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Hot-PBS Extract of *Vibrio vulnificus* Induces NF-κB Activation

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Research Article

Abstract

Background: *Vibrio vulnificus* causes necrotizing fasciitis and liver dysfunction. Although many studies have attempted to clarify the molecular mechanism by which *V. vulnificus* generates these symptoms, the mechanism remains poorly understood.

Methods and findings: We focused on determining the factor derived from *V. vulnificus* that induces inflammation. We found that an extract of *V. vulnificus* prepared using hot phosphate buffered saline (PBS) markedly induced the activation of NF-κB. The activation, however, was not caused by lipopolysaccharides, the capsule, peptidoglycans, or NOD1/NOD2 ligands, all of which are known to activate NF-κB.

Conclusion: A factor in a hot-PBS extract from *V. vulnificus* might activate NF-κB through an unknown receptor, and such a factor might play an important role in the etiology of diseases caused by *V. vulnificus*.

Keywords: *V. vulnificus*; Hot-PBS extract; Inflammation; NF-κB.

1. Introduction

Vibrio vulnificus is a gram-negative, curved, rod-shaped halophilic bacterium that causes severe wound infections and septicemia and is frequently fatal in hosts with liver disease or immunosuppression [1,2]. Septic shock, which occurs in fatal cases, has been attributed to a variety of virulence factors of *V. vulnificus,* such as bacterial Lipopolysaccharide (LPS), Capsular Polysaccharides (CPS), Hemolysin (VvhA) and Multifunctional Autoprocessing RTX Toxin (MARTX) [3-7]. However, the role of these factors in the local inflammation caused by *V. vulnificus* remains unclear [1-13].

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcription factor that plays a central role in immune responses and is involved in numerous physiological processes, such as acute and chronic inflammation, cell proliferation, and apoptosis. In this study, we found that a hot-PBS extract from *V. vulnificus* markedly induced NF-κB activation, and we examined the role of this hot-PBS extract in NF-κB activation.

2. Materials and Methods

2.1 Bacterial and culture conditions

The wild-type *V. vulnificus* MO6-24/O strain was provided by Dr. Antia C. Wright (University of Florida, Gainesville, FL). To eliminate the effects of hemolysin and MARTX toxin, double mutants of *V. vulnificus* (Δ*vvhA*/Δ*rtxA*) were used. The isogenic *V. vulnificus* double mutants (Δ*vvhA*/Δ*rtxA*) were provided by Dr. T. Iida (Osaka University, Japan). The bacteria were cultured in 100 ml of terrific broth at 37°C for 13 h, with shaking.

2.2 Preparation of bacterial extract

Bacteria were harvested by centrifugation at 10,000 x*g* for 10 min, washed in phosphate buffer saline (PBS), resuspended in 10 ml of PBS and then heated at 65°C for 1 h. After centrifugation at 15,000 x*g* for 10 min, the supernatant was filtered through a 0.25 μm membrane filter. The filtrate was then stored at -80°C.

2.3 NF-κB activity

A reporter plasmid that expresses luciferase depending on the NF-κB activity level (pBVI-Luc) was transfected into the HEK293T cells using a control reporter plasmid that expresses β-galactosidase constitutively under the control of the promoter of elongation factor 1 (pEF-BOS-β-Gal) using

the calcium phosphate method. Eight hours after transfection, the cells were treated with the bacterial extract, and 16 hours after exposure to the extract, the cells were lysed. The luciferase activity in the cell lysate was measured using a light emission assay with a commercially available kit (Promega, WI) and the β-galactosidase activity was measured using a coloring assay with ortho-nitrophenyl-β-galactoside. To evaluate the relative activity of NF-κB in the cells, the luciferase activity was divided by the activity of β-galactosidase. To test the ability of bacterial extract to activate NF-κB through NOD1 or NOD2, pMX-HA-NOD1 or pMX-HA-NOD2, respectively, was transfected into HEK293T cells with the luciferase and β-galactosidase reporter plasmids and evaluate the NF-κB activityas described above [8].

