Mechanisms of Increased Survival in Lipopolysaccharide-Treated Mice With a Single Subcutaneous Capsaicin Challenge

Research Article

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Abstract

Background: Capsaicin, a pungent compound present in capsicum fruits, has various biological activities such as stimulation of transient receptor potential vanilloid-1 (TRPV-1) and/or inhibition of tumor necrosis factor alpha (TNF-α). This study aimed to reveal the mechanism involved in capsaicin-mediated high survival rate in LPS-treated mice.

Methods: In LPS-treated mice, a comparative survival rate study using a TRPV-1 agonist (capsaicin) and a TRPV-1 antagonist (capsazepine). DNA-binding activity of NF-κB and TUNEL staining was conducted in vivo. In LPS-treated RAW264 cells, TNF-α was measured by enzyme-linked immunosorbent assay.

Results: In the present study, the survival rate of mice treated with 20 mg/kg LPS was significantly improved from 7.7% to 92.3% after treatment with 4 mg/kg capsaicin and to 53.8% with 40 mg/kg capsazepine. In liver tissues, the LPS-induced increase in NF-κB DNA-binding activity was attenuated by capsaicin. The increased number of TUNEL-positive cells observed in the alveolar epithelial and liver parenchymal cells of LPS-treated mice was diminished by capsaicin treatment. In RAW264 cells, mouse leukemic monocytes, capsaicin inhibited the release of TNF-α from LPS stimulation, by attenuating NF-κB DNA-binding activity.

Conclusion: The improvement in the survival rate (92.3% - high) by capsaicin administration in LPS challenge could be explained not only by TRPV-1 activity, but also by the pleiotropic effect which inhibits TNF-α release from macrophages. Thus, capsaicin may be a promising treatment option for LPS-related diseases such as severe sepsis or septic shock.

Keywords: Capsaicin, Capsazepine, Lipopolysaccharide, Nuclear Factor-Kappa B.

Introduction

Absolute mortality in severe sepsis has been decreased from 35.0% to 18.4% [1]. However, when if early aggressive treatment is not started (example; after suspected less than 6 h), the outcome is usually poor. Severe sepsis and septic shock are explained not only by TRPV-1 activity, but also by the pleiotropic effect which inhibits TNF-α release from macrophages. Therefore, capsaicin may be a promising treatment option for LPS-related diseases such as severe sepsis or septic shock.
reported that capsaicin (4 mg/kg, subcutaneously; s.c.) inhibits the overexpression of TNF-α, IL-1β, and NOx levels and has the potential to improve survival rate (from 0% to 91.7%) in LPS (20 mg/kg, intraperitoneally; i.p.) treated mice [4].

Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), a pungent compound present in capsicum fruits, is synthesized by the addition of a branched-chain fatty acid to vanillylamine.

Capsaicin and several related compounds are called capsaicinoids and are produced as secondary metabolites by chili peppers. Pure capsaicin is a volatile, hydrophobic, colorless, odorless, and crystalline to waxy compound. Capsaicin has various biological activities including transient receptor potential vanilloid-1 (TRPV-1) activation [9, 10], and antioxidant [11, 12], antibacterial [13], and anti-inflammatory effects [14, 15]. It is used as an ingredient in spicy foods and in oral dietary supplements, as an analgesic in topical ointments, nasal sprays, and dermal patches. It is typically used to treat post-herpetic neuralgia, painful diabetic neuropathy, pain from osteoarthritis [16], pruritus [17], and in pepper spray aerosols [18] in clinical settings.

Capsaicin has a reported additive effect on the inhibition of gastric acid secretion, through activation of TRPV-1 in vivo [19]. Capsaicin has also been reported to suppress TNF-α production by LPS-stimulated macrophages in vitro by downregulating nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) through peroxisome proliferator-activated receptor gamma (PPARγ) ligand-like activity [11, 20]. With respect to drug interactions, capsaicin upon large and long-term administration decreases the absorption of digoxin by inducing CYP3A2 mRNA expression via indirect activation of PXR in LPS-treated rats [21]. Moreover, pre-treatment of rats with capsaicin resulted in decreased bioavailability of simvastatin and galantamine [22, 23]. Additionally, other studies indicate that capsaicin at nontoxic doses (less than 100 μM), causes functional and structural changes in Caco-2 cell monolayers [24].

