Endothelium-Derived 5-Methoxytryptophan Is a Circulating Anti-Inflammatory Molecule That Blocks Systemic Inflammation

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Rationale: Systemic inflammation has emerged as a key pathophysiological process that induces multiorgan injury and causes serious human diseases. Endothelium is critical in maintaining cellular and inflammatory homeostasis, controlling systemic inflammation, and progression of inflammatory diseases. We postulated that endothelium produces and releases endogenous soluble factors to modulate inflammatory responses and protect against systemic inflammation.

Objective: To identify endothelial cell–released soluble factors that protect against endothelial barrier dysfunction and systemic inflammation.

Methods and Results: We found that conditioned medium of endothelial cells inhibited cyclooxygenase-2 and interleukin-6 expression in macrophages stimulated with lipopolysaccharide. Analysis of conditioned medium extracts by liquid chromatography–mass spectrometry showed the presence of 5-methoxytryptophan (5-MTP), but not other related tryptophan metabolites. Furthermore, endothelial cell–derived 5-MTP suppressed lipopolysaccharide-induced inflammatory responses and signaling in macrophages and endotoxemic lung tissues. Lipopolysaccharide suppressed 5-MTP level in endothelial cell-conditioned medium and reduced serum 5-MTP level in the murine sepsis model. Intraperitoneal injection of 5-MTP restored serum 5-MTP accompanied by the inhibition of lipopolysaccharide-induced endothelial leakage and suppression of lipopolysaccharide- or cecal ligation and puncture–mediated proinflammatory mediators overexpression. 5-MTP administration rescued lungs from lipopolysaccharide-induced damages and prevented sepsis-related mortality. Importantly, compared with healthy subjects, serum 5-MTP level in septic patients was decreased by 65%, indicating an important clinical relevance.

Conclusions: We conclude that 5-MTP belongs to a novel class of endothelium-derived protective molecules that defend against endothelial barrier dysfunction and excessive systemic inflammatory responses. (Circ Res. 2016;119:222-236. DOI: 10.1161/CIRCRESAHA.116.308559.)

Key Words: 5-methoxytryptophan ■ capillary permeability ■ inflammation ■ sepsis ■ shock

Systemic inflammation has emerged as a key pathophysiological process that induces multiorgan injury and causes serious human diseases.1–4 Sepsis is a prototypic systemic inflammatory syndrome characterized by overexpression of proinflammatory mediators, such as cytokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), and concurrent activation of innate immune cells such as neutrophils and macrophages.1,2,5–8 Sepsis is a serious human disease with high morbidity and mortality, which has major health and socioeconomic impacts. Most cases of sepsis are caused by uncontrolled infections or extensive tissue damage (such as caused by burns or multiple injuries). During sepsis, bloodborne bacterial pathogens trigger inappropriate activation and dysfunction of immune system to cause systemic inflammation and collateral tissue damage that lead to severe disorders.
such as septic shock, intravascular coagulation, endothelial injury, microvascular leakage, and multiple organ failure. However, treatment of systemic inflammatory syndrome and sepsis is limited to fluid administration, oxygen supplement, antibiotics, and other supportive measures. Specific therapeutic approaches targeting proinflammatory cytokines, coagulation cascade, lipopolysaccharide, or immune responses are unsuccessful or have marginal efficacy. New therapeutic strategies are needed to conquer this serious human illness.

Abnormal activation of innate immune system has been implicated in the development of inflammatory disorders such as septic shock and multiple organ failure. Toll-like receptors (TLRs) play a critical role in regulating immune response and maintaining immune homeostasis. Inappropriate activation of TLR signaling by pathogen components and endogenous harmful molecules is a major contributor to systemic inflammatory syndrome. In particular, activation of TLR4 by lipopolysaccharide is thought to be an important trigger of inflammatory disorders such as septic shock, intravascular coagulation, endothelial barrier dysfunction. Furthermore, 5-MTP inhibits lipopolysaccharide-induced COX-2 and iNOS expression and an array of cytokines and chemokine productions in macrophages. Administration of 5-MTP to murine sepsis models restored serum 5-MTP and rescued mice from sepsis-induced mortality and lung and spleen damages.

**Methods**

An expanded Methods is available in the Online Data Supplement.

**Reagents**

Brefeldin A, monensin, 2-(4-fluoro-benzoylamino)-benzoic acid methylester, nocardazole, and 2-aminobicyclo(2,2,1)-heptane-2-carboxylic acid were purchased from Sigma-Aldrich.

**Preparation of 5-MTP**

Two different sources of 5-MTP have been used in this study. DL-5-MTP was purchased from Sigma-Aldrich, and L-5-MTP was from custom-synthesized by AstaTech (AstaTech Inc, Bristol, PA). Purity of L-5-MTP and DL-5-MTP was verified by liquid chromatography–mass spectrometry.

For in vitro drug application, 50 mmol/L of DL- or L-5-MTP stock solution was prepared in dimethyl sulfoxide in blown tube, and stocked at −20°C or −80°C. For in vivo experiments, DL-5-MTP or L-5-MTP was freshly dissolved in saline (0.5 mL, pH adjusted to 7.4) and injected into animals at the concentration of 23.4 or 100 mg/kg.

**Metabolomic Analysis**

The metabolite analysis was performed using ultraperformance liquid chromatography (Acquity UPLC System; Waters Corporation) coupled with a Xevo-TOF mass spectrometer (Waters Corporation). The detailed procedures are provided in the Methods in the Online Data Supplement.

**Induction of Endotoxemia in Mouse Models**

Endotoxemia was induced in C57BL/6 mice by lipopolysaccharide injection or cecal ligation and puncture (CLP). The detailed procedures are provided in the Methods in the Online Data Supplement. Mouse experiments were approved by the Institutional Animal Care and Use Committee, National Health Research Institutes.
Human Subjects
The human study was performed from May 2013 to August 2013 in the Division of Infectious Diseases and Tropical Medicine, Tri-Service General Hospital, Taipei, Taiwan. Fifty patients with sepsis syndrome without shock (29 men and 21 women with a mean age of 64.0±19.6 years) and 15 septic shock patients (10 men and 5 women with a mean age of 74.4±11.5 years) were enrolled in the study and sepsis syndrome was diagnosed based on clinical and laboratory findings, following the guidelines of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference. \(^{16}\)

Thirty age- and sex-matched healthy subjects were assessed as controls. The human study was approved by the Ethics Committee of Tri-Service General hospital (TSGH IRB number: 2-101-05-024). All patients signed informed consent.

Cytokine-Specific ELISA
Cytokine levels in the culture supernatants and serum were determined in microtiter plates (96 wells) by a specific sandwich ELISA (Biosource or eBioscience) as previously described.\(^ {17,18}\)

Western Blot Analysis
Cellular proteins were resolved with 5% to 20% SDS-PAGE and transferred to nitrocellulose membranes. They were blotted with specific antibodies as previously described.\(^ {19}\)

Immunoprecipitation Assays
Cells were lysed in cold lysis buffer supplemented with protease inhibitor mixture (Sigma-Aldrich). After homogenization, the lysates were incubated with anti-peroxiredoxin 1 (anti-Prdx 1) Abs (2 mg/mL) or IgGs (2 mg/mL) and protein A-Sepharose beads (Thermo) overnight at 4°C. The pellet containing immunocomplexes was collected by centrifugation, and then the pellet was washed with cold lysis buffer. After 3 washes, recovered immunocomplexes were solubilized in SDS sample buffer and separated on 4% to 20% Novex Tris-Glycine gel (Invitrogen Life Technologies). Phospho-p38 (p-p38) mitogen-activated protein kinase (MAPK) was detected on Western blotting with a specific Ab.

Caspase-3 Activity Assay
Caspase-3 activity was determined by the cleavage of the fluorometric substrate z-DEVD-AMC (Upstate Biotechnology) as previously described.\(^ {20}\)

Statistical Analysis
All values were given as mean±SD. \(t\) test was used to determine the statistical significance of difference between treatment and control groups, whereas 1-way ANOVA was used to analyze multiple groups. \(P\) values of <0.05 were considered statistically significant. The diagnostic performance of 5-MTP, high-sensitive C reactive protein (hs-CRP), and interleukin 1 beta (IL-1β) was determined using area under the receiver operating characteristic curve (AUROC) analysis to assess the overall discriminatory power of these assays in predicting sepsis syndrome. The 95% confidence intervals and optimal cutoff points yielding maximum sums of sensitivity and specificity from the AUROC curves for each assay were also computed. Data were analyzed with the SPSS statistical software 18.0 (SPSS Inc., Chicago, IL).

