation and anion analyses, the CE-MS adapter kit includes a capillary cassette that enables thermostatic control of the capillary. The CE-ESIMS sprayer kit simplifies coupling of the CE system with the MS systems and is equipped with an electrospray source. For system control and data acquisition, we used G2201AA Agilent ChemStation software for CE and Agilent MassHunter software for TOF-MS. For anion analysis, the original Agilent SST316Ti stainless steel ESI needle was replaced with a SST316Ti stainless steel and platinum needle, which was passivated using 1% formic acid and 20% isopropanol aqueous solution at 80°C for 30 min.

For cationic metabolite analysis using CE-TOFMS (Soga et al. 2006), the samples were separated in fused silica capillaries (50 μ m i.d. \times 100 cm total length) filled with 1 mol/l formic acid as the reference electrolyte. Sample solutions were injected at 50 mbar for 3 s and a voltage of 30 kV was applied. The capillary temperature was maintained at 20°C and the temperature of the sample tray was kept below 5°C. The sheath liquid, composed of methanol/water (50% v/v) and 0.1 μ mol/l hexakis (2,2-difluoroethoxy) phosphazene (Hexakis), was delivered at 10 μ l/min. ESI-TOF-MS was conducted in the positive ion mode. The capillary voltage was set at 4 kV and the flow rate of nitrogen gas (heater temperature = 300°C) was set at 10 psig. In TOF-MS, the fragmentor, skimmer and OCT RF voltages were set at 75, 50 and 125 V, respectively. Automatic recalibration of each acquired spectrum was performed using reference masses of reference standards {[¹³C isotopic ion of protonated methanol dimer (2MeOH + H)]⁺, *m/z* 66.063061} and ([protonated Hexakis (M + H)]⁺, *m/z* 622.02896). Mass spectra were acquired at the rate of 1.5 cycles/s over a *m/z* range of 50–1,000.

For anionic metabolite analysis using CE-TOFMS (Soga et al. 2009), a commercially available COSMO(+) capillary (50 μ m i.d. \times 110 cm, Nacalai Tesque, Kyoto, Japan), chemically coated with a cationic polymer, was used for separation. A 50-mmol/l ammonium acetate solution (pH 8.5) was used as the electrolyte to achieve separation. Before the first use, a new capillary was flushed successively for 10 min each with the running electrolyte (pH 8.5), 50 mmol/l acetic acid (pH 3.4), and then the electrolyte again. Before each injection, the capillary was equilibrated for 2 min by flushing with 50 mM acetic acid (pH 3.4) and then for 5 min by flushing with the running electrolyte. A sample solution (30 nl) was injected at 50 mbar for 30 s, and a voltage of -30 kV was applied. The capillary temperature was maintained at 20°C and the sample tray was cooled below 5°C. An Agilent 1100 series pump equipped with a 1:100 splitter was used to deliver 5 mM ammonium acetate in 50% (v/v) methanol/water, containing 0.1 μ M Hexakis to the CE interface at 10 μ l/min. Here,

it was used as a sheath liquid around the outside of the CE capillary to provide a stable electrical connection between the tip of the capillary and the grounded electrospray needle. ESI-TOF-MS was conducted in negative ionization mode with the capillary voltage set to 3,500 V. For TOF-MS, the fragmentor, skimmer and Oct RF voltages were set at 100, 50 and 200 V, respectively. The flow rate of the drying nitrogen gas (heater temperature = 300°C) was 10 l/min. Automatic recalibration of each acquired spectrum was performed using reference masses of reference standards {[¹³C isotopic ion of deprotonated acetic acid dimer (2 CH₃COOH-H)]⁻, *m/z* 120.038339}, and {[Hexakis-deprotonated acetic acid (CH₃COOH-H)]⁻, *m/z* 680.03554}. Exact mass data were acquired at a rate of 1.5 spectra/s over a *m/z* range of 50–1,000.