2.4 Preparation of LPS, CPS and peptidoglycan

Lipopolysaccharide (LPS), Capsular Polysaccharide (CPS), and peptidoglycan were prepared according to the methods described by Westphal and Lüderitz [9], Reddy et al. [10] and Gan et al. [11], respectively. Each extract was subjected to periodic acid-Schiff staining.

2.5 Enzyme treatment

The hot-PBS extracts were treated with protease K, DNase I, lysozyme and lipase at a concentration of 100 μg/ml each at 37°C for 1 h and the resulting abilities of the extracts to induce NF-κB were examined.

2.6 Ultrafiltration and anion-change column chromatogram

Ultrafiltration was performed using commercially available centrifugal filter devices (Merck Millipore, MA). Gel filtration and anion exchange column chromatography were performed using the Sephadex G25 (Sigma Aldrich, MO) (1 kDa-5 kDa cut-off) (150 mm × 10 mm) and the Q-Sepharose Fast Flow (GE Healthcare, IL) (100 mm × 15 mm), respectively.

3. Results

We found that hot-PBS extract from *V. vulnificus* activates NF-κB in HEK293T cells in a dose-dependent manner (Figure 1a). For the full activation of NF-κB, heat treatment of the bacterial suspension was required (Figure 1b), and the bacteria had to