Results

ECs Produce 5-MTP
ECs are considered to play a key role in controlling systemic inflammation and progression of inflammatory diseases.\(^ {21}\) We hypothesized that ECs release soluble factors to modulate inflammatory responses. To determine whether human umbilical vein endothelial cells (HUVECs) release soluble molecules into the conditioned medium (CM), we evaluated the effect of HUVEC-CM on lipopolysaccharide-induced COX-2 expression and IL-6 production in RAW264.7 cells. HUVEC-CM inhibited lipopolysaccharide-induced COX-2 protein expression and IL-6 level (Online Figure IA and IB). In addition, CM from human aortic ECs, human pulmonary artery ECs, and human coronary artery ECs also significantly suppressed lipopolysaccharide-induced IL-6 production in RAW264.7 cells (Online Figure IC and ID). These results revealed that the CM of different types of ECs contains endogenous molecules to block lipopolysaccharide-induced COX-2 and IL-6 expression. We further determined soluble molecules in the CM by liquid chromatography–mass spectrometry. HUVEC-CM and control medium were filtered through 3K membrane, the filtrates were collected and applied to UPLC-QTOF mass spectrometer. Analysis of the spectra of mass spectrometry reveals striking differences between HUVEC-CM and control medium. Two major peaks at \(m/z\) 235.1 and 218.1 were detected in HUVEC-CM, but not in control medium. These 2 peaks matched the profile of pure 5-MTP (Figure 1A). To ensure that \(m/z\) 235.1 is 5-MTP, we analyzed daughters of \(m/z\) 235.1 and compared their profile with pure 5-MTP. The daughter profile of \(m/z\) 235.1 matched that of pure 5-MTP (Figure 1B). We did not detect \(m/z\) peaks matching serotonin (\(m/z\) 177.2), melatonin (\(m/z\) 233.3), or 5-methoxytryptamine (\(m/z\) 191.2). As 5-MTP is a key component of soluble molecules, we assessed the involvement of 5-MTP in controlling IL-6 expression. IL-6 suppression was abrogated by anti-5-MTP antibodies, but not by control IgG (Figure 1C). To confirm the presence of 5-MTP in CM, we analyzed 5-MTP in the CM by ELISA and detected comparable amounts of 5-MTP in human aortic EC-CM, human pulmonary artery EC-CM, human coronary artery EC-CM, and HUVEC-CM (Figure 1D). 5-MTP was undetectable in fresh medium containing fetal bovine serum. These results indicate that ECs release 5-MTP but not other tryptophan metabolites into the CM that accounts for COX-2 and cytokine-suppressing actions.

Fluorescence microscopy was used to determine the intracellular location of 5-MTP in HUVECs. Immunofluorescence analysis reveals cytoplasmic staining with endoplasmic reticulum (ER) staining pattern (Online Figure IIA), suggesting that 5-MTP may be secreted by an ER to Golgi vesicle–mediated secretory route. To further dissect the secretory route, we treated HUVECs and human aortic ECs with inhibitors that disrupt the extracellular milieu via a vesicle-mediated-ER to Golgi secretory pathway. The detailed trafficking mechanism is being investigated.

Lipopolysaccharide Suppresses Endothelial Production of 5-MTP
Lipopolysaccharide causes endothelial damage by disrupting endothelial barrier function and inducing inflammation.\(^ {22}\) We
Figure 1. A soluble factor in endothelial cell (EC)–conditioned medium (CM) inhibits lipopolysaccharide (LPS)–induced expression of cyclooxygenase-2 and proinflammatory cytokines in macrophages. A, Representative mass spectra (retention time, 4.48 min; m/z 100–280) of human umbilical vein endothelial cell (HUVEC)-CM, control medium M200 or pure L-5-5-methoxytryptophan (MTP).
B, Analysis of daughter profiles of m/z 235.1 of HUVEC-CM MS1 spectra vs that of pure L-5-MTP by liquid chromatography–mass spectrometry (MS) MS (MS2). Metabolomic analysis of HUVEC-CM was performed using UPLC–QT of MS. The experiments in A and B were repeated 3× with similar results. C, RAW264.7 cells were incubated in HUVEC-CM or control medium (M200) with or without LPS in the presence or absence of anti-IgG or anti-5-MTP antibodies for 24 h. Interleukin (IL)-6 level in culture supernatants was measured by ELISA. D, 5-MTP concentrations in control medium (endothelial cell growth medium-2 [EGM2], EGM2-MV, or M200) or CM from human aortic ECs (HAECs), human pulmonary artery ECs (HPAECs), human coronary artery ECs (HCAECs), and HUVECs. E, 5-MTP concentrations in control medium (M200) or CM from HUVECs treated with different amounts of LPS for 24 h. F, HUVECs were stimulated with different amounts of LPS for 24 h. Cell lysates were immunoblotted with antibodies for tryptophan hydroxylase (TPH)-1 or β-actin. Data in C–F represent mean±SD of at least 3 independent experiments.
wondered whether the endothelial damaging effect could be attributable to suppression of 5-MTP production. To address this, we measured CM 5-MTP in HUVECs treated with or without lipopolysaccharide. Lipopolysaccharide suppressed 5-MTP in a concentration-dependent manner and completely inhibited 5-MTP level at 100 ng/mL lipopolysaccharide (Figure 1E). We previously reported that tryptophan hydroxylase 1 (TPH-1) is a key enzyme in 5-MTP synthesis and TPH-1 silencing with siRNA causes reduction of 5-MTP. We wondered whether lipopolysaccharide suppresses 5-MTP through inhibition of TPH-1 expression. TPH-1 proteins were detected in HUVECs that were reduced by lipopolysaccharide in a concentration-dependent manner (Figure 1F). These results suggest that lipopolysaccharide inhibits 5-MTP synthesis by suppressing TPH-1 that results in reduced release of 5-MTP.

**Lipopolysaccharide Reduces 5-MTP in Murine Tissues and Blood**

To confirm that 5-MTP is suppressed by lipopolysaccharide in vivo, we determined 5-MTP expression in murine aorta by immunohistochemical examination. 5-MTP staining was detected in EC, vascular smooth muscle cells, and adventitial cells of normal aorta that was diminished by lipopolysaccharide treatment (Figure 2A). 5-MTP was also detected in vascular cells of pulmonary arteries and renal arteries (Online Figure IIIA and IIIB). We also detected 5-MTP staining in the epithelial cells of terminal bronchiole of lungs (Online Figure IIC) as well as renal epithelial cells (Online Figure IID). We next determined whether 5-MTP is detected in the circulating blood and whether its level is suppressed by lipopolysaccharide treatment. We injected lipopolysaccharide (60 mg/kg) into mice and analyzed 5-MTP in the serum of lipopolysaccharide-treated mice. 5-MTP was detected in saline-treated mice (mean, 0.19±0.05 μmol/L; n=8), which was reduced after lipopolysaccharide treatment (mean, 0.04±0.05 μmol/L; n=8; Figure 2B). These results indicate that 5-MTP is produced in vascular cells, pulmonary cell, and renal epithelial cell. Furthermore, vascular ECs release 5-MTP into the circulating blood. Lipopolysaccharide inhibits 5-MTP production, thereby reducing circulating 5-MTP concentrations.

**Serum 5-MTP Is Reduced in Human Septic Syndrome**

The novel finding that murine blood contains detectable 5-MTP (Figure 2B) prompted us to determine whether human blood has measurable 5-MTP. 5-MTP concentration in human serum is several folds higher than that in murine serum (1.05±0.39 μmol/L; n=30; Figure 2C; Table). To determine the clinical relevance of 5-MTP, we further measured serum 5-MTP in patients with clinical evidence of sepsis without shock. In the enrolled 50 septic patients, the serum levels of hs-CRP (98.2±74.3 versus 0.5±0.5 mg/L; P<0.001) and IL-1β (1327±2445 versus 526±175 pg/mL; P=0.025) were significantly higher than healthy subjects. Interestingly, the serum 5-MTP concentration of septic patients was markedly reduced with a mean value of 0.37±0.15 μmol/L (Figure 2C; Table). To assess whether serum 5-MTP level changes with progression of sepsis, we measured serum 5-MTP in 15 patients who had septic shock. The serum 5-MTP level of patients in septic shock (0.30±0.39 μmol/L) was slightly lower than that without shock. However, the difference is not statistically significant (Figure 2C). These results suggest that reduction of serum 5-MTP in septic syndrome may reflect systemic inflammation and may be a marker of sepsis.

As serum hs-CRP and IL-1β levels were reported to be a diagnostic marker of sepsis, we used AUROC to determine the discriminative power of 5-MTP, hs-CRP, and IL-1β in this group of patients (Figure 2D; Online Table I). The AUROC value of 5-MTP (0.958; 95% confidence interval, 0.919–0.997) was comparable with the value of hs-CRP (0.995; 95% confidence interval, 0.987–1.000) and significantly greater than that of IL-1β (0.696; 95% confidence interval, 0.581–0.810). Cutoff levels of hs-CRP and IL-1β with the optimum diagnostic efficiency derived from the AUROC curves were 2.73 mg/L (sensitivity 100%; specificity 93.9%) and 467.29 pg/mL (sensitivity 53.3%; specificity 78%). Using serum 5-MTP level of 0.63 μmol/L as a cutoff, the sensitivity was 83% and the specificity was 94% (Figure 2D; Online Table I). These results suggest that 5-MTP could serve as a predictive biomarker of sepsis.