2.8 Sample preparation for LC-TOFMS

To 30 µl of the extracellular samples, 270 µl of isopropyl alcohol containing an internal standard (2.2 µmol/l of camphor-10-sulfonic acid) was added with shaking. The mixture was centrifuged at 20,400×*g* for 10 min at 4°C, and the supernatant was then transferred to another tube and vacuum dried at 35°C. The samples were mixed with chloroform, methanol, and Milli-Q water in a volume ratio of 2:4:1 containing 20 µmol/l of 3,5-di-*tert*-butyl-4-hydroxyhydrocinamate, centrifuged at 20,400×*g*, for 5 min at 4°C, and 25 µl of the supernatant was used for LC-TOFMS analysis.

2.9 LC-TOFMS parameters

The LC system was an Agilent 1290 infinity HPLC (Agilent Technologies). The Acquity UPLC[®] BEH C18 (1.7 µm), ϕ 2.1 × 50 mm column was purchased from Waters (Tokyo, Japan), and was maintained at 50°C. The mobile phase consisted of 0.5% acetic acid/water as eluent A and isopropanol as eluent B. A gradient of 1–99% B over 12 min was used, followed by isocratic elution at 99% for a further 17 min. The flow rate was 0.3 ml/min and the injection volume was 1 µl.

MS data were acquired on a 6530 Accurate-Mass Q-TOF LC/MS using the dual spray ESI of G-3251A (Agilent). Samples were analyzed by both positive and negative ion electrospray mass spectrometry. The MS conditions used were as follows: gas temperature 350°C, drying gas 10 l/min, nebulizer 30 psig, fragmentor 200 V, skimmer 90 V, OCT1 RF V_{pp} 250 V, scan range *m/z* 100–1,600 and nozzle voltage 1,000 V. The capillary voltages were 3.5 and 4.0 kV for negative and positive mode, respectively.

2.10 Determination of free amino acids

The culture medium (0.1 ml) was mixed with 0.1 ml of 10% trichloroacetic acid (Wako). After centrifugation for

5 min at 21,000×*g* at 4°C, the deproteinized supernatant was collected and stored at –30°C. The supernatants (20 µl) were analyzed using a JLC-500/V amino acid analyzer (JEOL, Tokyo, Japan) and amino acids were detected by the ninhydrin reaction (Yamazaki et al. 2007).

2.11 Data and statistical analysis

Raw data were analyzed using our proprietary software called MasterHands, which detected all possible peaks, eliminated noise and redundant features, and generated the aligned data matrix, including annotated metabolite identities and relative areas (peak areas normalized via comparison with internal standards) (Sugimoto et al. 2010b). Concentrations were calculated using external standards based on relative areas. Student's *t* test (two-tailed) was used for statistical comparisons.

3 Results and discussion

3.1 Overview of the metabolomic profiles

In total, CE-TOFMS identified 44 charged metabolites secreted by macrophages (Fig. 1; Table 1). Prostaglandin E₂ and F₂α were observed by LC-TOFMS as they were not charged and therefore not detected by CE-TOFMS. However, because of their similar molecular structures, these two metabolites could not be adequately resolved by LC-TOFMS, so only their combined production could be calculated and used in subsequent analyses.

3.2 Validation of the amino acid profiles determined by CE-TOFMS and the amino acid analyzer

The quantified amino acids were validated by comparing the amino acids profiles consistently obtained by CE-TOFMS with those obtained using an amino acid analyzer (Fig. 2). The metabolites present at high concentrations, such as glycine, alanine and threonine, showed slightly greater discrepancy between the profiles determined by the two methods than metabolites present at low concentrations. Nevertheless, reasonably high overall correlation coefficients were obtained for the unstimulated and LPS-stimulated macrophages, which were $R = 0.855$ ($P < 0.0001$, Pearson correlation) and $R = 0.928$ ($P < 0.0001$, Pearson correlation), respectively.