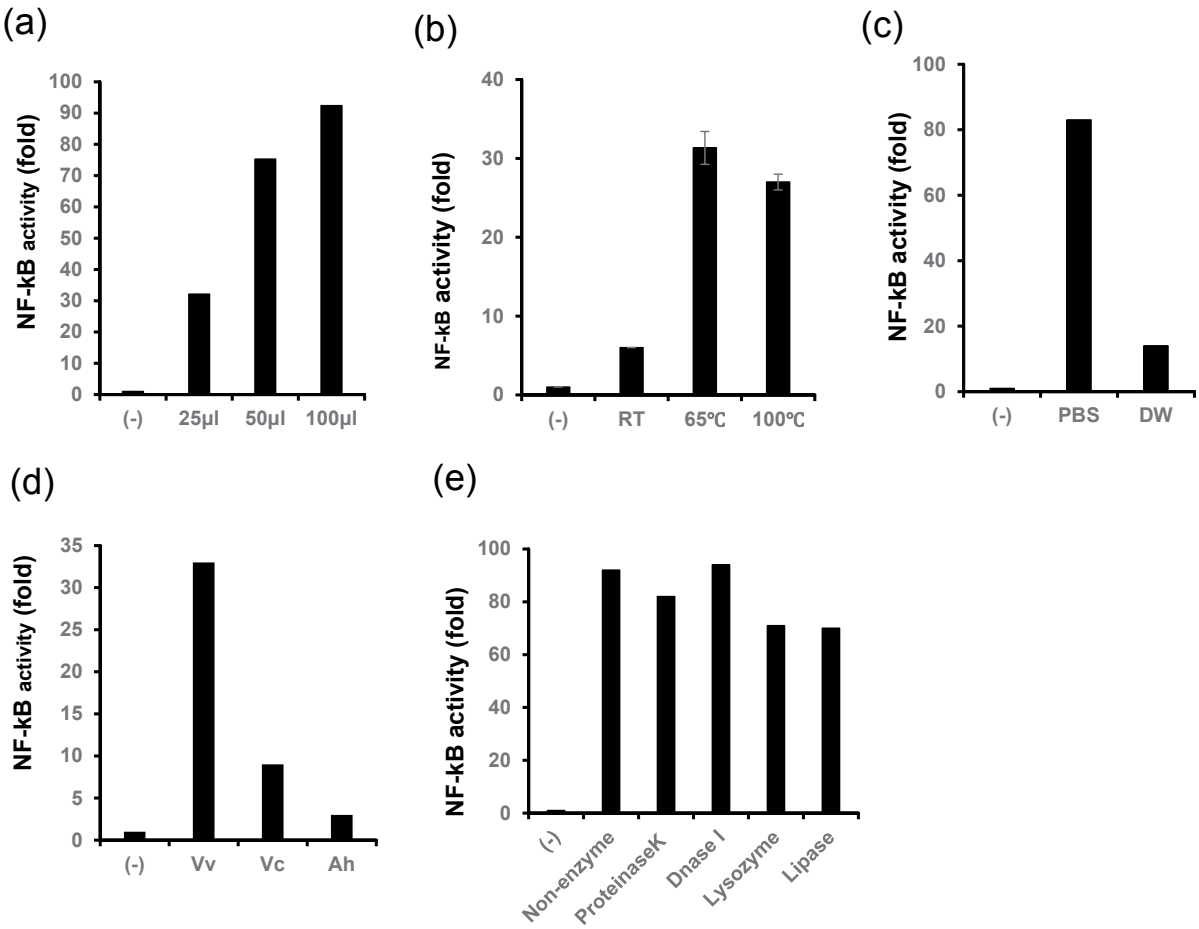

Figure 1. NF-κB activity of hot-PBS extract of *V. vulnificus*. (a) Volume dependent NF-κB activation. (b) Effect of temperature on heat extraction. *V. vulnificus* was heated at room temperature (RT), 65°C and 100°C for 1 h. (c) Effect of phosphate buffer saline (PBS) on the heat extract. *V. vulnificus* was suspended in distilled water (DW) or PBS and heated at 65°C for 1 h. (d) Effect of hot-PBS extracts of *V. vulnificus* (Vv), *V. cholerae* (Vc) and *A. hydrophila* (Ah). (e) Effect of enzyme treatment on hot-PBS extract. The extract was incubated with each enzyme (100 μg/ml) at 37°C for 1 h.

be suspended in PBS instead of pure water (Figure 1c). The hot-PBS extract of *V. vulnificus* induced a stronger activation of NF-κB than those of *Vibrio cholera* or *Aeromonas hydrophila* (Figure 1d). The ability of the hot-PBS extract to induce NF-κB was not affected by treatment with protease K, DNase I, lysozyme or lipase (Figure 1e).

We next examined whether LPS, CPS, or peptidoglycan prepared from *V. vulnificus* was capable of activating NF-κB to the same extent as the hot-PBS extract. Even though 5 μg of these compounds were used to stimulate the HEK293T cells, none of these compounds activated NF-κB to the same extent as the hot-PBS extract (Figures 2a-2c).

To estimate the molecular weight of the factor responsible for the NF-κB activation, we subjected the extract to size fractionation using ultrafiltration. As the activity was retained after passage through a 3kDa cut-off membrane (Figure 3a), the molecular weight of the NF-κB activator was estimated to be less than 3 KDa.

Gel filtration column chromatography using Sephadex G-25 (Figure 3b) showed that the size of the molecule responsible for the NF-κB activation was 3 kDa or smaller, which was similar to the size estimated using ultrafiltration.

Next, we tested whether the molecule could be fractionated using anion exchange column chromatography using Q-Sepharose Fast Flow anion exchange resin. As a result, the NF-κB activating factor was shown to bind to the column in PBS and to be eluted by a phosphate buffer containing 0.6 M NaCl (Figure 3c).

Since HEK293T cells are considered to express a certain amount of NOD1 and NOD2 protein, the *V. vulnificus* extract might activate NF-κB through endogenous NOD1 or NOD2 in HEK293T cells. Consequently, we examined whether the *V. vulnificus* extract activates NOD1 or NOD2. As shown in Figure 4, 100000 ng/ml of iE-DAP (NOD1 ligand) or MDP (NOD2 ligand) induced about a 4-fold activation of NF-κB in the plain HEK293T cells, while the same concentration of iE-DAP or MDP induced a more than 20-fold activation in the HEK283T cells that are forced to express NOD1 or NOD2, respectively. As 1.5 μl of fraction 11 induced about a 4-fold activation in the plain HEK293T cells, if the activation is mediated by NOD1 or NOD2, then the same amount of fraction 11 should induce a 20-fold activation in HEK293T cells expressing NOD1 or NOD2, respectively. However, 1.5 μl of fraction 11 induced only a 7-fold and a 6-fold activation of NF-κB in cells expressing NOD1 and NOD2, respectively. Thus, NF-κB activation by fraction 11 in HEK293T cells cannot be explained by endogenous NOD1 and NOD2 alone.