**5-MTP Rescues Lipopolysaccharide-Induced Endothelial Barrier Disruption**

Disruption of endothelial barrier function is a crucial step in sepsis. Lipopolysaccharide-induced endothelial barrier disruption results in uncontrolled passage of proinflammatory cells and blood solutes into vascular wall and tissues that trigger systemic inflammation and vasorelaxation. Because lipopolysaccharide suppresses endothelial 5-MTP production, we reasoned that exogenous administration of 5-MTP rescues endothelial barrier function. Addition of 5-MTP to culture medium reduced lipopolysaccharide-induced endothelial permeability in a concentration-dependent manner (Figure 3A). Furthermore, 5-MTP inhibited macrophage transmigration induced by lipopolysaccharide at concentrations comparable with those blocking permeability of FITC-dextran (Figure 3B). To confirm the in vitro findings, we determined the protective effect of 5-MTP on pulmonary microvascular permeability as measured by extravasation of Evans blue. Lipopolysaccharide injection caused a significant increase in Evans blue leakage into the mouse lung (Figure 3C), whereas this increase was prevented by 5-MTP administration. These results suggest that endothelium-derived 5-MTP controls vascular permeability and defends against inflammatory cell transmigration through endothelium into the vascular wall and underlying tissues.

**5-MTP Inhibits Lipopolysaccharide-Induced COX-2 Expression and Cytokine Production in Macrophages**

Given that dysregulation of COX-2 and cytokine production by innate immune cells is one of the important systemic inflammation hallmarks, we thus determined whether 5-MTP suppresses lipopolysaccharide-induced COX-2 expression and cytokine production in macrophages. The results revealed that 5-MTP blocked lipopolysaccharide-induced COX-2 protein expression and promoter activity in a concentration-dependent manner in RAW264.7 cells (Online Figure IVA and IVB). 5-MTP also inhibited lipopolysaccharide-induced
COX-2 expression in primary mouse peritoneal macrophages (Online Figure IVC). 5-MTP inhibited lipopolysaccharide-induced IL-6 promoter activity as well as IL-6 and tumor necrosis factor-alpha (TNF-α) proteins in RAW264.7 cells in a concentration-dependent manner (Figure 4A) comparable with its inhibition of COX-2 expression (Online Figure IVA and IVB). Furthermore, it inhibited IL-6, IL-1β, and TNF-α production in peritoneal macrophages (Figure 4B). As serotonin, 5-methoxytryptamine and tryptophan share an indole backbone with 5-MTP, we evaluated the effect of these compounds on lipopolysaccharide-induced COX-2 and IL-6 expressions. Neither serotonin nor 5-methoxytryptamine or
tryptophan exerted an effect on COX-2 and IL-6 expression (data not shown). Melatonin (N-acetyl-5-methoxytryptamine) was reported to inhibit COX-2 expression, albeit at millimolar concentrations. These results imply that the 5-methoxyindole-3-propionic acid moiety is required for inhibiting lipopolysaccharide-induced COX-2 and cytokine production (Online Figure VA).

To elucidate the molecular mechanisms by which 5-MTP attenuates lipopolysaccharide-induced cytokine and COX-2 expression, we assessed the effects of 5-MTP on lipopolysaccharide-induced kinase activation in RAW264.7 cells. Lipopolysaccharide induced phosphorylation of p38 MAPK and extracellular signal-regulated kinase (ERK)1/2 within 30 minutes and lasted for at least 8 hours (Online Figure VIA). Interestingly, 5-MTP dose-dependently blocked p38 MAPK phosphorylation but not ERK1/2 phosphorylation in lipopolysaccharide-treated RAW264.7 cells (Figure 4C). Prdx 1 was reported to enhance p38 MAPK activity and promoting pancreatic cancer cell invasion by directly binding to activated p-p38 MAPK. We wondered whether 5-MTP inhibits p38 MAPK activation by disrupting the interaction between Prdx 1 and p-p38. To examine this possible mechanism, RAW264.7 cells treated with 5-MTP or vehicle followed by lipopolysaccharide were lysed and the lysates were subjected to immunoprecipitation using a selective Prdx 1 antibody or nonimmune IgG. Phosphor-p38 and Prdx 1 in the immune complex were analyzed by Western blotting. Phosphor-p38 was complexed with Prdx 1 at basal cell state that was increased by lipopolysaccharide (Online Figure VIB). 5-MTP reduced p-p38 interaction with Prdx 1 in untreated and lipopolysaccharide-treated cells without an apparent effect on Prdx 1 level in the immune complex (Online Figure VIB). 5-MTP reduced p-p38 interaction with Prdx 1 in untreated and lipopolysaccharide-treated cells without an apparent effect on Prdx 1 level in the immune complex (Online Figure VIB). To further determine whether 5-MTP controls Prdx 1 expression in macrophages, RAM264.7 cells were stimulated with lipopolysaccharide in the presence of different concentrations of 5-MTP (1, 10, and 50 μmol/L) for 1 hour. 5-MTP did not affect Prdx 1 expression in lipopolysaccharide-treated RAW264.7 cells nor did it affect the basal Prdx 1 level (Online Figure VIC and VID). These results suggest that 5-MTP suppressed p38 MAPK activation by inhibiting interaction between Prdx 1 and p-p38 MAPK.

Activation of p300 histone acetyltransferase (HAT) plays an important role in transcriptional coactivation of NF-κB and expression of inflammatory genes, such as COX-2 and iNOS. Lipopolysaccharide activated p300 HAT in peritoneal macrophages at 4 hours and persisted for 24 hours, and 5-MTP significantly blocked p300 HAT activation up to 24 hours (Figure 4E).

Table. Serum Level of 5-MTP, hs-CRP, and IL-1β in Septic Patients and Healthy Donors

<table>
<thead>
<tr>
<th></th>
<th>Septic Patients (n=50)</th>
<th>Healthy Donors (n=30)</th>
<th>P Value</th>
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<tbody>
<tr>
<td>5-MTP, μmol/L</td>
<td>0.37±0.15</td>
<td>1.05±0.39</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>hs-CRP, mg/L</td>
<td>98.2±74.3</td>
<td>0.5±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td>1327±2445</td>
<td>526±175</td>
<td>0.025</td>
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5-MTP indicates 5-methoxytryptophan; hs-CRP, high-sensitive-C reactive protein; and IL, interleukin.

Figure 3. 5-methoxytryptophan (5-MTP) rescues lipopolysaccharide (LPS)–induced endothelial barrier disruption. A, After pretreating human umbilical vein endothelial cells (HUVECs) with different concentrations of L-5-MTP for 30 min, HUVEC monolayer was stimulated with LPS or TE buffer for 1 h, and dextran flux was measured. B, BCECF-AM-labeled peritoneal macrophages were seeded onto a HUVEC monolayer treated with vehicle or LPS for 1 h with or without L-5-MTP. After 4-h incubation, transmigrated macrophages were counted. Data in A and B represent mean±SD of at least 3 independent experiments. C, Mice were injected with saline or L-5-MTP (23.4 mg/kg) for 30 min before they were challenged with LPS (60 mg/kg) for 24 h (n=8 mice per group). Pulmonary microvascular permeability was assessed by Evans blue accumulation in the lungs as described in the Methods in the Online Data Supplement. The error bar, mean±SD (n=8 mice per group).
Taken together, these results indicate that 5-MTP inhibits cytokine and COX-2 expression in macrophages by blocking activation of p38 MAPK, NF-κB, and p300 HAT.

Results from signaling and transcriptional studies suggest that 5-MTP may act via a receptor. To begin to address this, we explored the possibility of a membrane receptor by using fluorescence CFTM488-labeled 5-MTP. Because RAW264.7 cells are a target cell of 5-MTP and do not produce 5-MTP, we incubated RAW264.7 cells with fluorescence CFTM488-conjugated 5-MTP and excessive unlabeled 5-MTP at increasing concentrations for 120 minutes. After extensive washing with PBS, cells were subjected to analysis with flow cytometry. When incubated with fluorescent-5-MTP alone, >60% of cells were fluorescence positive, which was reduced to ≈30% when coincubated with 5-MTP in excess by 3-folds over the fluorescent-5-MTP (Online Figure VIII). These preliminary data suggest that the anti-inflammatory action of 5-MTP could be mediated by a membrane receptor. Further studies are needed to identify the receptor.