3.3 Stimulatory effect of LPS on macrophages

NO production by stimulated RAW264.7 cells was significantly increased from 0.20 to 0.66 nmol/10⁶ cells/h (3.3-fold increase, $P = 0.0002$) (Fig. 3a), and TNF-α production was also significantly increased from 21.7 to 682.5 pg/10⁶ cells/h

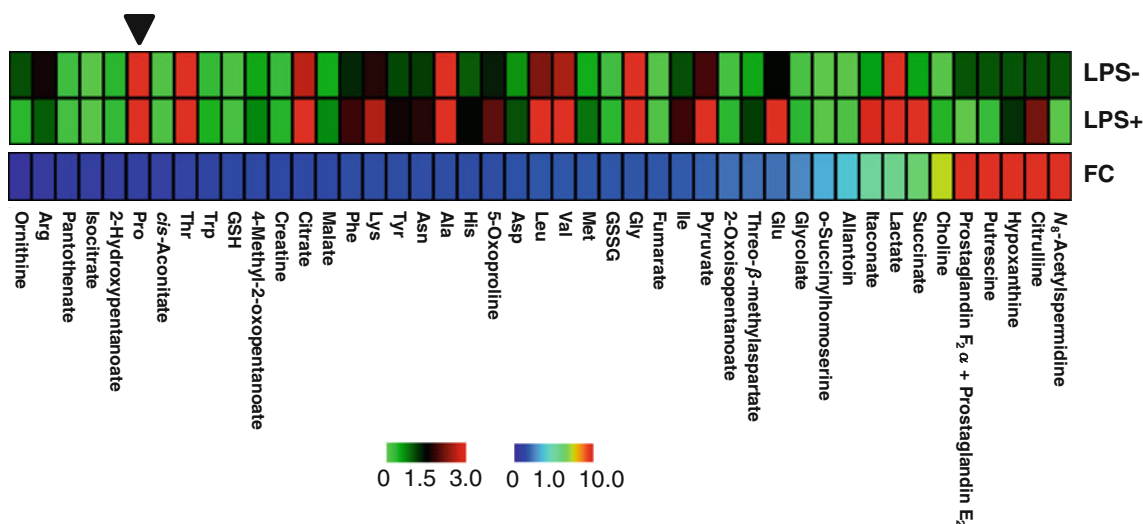


Fig. 1 Heatmap and bar graph visualization of the quantified metabolites. Heatmap showing the quantified metabolites using a green–black–red scheme, and the fold-change of metabolites in LPS+

(31.5-fold increase, $P = 0.0151$) (Fig. 3b). Citrulline production, which accompanies NO production at a molar ratio of 1:1, is generated from arginine by inducible NO synthase (Paradise et al. 2010). It was only observed for stimulated RAW264.7 cells (2.19 nmol/10⁶ cells/h, $P = 3.0 \times 10^{-5}$) (Table 1). Since NO is labile, citrulline levels are a more reliable guide to the levels of NO synthase activity. These data confirmed that LPS activated the RAW264.7 cells.

Prostaglandin E₂ and F_{2α}, detected by LC-TOFMS, are central molecules in inflammatory processes. The inflammatory response of macrophages induces prostanoid synthesis by (1) inducing mobilization of the fatty acid substrate arachidonate from membrane phospholipids, (2) promoting prostaglandin H₂ production from arachidonate by upregulating cyclooxygenases 1 and 2 (COX-1/2), and (3) converting prostaglandin H₂ to specific prostanoids, such as prostaglandin E₂ and F_{2α} (Noguchi and Ishikawa 2007). The production of prostaglandin E₂ and F_{2α} (4.6×10^{-3} nmol/10⁶ cells/h, $P = 1.2 \times 10^{-6}$) was only observed in stimulated RAW264.7 cells, again confirming the activation of these macrophages.

3.4 Amino acid profiles of stimulated macrophages

3.4.1 Glycine, serine and cysteine production

In amino acid profiles, glycine production was the highest of all amino acids detected (LPS– vs. LPS+: 17.1 vs. 29.5 nmol/10⁶ cells/h, $P = 0.073$) (Table 1), which is consistent with previous studies using LPS-stimulated mouse macrophages and regular culture medium (DMEM + 10% FBS) (Nishiyama et al. 2010). Glycine has been reported to stimulate the production of

versus LPS– cells. Where metabolites were not detected in LPS– cells, a 10-fold change was allocated. Proline, marked with triangle, showed no change. FC fold change