To elucidate the relation between an improved survival rate after LPS challenge due to capsaicin administration and TRPV-1 activation, a comparative survival rate study of the TRPV-1 agonist, capsaicin and the TRPV-1 antagonist, capsazepine (CPZ) [25, 26] was conducted. Furthermore, to clarify the interference between capsaicin and NF-κB, the DNA-binding activity of NF-κB was measured. Morphological changes were observed using HE staining under the light microscope upon LPS challenge in vivo (mice) and TNF-α levels were determined in vitro (RAW264 cells).

Materials and Methods

Reagents

LPS (Escherichia coli, 055:B55, Lot No. 114K4107) and CPZ (TRPV-1 antagonist) were purchased from Sigma-Aldrich, and capsaicin (98% pure) was provided by Maruishi Pharmaceutical Co., Ltd. Sevoflurane was purchased from Maruishi Pharmaceutical Co., Ltd. The other reagents used were commercially available ultra-pure grade chemicals.

Animals

Male BALB/c mice (aged 8- to 10-week-old; weight, 21-26 g; Japan SLC, Inc.) were used for these studies. They were acclimated for at least one week to controlled environmental conditions (temperature, 24°C ± 1°C; humidity, 55% ± 10%; light cycle, 6:00-18:00 h) with free access to solid food (NMF, Oriental yeast Co., Ltd.) and water. All experimental procedures were approved according to the guidelines for the use of experimental animals, and were conducted in the animal facility established by the ethics committee of Osaka University of Pharmaceutical Sciences (approved on 17 March 2009, No. 09014). At the end of the experiment, mice were euthanized with sevoflurane.

Treatment of mice

LPS was dissolved to a concentration of 1 mg/mL in physiological saline (Fuso Pharmaceutical Industries Ltd.). Capsaicin and CPZ were dissolved in physiological saline with 1% ethanol and 1% Tween-20. LPS (20 mg/kg) was administered i.p., and capsaicin (4 mg/kg), and CPZ (40 mg/kg) were administered s.c. in the dorsal region of mice 5 min after LPS administration.

Survival rate experiments

Mice were divided into the following eight treatment groups for the survival rate experiments: vehicle (physiological saline, i.p. + physiological saline, s.c., n = 13), LPS (LPS, i.p. + physiological saline, s.c., n = 13), capsaicin (physiological saline, i.p. + capsaicin, s.c., n=13), CPZ (physiological saline, i.p. + CPZ, s.c., n = 13), capsaicin + CPZ (physiological saline, i.p. + capsaicin and CPZ, s.c., n = 13), LPS + capsaicin (LPS, i.p. + Capsaicin, s.c., n=13), LPS + CPZ (LPS, i.p. + CPZ, s.c., n = 13), and LPS + capsaicin + CPZ (LPS, i.p. + capsaicin and CPZ, s.c., n = 13). Mice were monitored for mortality every 24 h for 7 days.

Preparation of embedded samples

Each mouse was anesthetized with sevoflurane and restrained in a supine position. The heart was exteriorized and the left ventricle was cannulated for perfusion fixation. Physiological saline (20 mL) was perfused through the cannula, followed by 20 mL of 10% buffered formalin solution (Wako Pure Chemical Industries, Ltd.). After the perfusion fixation, the lungs and liver were removed. For paraffin embedding, fixation was performed for more than 24 h in 10% buffered formalin and tissues were then embedded in paraffin blocks for sectioning. The paraffin blocks were cut into 5-μm sections with a rotary microtome (Leica RM2125RT; Leica Microsystems Co., Ltd.). For frozen embedding, fixation was performed for more than 12 h in 30% sucrose and tissues were embedded in the Optimal Cutting Temperature compound ( Sakura Finetechical Co. Ltd.) for sectioning. The frozen embedded samples were cut into 7-μm sections with a cryotome (Leica CM1850, Leica Microsystems Co., Ltd.).

HE and TUNEL stains

Paraffin sections were washed with tap water for 1.5 hours, dehydrated using standard methods, and stained with Mayer's H&E. TUNEL stain was performed using an Apoptosis in situ Detection Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stained tissues were observed and photographed with a light mi-
croscope (BX51; Olympus Co., Ltd.).