5-MTP Protects Against Lethal Endotoxemia in Mice

Suppression of endothelial 5-MTP production by endotoxemia could contribute to endothelial hyperpermeability and uncontrolled macrophage overproduction of proinflammatory mediators. We determined whether exogenous 5-MTP administration rescues mice from lipopolysaccharide-induced sepsis-like syndrome. Mice were injected with 5-MTP (23.4 mg/kg) or vehicle for 30 minutes, followed by lipopolysaccharide (60 mg/kg). To ensure that intraperitoneal 5-MTP administration increases serum 5-MTP, we analyzed serum 5-MTP level at 24 and 72 hours after 5-MTP injection. Serum 5-MTP was...
elevated several-fold over the basal level at 24 hours after 5-MTP administration and returned to basal level at 72 hours (Online Figure IX). Mice treated with lipopolysaccharide started to die at day 1 (Figure 5A), and >65% of mice died at day 3 (Figure 5A), correlating with a significant decrease in serum 5-MTP (Figure 2B). By contrast, none of the mice...
treated with 5-MTP died at 24 hours and only 20% of endotoxemic mice died at 72 hours, whereas no mortality was observed in control groups (saline or 5-MTP alone; Figure 5A). To confirm the protective effect of 5-MTP in endotoxemia, we used CLP model in mice. 5-MTP-afforded protection was also observed in mice challenged with CLP. It significantly improved survival from 0% to 44.4% at day 2. This improved survival was maintained up to day 6 (Figure 5B). 5-MTP had a marginal protective effect on body weight loss caused by lipopolysaccharide (Online Figure XA). Examination of lung tissues revealed that lipopolysaccharide induced time-dependent progression of polymorphonuclear leukocyte infiltration, alveolar septal wall thickening, interstitial edema, and alveolar congestion, consistent with pathological changes of sepsis (Figure 5C). Furthermore, 5-MTP significantly reduced leukocyte infiltration at all time points (Online Figure XB). 5-MTP significantly alleviated these pathological changes caused by lipopolysaccharide (Figure 5C). Effect of 5-MTP on reducing neutrophil infiltration in lipopolysaccharide-treated mice was confirmed by staining with Ly-6G, a neutrophil surface marker (Figure 5D) and measuring myeloperoxidase activity (Online Figure XC). At 24 hours after lipopolysaccharide administration, there was a significant increase in total cell number in the bronchoalveolar lavage fluid, which was reduced by 5-MTP in a dose-dependent manner (Online Figure XD).

We next investigated the effect of 5-MTP on pulmonary cell apoptosis. Lipopolysaccharide treatment resulted in a significant increase in apoptosis as demonstrated by increased cleaved caspase-3 at 12 and 16 hours, which was reduced by 5-MTP (Figure 5E). 5-MTP also prevented lipopolysaccharide-induced caspase-3 cleavage in spleen cells (Online Figure XIA). In lipopolysaccharide-challenged mice, the spleen weight/body weight ratio was markedly increased when compared with that of saline-treated mice, consistent with severe spleen edema. 5-MTP administration ameliorated spleen edema in a dose-dependent manner (Online Figure XIB).

5-MTP Suppresses COX-2 and iNOS Expression in Lung Tissues and Reduces Blood Levels of Cytokines and Chemokines in Lipopolysaccharide-Treated Mice

COX-2 and iNOS levels were progressively increased after mice had been infused with lipopolysaccharide from 12 to 24 hours, and 5-MTP blunted COX-2 and iNOS overexpression in a dose-dependent manner (Figure 6A and 6B). Effect of 5-MTP on lipopolysaccharide-induced cytokine production by macrophage in vivo was evaluated in lipopolysaccharide-treated mice. At 8 hours after lipopolysaccharide treatment, peritoneal macrophages were collected and their production of IL-1β and IL-6 was measured. Surge of macrophage production of IL-1β and IL-6 in lipopolysaccharide-treated mice was abated to the basal level by 5-MTP pretreatment (Figure 6C). As elevation of proinflammatory cytokines and chemokines in circulating blood creates cytokine storm, which is detrimental to multiple organs including lungs, we measured key cytokines and chemokines. Serum levels of IL-1β, TNF-α, IL-6, and interferon-γ were increased in lipopolysaccharide-treated mice in a dose- and time-dependent manner (Figure 7A; Online Figure XII). Pretreatment with 5-MTP dose-dependently prevented elevation of cytokines (Figure 7A; Online Figure XII) and chemokines such as CXCL1 (chemokine [C-X-C motif] ligand 1), MCP-1 (monocyte chemotactic protein-1), RANTES (regulated on activation, normal T-cell expressed and secreted), and Eotaxin at 24 hours after lipopolysaccharide injection (Figure 7B).

To confirm the involvement of NF-κB and p300 HAT in COX-2 and iNOS overexpression as well as cytokine overproduction, we analyzed p-p65 and p300 HAT activity in lung tissues. Lipopolysaccharide-induced p-p65 and p300 HAT activity in lung tissues was blocked by administration of 5-MTP in mice (Figure 7C and 7D). CLP in mice produces sepsis-like syndrome with systemic inflammation. We evaluated the effect of 5-MTP on blood cytokine levels in this model. 5-MTP administration resulted in marked reduction in serum IL-1β and IL-6 (Figure 7E). These results indicate that 5-MTP suppresses the production of cytokines and chemokines, and thereby reduces their levels in circulating blood.

5-MTP Reduces Serum Kynurenine Levels

Lipopolysaccharide-induced renal indoleamine 2,3-dioxygenase overexpression and the consequent increase in blood levels of kynurenine were reported to cause vasodilatation and contribute to arterial hypotension, a fatal manifestation of sepsis. We evaluated the effect of 5-MTP on kynurenine levels in lipopolysaccharide-treated mice. Consistent with the reported results, serum kynurenine level was increased by >3-fold in lipopolysaccharide-treated mice (Figure 7F). 5-MTP pretreatment reduced it to the basal level (Figure 7F). These results indicate that 5-MTP is effective in controlling overexpression of proinflammatory mediators and suppression of the circulating levels of proinflammatory cytokines and vasodilating kynurenine.

Discussion

Tryptophan, an essential amino acid supplied by dietary proteins, is used for protein synthesis and serves as a precursor for a large number of metabolites that are important in human nutrition and metabolism. In the present study, we used comparative metabolomics to identify an endothelium-derived tryptophan metabolite, 5-MTP, which plays a critical role in controlling systemic inflammatory responses. Our results show for the first time that 5-MTP is detected in human and murine circulating blood. Given its potent anti-inflammatory properties, 5-MTP may serve as a circulating autacoid to defend against systemic inflammation. Our results further show that 5-MTP production by ECs is suppressed by lipopolysaccharide, and the blood level of 5-MTP in the mouse model was reduced by lipopolysaccharide treatment. Importantly, serum 5-MTP level is reduced in sepsis patients with or without shock. Although human sepsis is not necessarily all because of lipopolysaccharide endotoxins, lipopolysaccharide plays a key role in sepsis in a majority of patients. Reduction of 5-MTP in sepsis patients is likely because of the suppression of 5-MTP production by lipopolysaccharide. Thus, lipopolysaccharide creates a 5-MTP deficiency state that results in abrogation of control of macrophage activation and cytokine overproduction. The critical role that 5-MTP plays in maintaining homeostasis of inflammation is supported by its rescue of mice from lipopolysaccharide- and CLP-induced excessive inflammatory responses, organ damage,
and mortality. Our findings indicate that intraperitoneal administration of 5-MTP raises blood 5-MTP in the murine sepsis model to combat excessive inflammation and protect against apoptosis, lung injury, and death. This protective effect of 5-MTP is in contrast to the unfavorable hypotensive effect of another endothelium-released tryptophan metabolite, kynurenine, in experimental endotoxemia.\(^{11}\) 5-MTP was reported to increase the expression of cytoprotective factors in rat cardiomyocytes subjected to \(\text{H}_2\text{O}_2\) insults, supporting the tissue protective effect of 5-MTP.\(^{27}\)

COX-2 is a major proinflammatory mediator. Its expression in inflammatory cells such as monocytes and macrophages is stimulated by environmental stresses and proinflammatory cytokines. Cytokines enhance binding of NF-\(\kappa\)B, C/EBP\(\beta\), AP-1 (activator protein 1), and CREB-2 (cAMP response element binding protein 2) to their cognate motifs located within 500-bp

**Figure 6.** 5-methoxytryptophan (5-MTP) suppresses cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in lung tissues and reduces proinflammatory cytokines production in macrophages. Paraffin-embedded sections were prepared from lungs of mice injected with 60 mg/kg lipopolysaccharide (LPS) with or without saline or DL-5-MTP for the indicated time. A, COX-2 and (B) iNOS protein levels in lung tissues were determined by immunohistochemistry by staining with anti–COX-2 and anti-iNOS antibodies, respectively. Scale bars, 100 \(\mu\)m. C, Peritoneal macrophages were isolated from mice 8 h after LPS injection with saline or DL-5-MTP (23.4 mg/kg) and cultured with medium alone. After 24 h, proinflammatory cytokines in conditioned medium were measured by ELISA (n=3). IL indicates interleukin.
Figure 7. 5-methoxytryptophan (5-MTP) treatment suppresses cytokine storm and reduces serum kynurenine levels in endotoxemic mice. 

A. Mice were injected intraperitoneally with or without different concentrations of DL-5-MTP or saline for 30 min, followed by lipopolysaccharide (LPS) administration for the indicated time. Proinflammatory cytokine levels in serum were measured by ELISA (n=6 per group).