prostaglandin E₂ and COX-2 protein in interleukin-1β-stimulated human gingival fibroblast cells, suggesting its involvement in the pathogenesis of periodontitis (Rausch-Fan et al. 2005). On the other hand, glycine has been reported to inhibit the production of inflammatory cytokines by macrophages by blocking calcium channels (Rose et al. 1999), inhibiting TNF-α secretion and increasing IL-10 production (Xu et al. 2008), or via neutral amino acid transporters (Carmans et al. 2010). Glycine inhibited the growth of an endothelial cell line (Yamashina et al. 2007) and salivary gland-derived progenitor cells (Nakamura et al. 2009). Although these data are contradictory, they mostly suggest that glycine negatively regulates the growth and activation of various cultured cells. This metabolite is also the most abundant amino acid in the saliva (Takeda et al. 2009; Tanaka et al. 2010); however, the cells in the oral cavity that secrete glycine have not been identified. In association with glycine pathway, serine, a major source of glycine, was only detected by the amino acid analyzer, but was present at relatively low concentrations (Table 1; LPS– vs. LPS+: 1.0 ± 0.074 vs. 1.1 ± 0.12 nmol/10⁶ cells/h, 1.1 fold). Cysteine, which is synthesized from serine, was not observed, possibly due to the limited supply of serine.

3.4.2 Glutamate, glutamine, proline and arginine production

Glutamate, a key intermediate in amino acid metabolism, including biosynthetic and degradative pathways, is formed from α-ketoglutarate in the tricarboxylic acid (TCA) cycle. We found that glutamate production was increased by 2.2-fold ($P = 0.0043$) in activated macrophages, and its

Table 1 Profiled metabolites of RAW264.7 cells

Mode	Metabolite	LPS–		LPS+		P value	FC
		Mean	SD	Mean	SD		
C	Gly	17.1	7.79	29.4	4.21	0.0735	1.7
C	Ala	8.61	1.65	13.1	3.19	0.0988	1.5
C	Thr	7.50	0.594	9.13	0.501	0.0219	1.2
C	Pro	4.17	0.385	4.22	0.323	0.891	1.0
C	Val	2.50	0.424	4.18	0.361	6.41×10^{-3}	1.7
C	Leu	2.25	0.227	3.75	0.153	6.80×10^{-4}	1.7
C	Glu	1.48	0.0657	3.28	0.530	4.31×10^{-3}	2.2
C	Lys	1.72	0.231	2.54	0.337	0.0251	1.5
C	Cit	0.00	0.00	2.19	0.180	3.01×10^{-5}	N.A.
C	Ile	1.04	0.232	1.86	0.0675	4.19×10^{-3}	1.8
C	Phe	1.27	0.180	1.86	0.182	0.0162	1.5
C	Asn	1.14	0.194	1.72	0.222	0.0275	1.5
C	Tyr	1.07	0.182	1.61	0.101	0.0111	1.5
C	His	0.970	0.0954	1.48	0.186	0.0134	1.5
C	Asp	0.630	0.119	1.03	0.477	0.235	1.6
C	Arg	1.62	0.196	0.958	0.0862	6.01×10^{-3}	0.59
C	Met	0.482	0.160	0.822	0.0944	0.0340	1.7
C	Trp	0.334	0.0286	0.446	0.0218	5.81×10^{-3}	1.3
C	Hypoxanthine	0.00	0.00	1.22	0.0797	1.21×10^{-5}	N.A.
C	5-Oxoproline	1.34	0.213	2.05	0.310	0.0311	1.5
C	Choline	0.0554	2.52×10^{-3}	0.408	0.0101	5.13×10^{-7}	7.4
C	Creatine	0.287	0.0418	0.401	0.0696	0.0715	1.4
C	Ornithine	1.04	0.239	0.383	0.109	0.0123	0.37
C	Putrescine	0.00	0.00	0.315	0.0605	8.36×10^{-4}	N.A.
C	Allantoin	0.0342	0.415	0.129	2.41	0.950	3.8
C	<i>o</i> -Succinylhomoserine	0.0142	0.0480	0.0475	0.0318	0.373	3.3
C	<i>N</i> ₈ -Acetylspermidine	0.00	0.00	0.0277	2.52×10^{-3}	4.55×10^{-5}	N.A.
C	GSSG	0.217	0.0323	0.371	0.0667	0.0225	1.7
C	GSH	0.118	0.0233	0.164	0.0883	0.428	1.4
A	Glycolate	0.142	0.935	0.367	0.240	0.359	2.6
A	Pyruvate	1.91	0.958	3.66	0.632	9.38×10^{-3}	1.9
A	Lactate	6.65	3.36	37.0	7.58	2.33×10^{-3}	5.6
A	Fumarate	0.0676	0.110	0.117	0.0289	0.291	1.7
A	2-Oxoisopentanoate	0.171	0.238	0.370	0.0370	0.0875	2.2
A	Succinate	0.513	0.237	3.16	0.400	3.30×10^{-4}	6.2
A	2-Hydroxypentanoate	0.379	0.198	0.328	0.0575	0.525	0.86
A	Itaconate	0.549	0.269	2.86	0.254	1.18×10^{-4}	5.2
A	4-Methyl-2-oxopentanoate	0.509	0.256	0.711	0.0391	4.20×10^{-3}	1.4
A	Malate	0.479	0.243	0.680	0.0800	0.0363	1.4
A	Threo- β -methylaspartate	0.511	0.286	1.13	0.0967	1.33×10^{-3}	2.2
A	<i>cis</i> -Aconitate	0.114	0.0579	0.123	0.0154	0.562	1.1
A	Isocitrate	0.0520	1.31	0.0444	0.0386	0.761	0.85
A	Citrate	2.62	1.32	3.69	0.485	0.0357	1.4
A	Pantothenate	0.240	0.121	0.184	0.0315	0.0818	0.77