Estimation of NF-κB DNA-binding activity

Blood was collected from the axillary artery of anesthetized mice at 0.5, 1, 3, and 6 h after administration of physiological saline or LPS. After ligating the portal vein and hepatic artery, the hepatic vein was cut, and the portal vein was perfused with physiological saline to remove the blood from the liver. One-hundred milligrams of hepatic tissue was lysed in 1 mL of lysis buffer [10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1% dithiothreitol (DTT), 1% Protease Inhibitor Cocktail Set III, ethylenediaminetetraacetic acid (EDTA)-Free (Calbiochem)] before being homogenized with a homogenizer pestle. The homogenate was then centrifuged (20 min, 10,000×g, 4°C) and the resulting precipitate was dissolved in 140 µL of extraction buffer [20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1% DTT, 1% Protease Inhibitor Cocktail Set III, EDTA-Free] and incubated on ice (30 min). The suspension was then centrifuged (5 min, 18,000×g, 4°C), and the supernatant was collected as nuclear protein. DNA-binding activity of NF-κB in the liver tissues was measured using an NF-κB (p65) Transcription Factor Assay Kit (Cayman Chemical Company). The reaction was stopped by adding 100 µL/well of stop solution with gentle tapping. Within 5 min, absorbance at 450 nm was measured using a plate reader (Labsystems Multiscan MS; Dainippon Sumitomo Pharma Co., Ltd.). Data are represented as relative values with the assumption that DNA-binding activity of NF-κB in the vehicle group was 100%.

Cell culture

Mouse leukemic monocytes, RAW264 cells, were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 4.5 g glucose/L, Nacalai Tesque, Inc.), containing 10% fetal bovine serum (BioSource International Inc.), penicillin (50 IU/mL), and streptomycin (50 IU/mL) (PS, Life Technologies Corporation) at 37°C with 5% CO₂ in 25-cm² tissue culture flasks (Becton-Dickinson Ind.). The RAW264 cells were sub-cultured by treatment with 0.25% trypsin and (1 mM) ethylenediaminetetraacetic acid (EDTA) in a Ca²⁺- and Mg²⁺-free PBS solution after every 3 days. This involved detachment of cells from the stock cultures by trypsin digestion, washing once by centrifugation, re-suspension, and sub-culture in 10 mL medium in culture flasks at a concentration of 5 × 10⁵ cells/mL. The cultures were regularly observed under an inverted light microscope to monitor growth and contamination.

Estimation of TNF-α Concentration in RAW264 Cells Using Enzyme-Linked Immunosorbent Assay (ELISA)

RAW264 cells (5 × 10⁶ cells) were seeded on a 24-well plate and cultured for 3 days at 37°C with 5% CO₂. The medium was aspirated and washed with PBS. After washing, medium containing LPS (100 ng/mL) and capsaicin (100 µM) was added to the cells and incubated for 1 h at 37°C with 5% CO₂. After incubation, the medium from each well was collected and TNF-α concentration was determined by ELISA (Quantikine Immunoassay mouse TNF-α, R&D).

Statistical Analysis

The survival rate data were plotted as Kaplan-Meier curves and examined for significant differences between the various treatment groups using the log-rank test. Results are shown as mean ± standard deviation (SD). Statistical analysis was performed using Tukey’s test. A significant difference was defined as p < 0.05.

Results

Survival tests

The comparative survival rate test at 7 days after the LPS challenge, and capsaicin (TRPV-1 agonist), and CPZ (TRPV-1 antagonist)
Histological examination

Histological examination of the HE-stained sections of lung tissues (terminal bronchioles and alveolar epithelial cells) removed at 12 h after LPS challenge, showed increased numbers of neutrophilic leukocytes (stab cells and segmented leucocytes) (Figure 2A). In the liver tissues at 12 h after LPS challenge, increase in spread sinusoids and diffused neutrophilic leucocytes (stab cells and segmented leucocytes) was observed (Figure 2B). On the other hand, 12 h after LPS + capsaicin treatment, sinusoids and oil droplets were observed in the liver sections (Figure 2B). TUNEL-positive cells (TPCs) are indicated by brownish spots (Figure 3). The lung and liver tissues of the control and LPS + capsaicin-treated mice lacked TPCs, which were observed in the alveolar epithelial cells and liver parenchymal cells of the LPS group (Figure 3).