B. Chemokine level was measured by ELISA in the serum of saline or LPS-injected mice with or without saline or DL-5-MTP (23.4 mg/kg) after 24 h (n=10).

C. Phospho-p65 level in lung tissues was determined by an ELISA kit at 4 and 8 h after LPS injection with or without DL-5-MTP (23.4 mg/kg; n=6 mice per group).

D. p300 histone acetyltransferase (HAT) activity in lung tissues of mice (n=6 mice per group) at 8 to 24 h after LPS injection with or without DL-5-MTP (23.4 mg/kg) (Continued)
from the transcription start sites of COX-2 promoter.28 Here, we confirm that 5-MTP inhibits lipopolysaccharide-induced NF-κB and p300 HAT activation in macrophages and in lung tissues in vivo. Because p300 HAT is a master transcription co-activator for the expression of diverse proinflammatory genes through modifying chromatin structure and augmentation of multiple transactivator binding,26,29–31 control of systemic inflammation and multiorgan damage in lipopolysaccharide-induced sepsis is attributable to its inhibition of p300 HAT activation and the consequent reduction of NF-κB and other transactivator binding and decrease in cytokine, and chemokine as well as COX-2, and iNOS transcriptional activation and expression. These findings are clinically relevant as p300 was reported to be upregulated in sepsis.32 It is unclear how 5-MTP suppresses p300 HAT activity. Our preliminary data suggest that 5-MTP does not directly inhibit p300 HAT (data not shown). We suspect that 5-MTP inhibits p300 HAT by disrupting proinflammatory signal transduction in a receptor-dependent fashion. This hypothesis is further being tested.

NF-κB is a master activator of inflammatory gene transcription.29,33,34 On cellular activation, activated NF-κB (p65/p50) binds to the functional binding sites on the promoter/enhancer region of diverse genes such as COX-2, iNOS, and proinflammatory cytokines and chemokines.14,21,25,26,35–37 We show that 5-MTP blocks NF-κB activation that could account for its broad inhibitory actions on the proinflammatory cytokines and mediators. It has been shown that the inhibition of NF-κB is significantly correlated with improvement of survival from endotoxin shock and sepsis.38,39 Our results further demonstrate that 5-MTP blocks NF-κB by interrupting p38 MAPK signaling pathway. Although signaling kinases such as p38 MAPK and ERK1/2 are activated and involved in TLR-mediated responses,17,18,40 5-MTP selectively suppresses p38 MAPK activity which inhibited NF-κB transactivation and IL-6 and TNF-α production, whereas a higher concentration (15–50 μmol/L) is needed to inhibited cellular events such as prevention of endothelial barrier disruption or macrophage transmigration. The effective 5-MTP concentrations are higher than serum level. In our cellular experiments, we observed that 5-MTP at 5 to 10 μmol/L inhibited NF-κB transactivation and IL-6 and TNF-α production, whereas a higher concentration (15–50 μmol/L) is needed to inhibited cellular events such as prevention of endothelial barrier disruption or macrophage transmigration. The effective 5-MTP concentrations are higher than serum concentration but may be in the range of tissue 5-MTP concentrations. Thus, 5-MTP may play a physiological role in maintaining an inflammatory balance and preventing excessive inflammation and protecting cells and tissues from inflammatory damage.

5-MTP has been reported to be synthesized from l-tryptophan via 2 enzymatic steps: (1) a TPH that convert l-tryptophan to 5-hydroxytryptophan (5-HTP) and a hydroxyindole O-methyltransferase that produces 5-MTP.4 Intracellular l-tryptophan is catabolized by 2 major pathways: (1) direct conversion into kynurenine via indoleamine 2,3-dioxygenase or tryptophan 2,3-dioxygenase,43,44 and (2) conversion into an intermediary metabolite 5-HTP via TPH. 5-HTP is a common precursor of several biologically active molecules including serotonin, melatonin, and 5-MTP (Online Figure VB–VD). Two TPH isoforms have been identified and characterized. TPH-2 isoform is expressed in central nervous system, whereas TPH-1 is expressed in peripheral tissues such as vascular cells.45,46

In this study, we provide evidence that TPH1-derived 5-HTP is converted primarily and probably exclusively to 5-MTP in ECs as we do not detect serotonin, melatonin, kynurenine, or human cells release a large quantity of 5-MTP into the circulation. The cellular source of blood 5-MTP is not entirely clear, but ECs are likely to be the major contributor. This is supported by experimental data. First, CM of several types of cultured ECs contain comparable quantities of 5-MTP as analyzed by ELISA, indicating that ECs release a considerable amounts of 5-MTP into the CM. Second, 5-MTP is detected in the cytoplasm of cultured HUVECs by immunofluorescence microscopy. Finally, ECs in murine aorta, pulmonary, and renal blood vessels are stained positively for 5-MTP by immunohistochemical analysis. In addition to ECs, vascular smooth muscle cells and adventitial fibroblasts are also positively stained, suggesting that these 2 types of vascular cells may also contribute to 5-MTP in circulating blood. Additional studies will be needed to assess their 5-MTP production. Our results suggest that lipopolysaccharide suppresses EC production of 5-MTP thereby reducing the serum 5-MTP level. Drop of serum 5-MTP in sepsis may be aggravated by cytokines such as IL-1β and TNF-α which inhibited 5-MTP release in HUVECs (data not shown).

5-MTP is also detected in epithelial cell of lung and kidney tissues from normal mice. Together with our previous reports that human fibroblasts release 5-MTP into the CM,14,15 these findings indicate that 5-MTP is produced in a broad spectrum of cells including vascular ECs and smooth muscle cells, epithelial cells, fibroblasts, and other mesenchymal cells. Tissue 5-MTP level may thus be higher than the blood level. In our cellular experiments, we observed that 5-MTP at 5 to 10 μmol/L inhibited NF-κB transactivation and IL-6 and TNF-α production, whereas a higher concentration (15–50 μmol/L) is needed to inhibited cellular events such as prevention of endothelial barrier disruption or macrophage transmigration. The effective 5-MTP concentrations are higher than serum concentration but may be in the range of tissue 5-MTP concentrations. Thus, 5-MTP may play a physiological role in maintaining an inflammatory balance and preventing excessive inflammation and protecting cells and tissues from inflammatory damage.
5-methoxytryptamine on the metabolomic profile of HUVEC-CM. Our unpublished data reveal that HUVECs express TPH-1 and do not have detectable THP-2. Nor do they have detectable aromatic amino acid decarboxylase (data not shown). These results further support that Try→5-HTTP→5-MTP is the predominant if not the only 1-tryptophan catabolic pathway in ECs. Our findings indicate that this tryptophan metabolic pathway plays an important vasoprotective function through the regulation of vascular permeability and control of systemic inflammation. We show that 5-MTP is a circulating autacoid that provides a global control of production of proinflammatory cytokines and enzymes and defends against systemic inflammation and multiple organ failure.

Our findings indicate that 5-MTP has the potential for treating sepsis. Being an endogenously produced compound, 5-MTP has the advantage of having less unexpected adverse effects. Thus, 5-MTP will be a valuable lead compound for new inflammatory drug development. Another potential clinical application of 5-MTP is its use as a biomarker of sepsis and other systemic inflammatory disorders. Hence, it may be useful as a therapeutic biomarker for selecting sepsis patients for 5-MTP therapy.

Acknowledgments

This research was conducted under the Graduate Program of Biotechnology in Medicine sponsored by National Tsing Hua University and the National Health Research Institutes.

Sources of Funding

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Disclosures

None.

References

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**Novelty and Significance**

**What Is Known?**

- Endothelium plays a key role in controlling systemic inflammation and progression of inflammatory diseases.
- Tryptophan, an essential amino acid supplied by dietary proteins, serves as a precursor for a large number of metabolites that are important in human nutrition and metabolism.
- Human fibroblasts produce a novel tryptophan metabolite, 5-methoxytryptophan (5-MTP) and release it into the extracellular milieu to control cyclooxygenase-2 overexpression in inflammatory and cancer cells.

**What New Information Does This Article Contribute?**

- Vascular endothelial cells produce and release 5-MTP via the vesicular secretory pathway.
- Bacterial endotoxins such as lipopolysaccharide suppress 5-MTP production resulting in reduction of serum 5-MTP concentrations in mice and patients with sepsis.
- 5-MTP administration prevents endotoxin-induced surge of circulating cytokines and chemokines and reduces tissue cytokine and cyclooxygenase-2 expression, accompanied by alleviation of lung damage and mortality in murine sepsis models.
- 5-MTP inhibits endotoxin-induced p38 MAPK activation by disrupting binding of activated p38 to peroxiredoxin-1 and thereby destabilizing p38 MAPK activation.