Table 1 continued

Mode	Metabolite	LPS-		LPS+		P value	FC
		Mean	SD	Mean	SD		
N	Prostaglandin F ₂ α+ Prostaglandin E ₂	0.00	0.00	4.62 × 10 ⁻³	1.68 × 10 ⁻⁴	1.16 × 10 ⁻⁶	N.A.

Units are nmol/10⁶ cells/h. The metabolite concentration in LPS- cells was 0 nmol/10⁶ cells/h

SD standard deviation; FC fold-change; C cationic; A anionic; N neutral; NA not applicable

Fig. 2 Comparison of amino acid productions determined by CE-TOFMS and the amino acid analyzer for LPS- (a) and LPS+ macrophages (b). Units for both X- and Y-axis are nmol/10⁶ cells/h

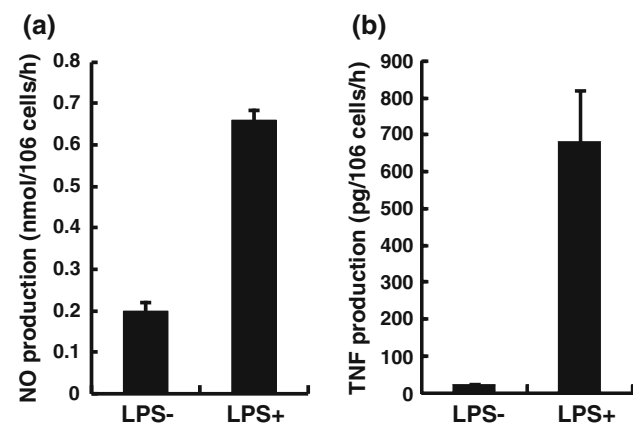
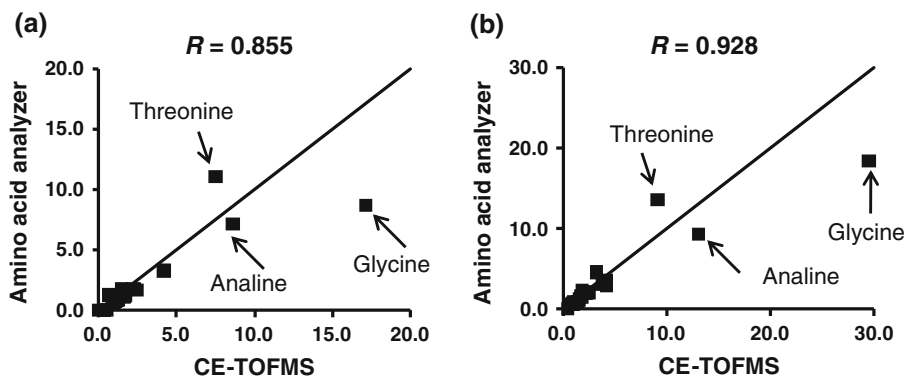