NF-κB DNA-binding activity

Compared with the vehicle group, both the LPS and LPS + capsaicin groups showed significantly higher NF-κB DNA-binding activity at 1 h after LPS challenge (p < 0.01, Figure 4). However, compared to the LPS group, the LPS + capsaicin group showed a significantly lower NF-κB DNA-binding activity at 1 h after LPS challenge (p < 0.05, Figure 4).

Discussion

Capsaicin has various biological activities including TRPV1 activation and is used as an analgetic in topical ointments and dermal patches, typically to treat post-herpetic neuralgia, painful diabetic neuropathy, and osteoarthritic pain in the clinical setting [16]. Capsaicin is reported to have an additive effect on the inhibition of gastric acid secretion, through the activation of TRPV1 in vivo [19]. However, most of these clinical trials of capsaicin have been called off at phase 3. We have previously performed a dose-response trial of LPS (10, 15, and 20 mg/kg, i.p.) and capsaicin (2, 3, 4, 5, 10 mg/kg, s.c.); capsaicin (4 mg/kg, s.c.) significantly improved the survival (92.3%) of mice treated with LPS (20 mg/kg, i.p.) [4]. Furthermore, we also reported that capsaicin inhibited both plasma-soluble TNF production and mRNA expression of TNF-α [27]. Macrophages can also release TNF-α in the soluble form (sTNF) [28] that elicits biological activities by binding to type 1 and 2 TNF receptors (TNF-R1 and TNF-R2) [29, 30, 31]. In this study, to investigate whether the improving action on survival rate by capsaicin in LPS challenge is via TRPV1 or not, a comparative test of capsaicin (TRPV1 agonist) vs. CPZ (TRPV1 antagonist) was performed. In the LPS group, mice died within 3 days in the present study (survival rate: 7.7%, 12 deaths of 13 participants). In contrast, the survival rate in the LPS + capsaicin group (92.3%) was significantly improved, compared to that in the LPS + CPZ group. However, survival rate in the LPS + capsaicin + CPZ group was 100% and that in the LPS + CPZ group was 53.8%, which could not be explained by TRPV1 action.

Figure 2. Hematoxylin and eosin (HE) staining. HE stain of lung tissue; the terminal bronchioles and alveolar epithelial cells (A) and of liver tissue (B) (Magnification; ×40). LPS, lipopolysaccharide; Cap, capsaicin.

A. Lung tissues

Vehicle control LPS LPS + Cap

B. Liver tissues

Vehicle control LPS LPS + Cap

TNF-α release from mouse leukemic monocytes, RAW264 cells

TNF-α release was measured after 1 h of LPS or capsaicin treatment using ELISA in RAW264 cells that are mouse leukemic monocytes. TNF-α release decreased significantly (p < 0.05) in the capsaicin group, whereas the LPS and LPS + capsaicin groups showed significantly increased levels of TNF-α (p < 0.01), compared to the control group. However, LPS + capsaicin treatment showed significantly decreased release of TNF-α compared to LPS treatment alone (p < 0.05, Figure 5).
Figure 3. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. TUNEL stain of lung (A) and liver (B) tissues (Magnification ×40). LPS, lipopolysaccharide; Cap, capsaicin.

A. Lung tissues (×40)

Vehicle control | LPS | LPS + Cap

B. Liver tissues (×40)

Vehicle control | LPS | LPS + Cap

Figure 4. The effect of capsaicin on DNA binding activity of nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) in liver tissues. In the vehicle group, mice were treated with physiological saline (10 mL/kg). In the lipopolysaccharide (LPS) group, mice were treated with LPS (20 mg/kg). In the capsaicin group, mice were treated with capsaicin (4 mg/kg) at 5 min after physiological saline (10 mL/kg) administration. In the LPS + capsaicin group, mice were treated with capsaicin (4 mg/kg) at 5 min after LPS (20 mg/kg) administration. 0 hour represents the vehicle control group. □, vehicle group; ■, LPS group; , capsaicin group; ■, LPS + capsaicin group. Data shows means ± SD (n = 5). **p < 0.01, compared with the vehicle group. †p < 0.05, compared with the LPS group.
promising treatment option for LPS-related diseases such as sepsis or septic shock. Thus, capsaicin may be responsible for the survival benefit observed following the LPS challenge in severe sepsis or septic shock.

Acknowledgement

The authors thank Dr. Akashi for the kind gift of RAW264 cells, and the Maruishi Pharmaceutical Co., Ltd. for providing Capsaicin.

References