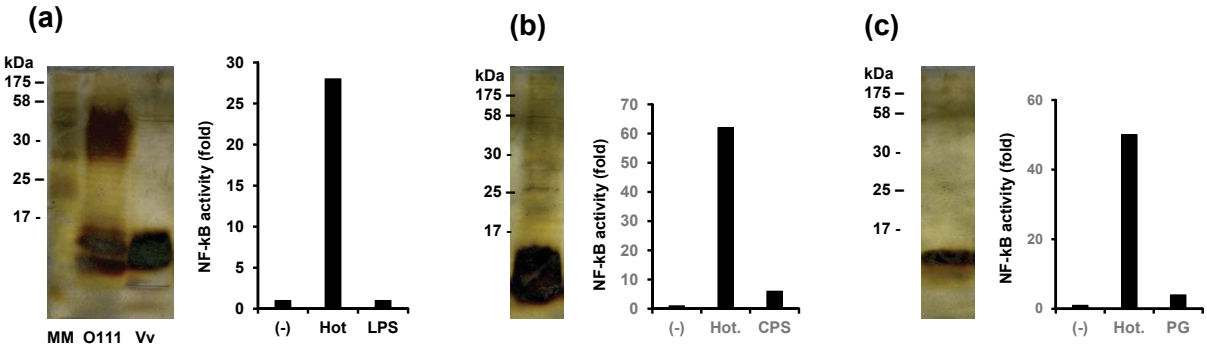

Figure 2. NF-κB activator characterization. (a) Effect of lipopolysaccharide (LPS), (b) capsular polysaccharide (CPS), and (c) peptidoglycan (PGN) isolate of *V. vulnificus* on NF-κB activation. Each isolate was used at a concentration of 50 μg/ml.

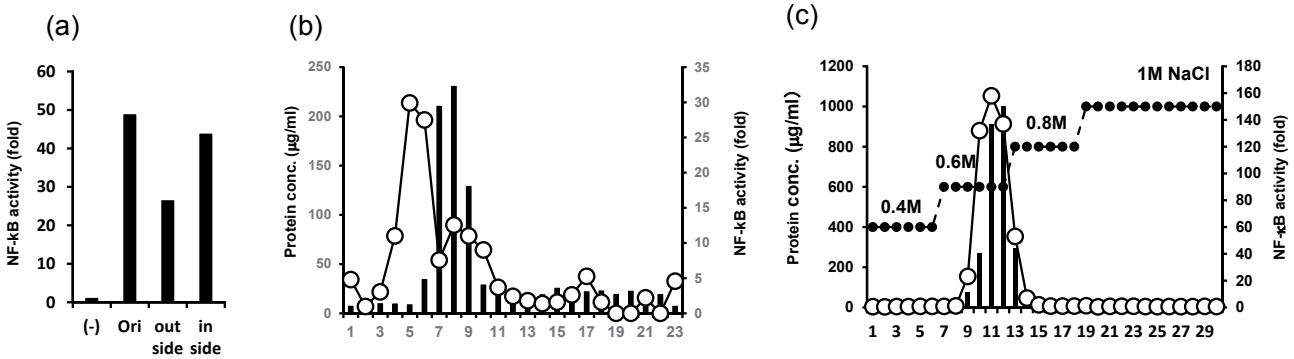

Figure 3. Molecular weight and charged state of hot-PBS extract. (a) Effect of ultrafiltration (3 kDa cut-off). The hot-PBS extract was passed through the ultrafiltration membrane, and the activities of the outer (out) and inner (in) solutions were examined. (b) Profile of Sephadex G25 column profile. A 1 ml sample was added to the gel and 1 ml was collected at a flow rate of 0.1 ml/min with PBS. (d) Profile of Q-anion exchange column chromatography. Active fractions of the Sephadex G25 column were collected and added to the Q-anion exchange column, washed with PBS, and the adsorbed materials on the column were eluted using a stepwise NaCl gradient (0.4-1 M).