Fig. 3 Stimulation of NO (a) and TNF- α (b) production in LPS-activated RAW264.7 cells. Values are means \pm standard deviation of triplicate determinations

fold-change in production was the highest observed for any amino acid. Glutamate is an excitatory neurotransmitter and the glutamate receptor protein is present on the macrophage cell surface (Noda et al. 2000). Glutamate plays a number of roles in the immune system including removal of oxidants and regulation of immune response (Li et al. 2007). Increased glutamate production in LPS-activated macrophages has already been reported (Stuckey et al. 2005). Glutamate is also a substrate in the synthesis of γ -aminobutyrate (GABA), present in macrophages (Stuckey et al. 2005). However, in this study, GABA was not

detected in the culture supernatant of the activated macrophages.

Proline is synthesized from glutamate, but its concentration did not change, despite LPS stimulation (1.0-fold, $P = 0.891$). In contrast, significant decreases in arginine (0.59-fold, $P = 6.01 \times 10^{-3}$) and ornithine (0.37-fold, $P = 0.109$) were observed; ornithine is synthesized from arginine by arginase. Considering the excessive increase of citrulline upon macrophage activation, the arginine pathway seems to be highly activated and mainly used for citrulline synthesis,

3.4.3 Production of other amino acids

The production of alanine (1.5-fold, $P = 0.0988$), valine (1.7-fold, $P = 6.41 \times 10^{-3}$), and leucine (1.7-fold, $P = 6.80 \times 10^{-4}$) was increased to a similar extent by macrophage activation. The production of pyruvate, their precursor metabolite, was also elevated (1.9-fold, $P = 9.38 \times 10^{-3}$). Thus, the change of these metabolites might be due to pyruvate elevation. Aspartate (1.6-fold), which is formed from the TCA component oxaloacetate by the action of transaminase, and its downstream metabolites such as asparagine, lysine, methionine, threonine, isoleucine (from 1.2 to 1.8-fold), showed similar upregulation (Fig. 4; Table 1). On the other hand, cysteine was undetected, probably because of its degradation during sample preparation before CE-TOFMS.

3.5 Metabolomic profile of carbohydrate metabolism

3.5.1 Lactate production

Lactate production in activated macrophages was significantly elevated, and this elevation was the largest observed of all metabolites (37.0 nmol/10⁶ cells/h, 5.57-fold increase, $P = 0.0023$). This finding is consistent with previous reports showing that glycolysis and lactate production were increased during osteoclast differentiation induced by receptor activator of nuclear factor [NB]- κ B ligand (RANKL) in RAW264.7 cells (Kim et al. 2007). The increase in lactate production, the end product of mono-oxidative glycolysis, indicates that the glycolysis pathway is activated. Enhanced glycolysis in macrophages is likely to be a survival strategy to cope with the low oxygen conditions commonly observed at inflammatory lesions (Roiniotis et al. 2009). Lactate is a signaling molecule that can activate macrophages by stimulating inflammatory pathways, such as the TLR4 and NF- κ B pathways (Samuvel et al. 2009; Nareika et al. 2005). Combined stimulation with LPS and lactate was shown to enhance macrophage activation more than LPS alone (Samuvel et al. 2009). The positive correlation between lactate production and macrophage activation observed here suggests an interaction between extracellular lactate and macrophages, possibly by a feed-forward loop, although further studies are necessary to confirm this postulation.

3.5.2 The balance between glycolysis and TCA cycle activation

Although less substantial than the change in lactate production, a significant increase in TCA cycle metabolite production was observed following macrophage activation. The levels of citrate, succinate, itaconate, malate and fumarate all increased following activation (Table 1). Rodriguez-Prados et al. (2010) observed upregulation of genes involved in glycolysis and downregulation of genes involved in the TCA cycle of LPS-activated macrophages. Indeed, the production of pyruvate in our study was significantly increased (1.9-fold, $P = 0.0094$). It is plausible that glycolysis was activated in activated macrophages under conditions where overall energy metabolism was enhanced. This implies that, even though genes in the TCA cycle were downregulated, the excessive activation of glycolysis caused a slight increase in several metabolites of the TCA cycle.