Endothelium plays a critical role in defending against systemic inflammation. It is unclear whether endothelial cells produce and release 5-MTP to control inflammatory responses. Here, we show that endothelial cells produce and release 5-MTP, accounting for a relatively high serum 5-MTP concentration. LPS reduces serum 5-MTP in endotoxemic mice and humans with sepsis. 5-MTP administration prevents surge of circulating cytokines and chemokines with the consequent alleviation of lung damage and reduction of sepsis-related mortality in endotoxemic mice. 5-MTP exerts its protective actions by blocking activation of a key signaling kinase in sepsis, ie, p38 MAPK. Furthermore, 5-MTP inhibits p38 MAPK activation by disrupting the interaction between activated p38 to peroxiredoxin-1. We conclude that endothelium-derived 5-MTP is a new class of circulating anti-inflammatory molecules which plays an important role in controlling systemic inflammation and is a valuable lead compound for new antisepsis drug development.
Endothelium-Derived 5-Methoxytryptophan Is a Circulating Anti-Inflammatory Molecule That Blocks Systemic Inflammation

Yi-Fu Wang, Yu-Juei Hsu, Hsu-Feng Wu, Guan-Lin Lee, Ya-Sung Yang, Jing-Yiing Wu, Shaw-Fang Yet, Kenneth K. Wu and Cheng-Chin Kuo

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An erratum has been published regarding this article. Please see the attached page for:
/content/119/6/e108.full.pdf

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/05/05/CIRCRESAHA.116.308559.DC1
In the *Circulation Research* article by Wang et al (Endothelium-Derived 5-Methoxytryptophan Is a Circulating Anti-Inflammatory Molecule That Blocks Systemic Inflammation. *Circ Res*. 2016;119:222–236. DOI: 10.1161/CIRCRESAHA.116.308559), the authors recently discovered an error in the lower panel of Figure 3B (LPS). This error has been corrected and the authors assert that it does not impact the conclusions of the article.

The authors apologize for this error, and the error has been noted and corrected in the online version of the article, which is available at [http://circres.ahajournals.org/content/119/2/222.full](http://circres.ahajournals.org/content/119/2/222.full).
SUPPLEMENTAL MATERIAL

Endothelium-derived 5-Methoxytryptophan Is a Circulating Anti-inflammatory Molecule That Blocks Systemic Inflammation

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Running title: 5-methoxytryptophan in systemic inflammation

Detailed Methods

Cell culture and treatment
Human umbilical vein endothelial cells (HUVEC) and mouse RAW264.7 macrophages were obtained from the American Type Culture Collection. Human aortic endothelial cells (HAECs), human pulmonary artery endothelial cells (HPAECs) and human coronary artery endothelial cells (HCAECs) were purchased from Lonza. HUVECs were cultured in M200 supplemented with 12% FBS and Low Serum Growth Supplement (LSGS) (Invitrogen). HAECs, HPAECs and HCAECs were incubated in EGM2 and EGM2-MV, respectively. RAW264.7 cells were grown in DMEM containing 10% FBS.1 Cells were typically pre-incubated with or without DL-5-MTP (Sigma-Aldrich) or L-5-MTP (AstaTech) for 30 min before LPS treatment, unless specified otherwise. The duration of LPS (100 ng/ml) treatment varied depending on the experiments.

For mouse peritoneal macrophage isolation, C57BL/6J mice were injected intraperitoneally with 2 ml of 4% thioglycollate. Four days after injection, the peritoneal cavity was washed with 5
ml of ice-cold RPMI1640 medium, and cells from the peritoneal exudates were collected by centrifugation, and suspended in RPMI1640 medium, and seeded on 15-cm dishes. They were allowed to adhere to the plastic surface for 4 h. Floating cells were washed out. Adherent cells were collected and used as peritoneal macrophages in the experiments. The cells were incubated with or without LPS (100 ng/ml) for the indicated time before each experiment, according to the protocol for the RAW264.7 cell experiments.

Preparation of 5-MTP
Two different sources of 5-MTP have been used in this study. DL-5-MTP was purchased from Sigma-Aldrich, and L-5-MTP was custom-synthesized by AstaTech (AstaTech Inc, Bristol, PA). Purity of L-5-MTP and DL-5-MTP was verified by LC-MS.

For in vitro drug application, 50 mM of DL- or L-5-MTP stock solution was prepared in DMSO in blown tube, and stocked at -20 or -80°C. For in vivo experiments, DL-5-MTP or L-5-MTP was freshly dissolved in saline (0.5 ml, pH adjusted to 7.4), and injected into animals at the concentration of 23.4 or 100 mg/kg.

5-MTP measurement
5-MTP was measured by a competitive ELISA in a 96-well microtiter plate coated with polyclonal rabbit anti-5-MTP antibodies (Abcam) using a coated buffer, 0.05 M carbonate-bicarbonate (pH 9.6) at 4°C overnight. After PBST washing and treatment with blocking buffer, mixture of 5-MTP-conjugated HRP generated by using an NH₂ peroxidase labeling kit (Abnova) and 5-MTP standards, HUVEC-conditioned medium or serum samples were added to the wells and incubated at 4°C overnight. The wells were washed and treated with a substrate tetramethylbenzidine at room temperature for 30 min. 0.1 N H₂SO₄ stop solution was added to each well, and the product was analyzed at 450 nm. The calibration curve was established by using pure 5-MTP at concentrations of 0.01-50 µM.

Fluorescence Microscopy
Cells were fixed by incubation in PBS containing 3.5% paraformaldehyde at room temperature for 10 min. Cells were washed extensively and blocked with Image-iT FX signal enhancer (Invitrogen) for 30 min at room temperature. Cells were subsequently permeabilized with 0.1% triton X-100 for 1.5 min at room temperature, then cells washed with PBS and stained with anti-5-MTP antibodies at room temperature. After 1 h staining, cells were washed and counter-stained with FITC-conjugated rabbit anti-mouse IgG for 1 h. All cells were visualized under a Leica fluorescence microscope.

Metabolomic analysis
The metabolite analysis was performed using ultraperformance liquid chromatography (Acquity UPLC System, Waters Corporation) coupled with an orthogonal time-of-flight mass spectrometer (Xevo TOF MS, Waters Corporation). For ultraperformance liquid chromatography (UPLC), a
1.7-µm (2.1 × 100 mm) C18 column (Acquity UPLC System; Waters Corporation) was used. The liquid chromatography separation was carried out at 40 °C with a flow rate of 0.2 mL/min using the following gradient for the analysis: 0–2 min 1% B, 2–6 min from 2% B to 80% B, 6–14 min 80% B, 14–15 min from 80% to 99% B and 17–20 min 1% B [solvent system A: water/formic acid (100:0.1, vol/vol); B: acetonitrile/ formic acid (100:0.1, vol/vol)]. The quadrupole-time of flight (QTof) mass spectrometry (MS) system (QTof-MS) was operated in positive electrospray ionization mode with a mass resolution larger than 10,000. Data were acquired by MassLynx software (Waters Corporation) in uncentroided format over a mass range of m/z 100–800 with scan duration of 0.5 s and an interscan delay of 0.1 s. The capillary and the cone voltage were maintained at 3,200 and 40 V and the desolvation and source temperature at 300 °C and 80 °C, respectively. Nitrogen was used as cone (25 L/h) and desolvation gas (800 L/h). For accurate mass measurement, the QTof-MS was calibrated with formic acid 0.1% (vol/vol) in acetonitrile/water (50:50, vol/vol), and the dynamic range enhancement mode was used for data recording. All analyses were monitored by using leucine-enkephaline ([M+H]+ ion at 556.2771 or [M-H]− ion at 554.2615) (Sigma- Aldrich) as lock spray reference compound at a concentration of 0.2 µg/mL in acetonitrile water (50:50, vol/vol) and a flow rate of 10 µL/min. The raw mass spectrometry data of all samples were processed using the MassLynx software (Waters Corporation).

**Induction of endotoxemia in a mouse model**

C57BL/6 mice (6–8 wks old) were treated intraperitoneally with saline or with different concentrations of 5-MTP (23.4 or 100 mg/kg) for 30 min before intraperitoneal administration of LPS (60 mg/kg). Animals were monitored for survival and other clinical signs including ruffled fur, lethargy, diarrhea, and body weight loss. Some animals were sacrificed at different times after LPS injection. Blood samples, peritoneal exudates, lungs, and spleens were collected.

Polymicrobial sepsis in mice was induced by puncture of the cecum (CLP) surgery. Mice were anesthetized with 3% inhaled isoflurane, and an abdominal incision was performed. The cecum was identified, ligated with 4-O silk suture, and punctured once with a 21-gauge needle. The cecum was then returned to the peritoneal cavity, and the abdominal incision was closed in two layers with 6-O silk sutures. Sham-operated mice were treated cecal manipulation only. About 30 min after surgery, CLP mice received saline or 5-MTP by intraperitoneal injection. CLP-treated and sham-treated mice were killed and blood samples were collected for cytokine analysis 24 h after surgery.

Mouse experiments were approved by the Institutional Animal Care and Use Committee, National Health Research Institutes.