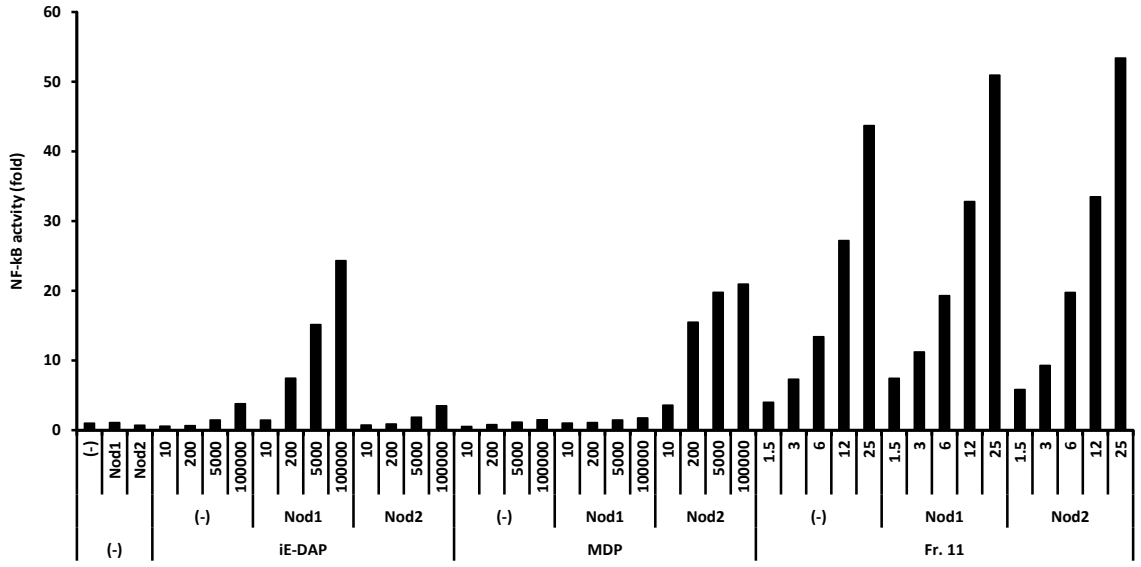

Figure 4. Nod-independent activation of NF-κB by *V. vulnificus* heat extract. Either NOD1 or NOD2 or pMX-HA-NOD1 or pMX-HA-NOD2 was transfected into HEK293T cells using luciferase and β-galactosidase reporter plasmids, respectively, and iE-DAP (D-gamma-Glu-mDAP), MDP (Muramyl Dipeptide) or fraction 11 were added.

4. Discussion and Conclusion

NF-κB is activated in response to various stresses and stimuli received from the surrounding environment and controls genes such as immunity, inflammation, carcinogenesis, programmed death and infection. Therefore, the overactivation of NF-κB is expected to be involved in pathogenicity. Humans with underlying diseases such as liver disease and diabetes can develop lethal systemic diseases such as sepsis and necrotizing fasciitis. People with chronic liver disease are known to have activated NF-κB and NF-κB activation by the hot-PBS extract may exacerbate their conditions. The causative component in the hot-PBS extract is likely activated by the presence of fever in the patient and bacterial death caused by the infection, and this component may contribute to the development of severe necrotizing fasciitis Furthermore, the present study also suggested that NF-activating receptors other than NOD1 and NOD2 might exist. Future studies should attempt to isolate the activator from the hot-PBS extract. Once this goal has been achieved, the following items will require investigation: 1) structure determination, 2) contribution to pathogenicity and possible treatment targets, 3) search for receptors and construction of new innate immune mechanism, and 4) possibility of physiological activity and immunological control in animal models.

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