3.6 Oxidative stress

Barnes et al. (2009) found that the levels of hypoxanthine, inosine, xanthine, guanosine and guanine in gingival

crevicular fluid (GCF) were elevated in inflammation sites compared with healthy sites in a human oral cavity. Their findings suggest accelerated activity of the purine degradation pathway and the production of reactive oxygen species causing marked cellular oxidative stress. In this study, we only detected hypoxanthine in activated macrophages (1.22 nmol/10⁶ cells/h, $P = 1.2 \times 10^{-5}$).

GSH plays an anti-inflammatory role via its role as an antioxidant (Ghezzi 2011). The production of GSH was increased slightly (1.4-fold, $P = 0.43$), while the production of GSSG was significantly increased after activation in this study (1.7-fold, $P = 0.022$). The ratio of GSH and GSSG decreased slightly from 0.54 to 0.44 ($P = 0.64$) but this decrease was at levels below those considered significant, indicating no dramatic change in oxidative stress after treating macrophages with LPS. Allantoin, which is a uric acid oxidation product, and is therefore a marker for oxidative stress (Kand'ar et al. 2006), also showed no significant changes following exposure to LPS (3.8-fold, $P = 0.95$). These findings conflict with the elevated production of hypoxanthine, a marker for oxidative stress. Therefore, the profiling of cellular metabolites and integration of those data with the extracellular profiles determined in this study would be valuable.

3.7 Other metabolites secreted by macrophages

In addition to amino acids and metabolites related to energy metabolism, several other metabolites were also identified. The production of threo- β -methylaspartate, which is generated from oxaloacetate and NH₃ via threo-3-hydroxy-L-aspartate ammonia-lyase, was significantly increased following activation (2.2-fold, $P = 0.0013$). N₈-Acetylspermidine ($P = 4.5 \times 10^{-5}$, not detected in LPS- cells), a polyamine that generally indicates active cell growth, was only detected in activated macrophages. While the increases following activation were statistically significant ($P > 0.05$) for the following metabolites, there

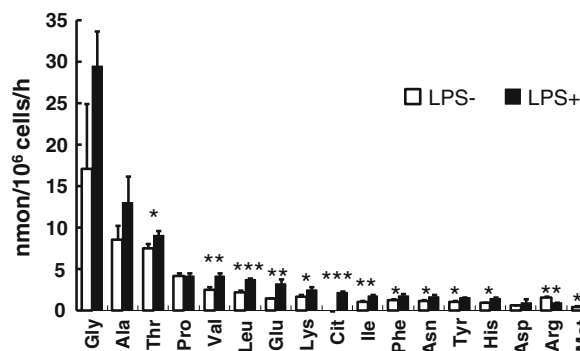


Fig. 4 Bar graph showing amino acid production profiles in LPS+ and LPS- cells. Values are means \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

were small increases in the production of *o*-succinylhomoserine, glyconate and 2-oxoisopentanoate, which showed high fold-changes compared with most amino acids (>1.7-fold) (Table 1). The biological relevance of these metabolites should be confirmed using other “-omics” data and cellular metabolite profiles.

4 Concluding remarks

In this study, we conducted CE-TOFMS-based metabolomics analysis to identify the metabolites secreted by LPS-stimulated macrophage-like RAW264.7 cells. Cellular activation was confirmed by the increased production of NO, citrulline, TNF- α and prostaglandins E₂ and F_{2 α} by the stimulated cells. In addition to large increases in glycine production, the production of lactate was also enhanced, indicating the activation of glycolysis. Increased production of several intermediates in the TCA cycle was also observed. Of oxidative stress-related metabolites, the production of hypoxanthine was particularly high, although the GSSH/GSH ratio was almost unchanged by LPS stimulation. To understand these conflicting results, studies using integrated cellular metabolomics profiles are needed to elucidate how the observed metabolite changes contribute to the subsequent inflammatory events in periodontal disease. Nevertheless, this report provides the first catalog of the various metabolites secreted by activated macrophages, and complements prior reports on the already well-studied pro-inflammatory proteins that are known to be secreted by macrophages.

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Conflicts of interest The authors have no conflicts of interest to declare.

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