**Human subjects**

The human study was carried out from May 2013 to August 2013 in the Division of Infectious Diseases and Tropical Medicine, Tri-Service General Hospital, Taipei, Taiwan. Fifty patients with sepsis syndrome without shock (29 male and 21 female with a mean age of 64.0 ± 19.6 years)
and fifteen septic shock patients (10 male and 5 female with a mean age of 74.4 ± 11.5 years) were enrolled in the study and sepsis syndrome was diagnosed based on clinical and laboratory findings, following the guidelines of the American College of Chest Physician/Society of Critical Care Medicine Consensus Conference.  

30 age and sex matched healthy subjects were assessed as controls. The human study was approved by the Ethics Committee of Tri-Service General hospital (TSGH IRB number: 2-101-05-024). All patients signed informed consent.

**Cytokine-specific ELISA**

Cytokine levels in the culture supernatants and serum were determined in microtiter plates (96-well) by a specific sandwich ELISA (Biosource) as previously described.  

**5-MTP binding assay**

RAW264.7 cells were incubated with 5-MTP conjugated with fluorescent CF<sup>TM</sup>488 (Biotum) generated by using an NH<sub>2</sub> peroxidase labeling kit (Abnova) in the presence of different concentrations of unconjugated 5-MTP for 120 min. After washing with PBS for 3 times, fluorescence positive cells were measured at 530 nm using flow cytometry (BD Biosciences) at an excitation wavelength of 488 nm and were expressed as the mean percentage of the fluorescence positive cells amount 10000 cells.

**Flux Assay**

HUVECs (6 × 10<sup>4</sup> cells/well) were seeded on the apical side of the polycarbonate membrane transwell plates (0.4-μm pores) in triplicate overnight, allowed to grow to full confluence for the formation of a monolayer. After pretreating HUVECs with different concentrations of 5-MTP for 30 min, cells were stimulated with TE buffer or LPS for 1 h, and then FITC-conjugated dextran (~70,000 Da) was added (10 μg/ml) to the basal chamber. After 30 min incubation, 100 μl of the medium from each chamber was collected, and the fluorescence intensity was measured using SpectraMax microplate fluorometer (Molecular Devices).

**Assessment of vascular Leakage**

In pulmonary microvascular permeability determination, Evans blue dye (20 mg/kg) was injected into the tail vein of mice treated with LPS or saline for 24 h in the absence or presence of 5-MTP 30 min before the termination of the experiment to assess vascular leak. Lungs were perfused, removed, and Evans blue was extracted from equal weights of lung tissues with 1 ml of formamide overnight at 55°C. The absorption of Evans blue was measured at 620 nm.

**Macrophage Transmigration**

Mouse peritoneal macrophages were incubated with BCECF-AM-HBSS buffer in an incubator for 1h. BCECF-AM-labeled mouse peritoneal macrophages were collected by centrifugation at 1100 rpm for 3 min. To assess macrophage transmigration, the BCECF-AM-labeled mouse peritoneal macrophages (1 × 10<sup>5</sup> cells/well) were seeded onto a HUVEC monolayer treated with
vehicle or LPS for 1 h in the absence or presence of 5-MTP in the upper chamber of 24-well transwell plates (8-µm pore size; Costar) in triplicate. The bottom chambers were filled with 12% FBS medium containing PDGF-BB (10 ng/ml) (Peprotech) as a chemoattractant. After 4 h incubation, cells from the apical side were removed by a cotton swab; the membrane was cut, fixed, and mounted onto glass slides with Prolong Gold antifade mounting medium, and the macrophages transmigrated through the HUVEC monolayer to the dorsal side of the membrane were observed, and fluorescence images were captured by fluorescence microscopy.

**Western blot analysis.** Cellular proteins were resolved with 5% to 20% SDS-PAGE and transferred to nitrocellulose membranes. They were blotted with specific antibodies as previously described.\(^4\)

**Promoter-luciferase reporter assay**

RAW264.7 macrophages were cotransfected with COX-2 promoter-luciferase, or IL-6 promoter-luciferase constructs as previously described\(^3\) or p5xNF-κB-luciferase (Stratagene) and pcDNA3.1-β-galactosidase plasmids using FuGENE 6 (Roche). After overnight transfection, the cells were incubated with or without LPS in the presence or absence of 5-MTP for 8 h. After treatment, cells were lysed, and luciferase activity was measured using an assay kit (Promega). β-galactosidase was used to normalize the data.

**HAT activity assay**

HAT activity was measured by HAT activity kit (BioVision, Milpitas) according to the manufacturer's instruction. In brief, p300 was immunoprecipitated from cell nuclear extracts or tissue extract protein with anti-p300 antibodies (Upstate, Temecula, CA). p300 immunoprecipitates were incubated with a reaction mixture containing HAT assay buffer and HAT substrate at 37°C for 1 h and the absorbance was taken at 450 nm.

**MPO activity assay**

Myeloperoxidase (MPO) activity of lung tissue was assayed by myeloperoxidase fluorometric detection kit (Enzo Life Sciences). In brief, lung tissue specimens were homogenized by TissueLyser II (Qiagen) in tissue protein extraction buffer (pH 7.4 25 mM Tris buffer, 150 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40 and 0.2% SDS), and centrifuged at 12,000 × g for 20 min at 4°C. The supernatants were removed and the pellets were homogenized by TissueLyser II for 30 seconds in 1 ml assay buffer with 0.5% hexadecyltrimethylammonium. The samples were frozen and thawed three times, and centrifuged at 8,000 × g for 20 min. Collected supernatants and standards were mixed with reaction cocktail (50 µM detection reagent and 20 µM hydrogen peroxide in 1X assay buffer) at room temperature in the dark. After 30 min incubation, MPO activity was determined by measuring the fluorescence intensity at excitation 530 nm and emission 600 nm in a fluorescent plate reader.
Caspase-3 activity assay
Caspase-3 activity was determined by the cleavage of the fluorometric substrate z-DEVD-AMC (Upstate Biotechnology) as previously described.\(^5\)

Histology and immunohistochemistry
For histological studies, the lungs were perfused with saline, and immersed in formalin for 24 h. Tissue blocks were placed in formalin, dehydrated in a graded series of ethanol, embedded in paraffin, cut into 3 µm-thick serial sections, and stained with haematoxylin and eosin for detecting inflammatory cells, alveolar congestion and alveolar septal wall thickness and interstitial edema.

Prior to immunohistochemical detection of COX-2, iNOS, Ly6g, or cleaved caspase-3 in lung sections, sections were deparaffinized with xylene, progressively rehydrated through graded alcohols. Antigen sites were retrieved by heating the sections on slides in pH 8 EDTA antigen retrieval (Trilogy; Cell Marque Corporation) in electric pressure cooker for 15 min. Sections were sequentially blocked by UltraVision hydrogen peroxide block (Thermo) for 10 min and UltraVision protein block for additional 5 min. All antibodies described hereafter were diluted in blocking buffer. Sections were incubated at room temperature for 2 h with primary antibody and then washed in PBST. The sections were incubated with primary antibody amplifier quanto (Thermo) for 10 min. After rinsing with PBST, the sections were incubated with HRP Polymer Quanto (Thermo) for 10 min and washed three times with PBST. COX-2, iNOS, Ly6g and cleaved caspase-3 were visualized by addition of DAB Quanto Chromogen: Substrate for 3 min. Tissues were counterstained with hematoxylin.

Kynurenine measurement
Kynurenine was measured using UPLC coupled with a Xevo-TQ mass spectrometer. Liquid chromatography was performed on Acquity ultra-performance liquid chromatography (UPLC) system (Waters) using a BEH C18 column (1.7 µm, 2.1 mm × 100 mm, Waters Corporation). UPLC linear gradient conditions were: 0–3 min, 1% B; 3–5 min, 30% B; 6–8.5 min; 99% B; and 9.5-12 min 99% A [solvent system A: water/formic acid (100:0.1, vol/vol); B: acetonitrile/ formic acid (100:0.1, vol/vol)]. The injection volume was 2 µL, and the column temperature was maintained at 35 °C. Mass spectrometry detection was performed by using a Xevo™ Triple Quadrupole MS (Waters Corporation) equipped with an electrospray ionisation (ESI) source operating in positive ionization mode. The online MS analysis was at the Multiple Reaction Monitoring (MRM) mode. Parameters for the cone energy and collision energy for Kynurenine are: parent ion m/z 209.13, daughter ion m/z 192.11, cone energy 14 V, collision 8 V. Quantification of kynurenine was done using Target Lynx software (Waters Corporation).

Statistical analysis
All values were given as mean ± S.D. T-test was used to determine the statistical significance of difference between treatment and control groups while one-way ANOVA was used to analyze multiple groups. \(P\) values of less than 0.05 were considered statistically significant. The
diagnostic performance of 5-MTP, hs-CRP and IL-1β was determined using area under the receiver operating characteristic curve (AUROC) analysis to assess the overall discriminatory power of these assays in predicting sepsis syndrome. The 95% confidence intervals (CIs) and optimal cutoff points yielding maximum sums of sensitivity and specificity from the AUROC curves for each assay were also computed. Data were analyzed with the SPSS statistical software 18.0 (SPSS Inc., Chicago, IL, USA).

References


Online Figure I. Soluble factors in EC-conditioned medium inhibit LPS-induced expression of COX-2 and pro-inflammatory cytokines in macrophages. (A) RAW264.7 cells were incubated in control medium M200 or conditioned medium (CM) collected from HUVECs cultured in M200 medium containing different level of FBS with or without LPS for 24 h. Cell lysates were immunoblotted with antibodies for COX-2 or β-actin. The experiments were repeated 3 times with similar results. (B) RAW264.7 cells were incubated in control medium M200 or HUVEC-CM containing 12% FBS with or without LPS for 24 h. (C) RAW264.7 cells were incubated in control medium EGM2, HAEC-CM or HPAEC-CM containing 12% FBS with or without LPS for 24 h. (D) RAW264.7 cells were incubated in control medium EGM2-MV or HCAEC-CM containing 12% FBS with or without LPS for 24 h. IL-6 level in culture supernatants was measured by ELISA. Data represent mean ± SD of at least 3 independent experiments.
Online Figure II

5-MTP release from ECs. (A) HUVECs were fixed and permeabilized. 5-MTP was stained with anti-5-MTP antibodies (green, FITC) and nuclei were stained with DAPI (blue). Inset shows high magnification view of indicated area. (B-C) 5-MTP concentrations in control medium or conditioned medium from HUVECs (B) or HAECs (C) treated with different concentrations of various inhibitors as indicated for 2 h. Data represent mean ± SD of 3 independent experiments.
Online Figure III

Online Figure III. Immunohistochemical staining of 5-MTP in mouse aorta, lung and kidney. Paraffin-embedded sections were prepared from lungs and kidneys of mice. 5-MTP distribution in (A) vascular cells of pulmonary arteries, (B) renal arteries, (C) epithelial cells of terminal bronchiole of lungs and (D) renal epithelial cells was determined by immunohistochemical staining of 5-MTP.
Online Figure IV

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<tr>
<td>COX-2</td>
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<tr>
<td>β-actin</td>
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B

Relative luciferase activity of COX-2 promoter

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C

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COX-2

β-actin
Online Figure IV. 5-MTP inhibits LPS-induced COX-2 expression in macrophages. (A) After pretreating RAW264.7 cells with different concentrations of DL-5-MTP for 30 min, cells were stimulated with LPS for 8 h. Cell lysates were immunoblotted with antibodies for COX-2 or β-actin. (B) RAW264.7 cells were transfected with COX-2 promoter-luciferase plasmid. After 24 h transfection, the cells were incubated with LPS for 8 h. Luciferase activity was measured. The error bars denote mean ± SD (n = 3). (C) Mouse peritoneal macrophages were stimulated with LPS with or without DL-5-MTP. After 8 h, COX-2 protein expression was determined by Western blot. The experiments in A and C were repeated 3 times with similar results.
Online Figure V

A

5-methoxy moiety
Carboxy moiety

B

Serotonergic neuron

C

Pineal cells

D

Fibroblasts

L-tryptophan
Tryptophan hydroxylase-1
5-hydroxytryptophan
Aromatic amino acid decarboxylase
Serotonin (5-HT)
5-hydroxytryptamine (5-HT)
N-acetyltransferase
N-acetyl-5-HT
5-hydroxyindole-O-methyltransferase
Melatonin
5-methoxytryptamine
Online Figure V. Schematic illustration of the three major metabolic pathways of Tryptophan (Trp) in representative cell types. (A) Highlight the 5-methoxy and the 3-propionic acid moieties of 5-methoxytryptophan (5-MTP) as required for its anti-inflammatory action. (B) Serotonin synthesis from Try is catalyzed by TPH-2 and AAA decarboxylases. (C) Melatonin is further converted from serotonin. Melatonin is deacetylated to yield the catabolite, 5-methoxytryptamine. (D) 5-methoxytryptophan synthesis from Try is catalyzed by TPH-1 and a HIOMT without the decarboxylase step.
Online Figure VI. 5-MTP decreases LPS-induced phosphorylation of p38 MAPK via inhibition of interaction of Prdx 1 with phosphorylated p38 MAPK. (A) Activation of TLR4 signaling induces kinase phosphorylation in RAW264.7 cells. Cells were treated with LPS for the indicated times. Cell lysates were immunoblotted with antibodies for phospho-p38 MAPK (p-p38), p38 MAPK, phospho-ERK1/2 or ERK1/2. (B) Upper panel, after pretreating RAW264.7 cells with L-5-MTP (10 µM) for 30 min, cells were stimulated with or without LPS for 1 h. Cells were lysed on ice, and the lysates were subjected to immunoprecipitation with anti-Prdx 1. The precipitated level of endogenous Prdx 1 and p-p38 was determined using immunoblotting with anti-Prdx 1 or anti-p-p38. Lower panel, total input was determined by immunoblotting. (C) After pretreating RAW264.7 cells with different concentrations of L-5-MTP for 30 min, cells were stimulated with LPS for 1 h. Cell lysates were immunoblotted with antibodies for Prdx 1 or β-actin. (D) RAW264.7 cells were treated with L-5-MTP (10 µM) for 1 h. Cell lysates were immunoblotted with antibodies for Prdx 1 or β-actin. The experiments in A-D were repeated 3 times with similar results. Error bars in B denote mean ± SD (n = 3).
Online Figure VI. 5-MTP decreases LPS-induced phosphorylation of NF-κB p65 in macrophages. (A) RAW264.7 cells with different concentrations of DL-5-MTP for 30 min, cells were stimulated with LPS for 4 h. NF-κB p65, phospho-NF-κB p65 (p-p65) (Ser536) or β-actin was determined by immunoblotting with specific antibodies. (B) Mouse peritoneal macrophages were treated with LPS with or without DL-5-MTP (50 µM) for the indicated time. Phospho-p65 in the lysates was analyzed by an ELISA kit.
Online Figure VIII. 5-MTP binds to RAW264.7 cell surface. RAW264.7 cells were incubated with 5-MTP conjugated with fluorescent CF<sup>TM</sup>488 and increasing concentrations of unconjugated 5-MTP for 120 min. After washing with PBS for 3 times, fluorescence positive cells were measured by flow cytometry. The error bars denote mean ± SD mean of 3 independent experiments.
Online Figure IX. Intraperitoneal 5-MTP administration increases mouse serum 5-MTP.
Serum 5-MTP concentrations in mice treated with saline or LPS with or without DL-5-MTP (23.4 mg/kg) for the indicated time were measured by competitive 5-MTP ELISA. The error bars denote mean ± SD (n = 3).
Online Figure X. Effect of 5-MTP on LPS-induced endotoxemia in a murine model. (A) Effect of 5-MTP on mouse body weight loss caused by LPS. Mice were injected with saline or DL-5-MTP (23.4 mg/kg) for 30 min before LPS (60 mg/kg) administration (n = 10). Body weight was determined at day 2 after LPS treatment. (B) Quantification of leucocyte infiltration per high power fields (x 400, 10 fields each) in lungs of mice injected with 60 mg/kg LPS with or without saline or DL-5-MTP for the indicated time. (C) 5-MTP suppresses LPS-induced MPO activity. MPO activity was determined in lung tissues of mice treated with saline or LPS in the presence or absence of DL-5-MTP (23.4 mg/kg) for 16 h (n = 6 mice/group). (D) Effect of 5-MTP on total cell number of bronchoalveolar lavage fluid (BALF) in LPS-injected mice. BALF was isolated from mice treated with LPS with or without various concentrations of DL-5-MTP for 24 h (n = 6 mice/group). Cell number in BALF was determined by trypan blue exclusion assay. The error bars denote means ± SD of at least 3 independent experiments.
Online Figure XI. 5-MTP prevents LPS-induced spleen cell apoptosis and spleen edema in endotoxemic mice. (A) Caspase-3 activity in splenocytes of mice treated with saline, LPS or LPS plus DL-5-MTP (23.4 mg/kg) for 16 h was measured by fluorogenic substrate as described in Materials and Methods. The error bar denotes mean ± SD (n = 6 mice/group). (B) Spleen weight/body weight (SW/BW) ratio was assessed (n = 6 mice/group).
Online Figure XII. 5-MTP reduces serum IL-12 level in LPS-induced endotoxemic mice. (A) Mice were injected intraperitoneally with or without different concentrations of DL-5-MTP or saline for 30 min, followed by LPS administration for the indicated time. (n = 6 per group). Pro-inflammatory cytokines level in serum was measured by ELISA. (B) Serum was isolated from mice treated with saline or LPS with or without DL-5-MTP (23.4 mg/kg) for 24 h (n = 10). IL-12p40 was measured by ELISA.
## Supplemental Table

### Online Table 1
Sensitivity and specificity of 5-MTP, hsCRP and IL-1\(\beta\) at optimum diagnostic cut-off values for sepsis

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<th>Cut-off</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<td>IL-1(\beta) (pg/ml)</td>
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