



Available online at [www.sciencedirect.com](http://www.sciencedirect.com)

SciVerse ScienceDirect

journal homepage: [www.e-jmii.com](http://www.e-jmii.com)



SHORT COMMUNICATION

# *Lactobacillus casei* cell wall extract directly stimulates the expression of COX2 independent of Toll-like receptor 2 in rat glial cells



Naotoshi Sugimoto <sup>a,\*</sup>, Kunio Ohta <sup>b</sup>, Takekatsu Saito <sup>b</sup>,  
Yuko Nakayama <sup>b</sup>, Taichi Nakamura <sup>b</sup>, Akiko Maeda <sup>b</sup>,  
Akihiro Yachie <sup>b</sup>

<sup>a</sup> Department of Physiology, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan

<sup>b</sup> Department of Pediatrics, Graduate School of Medical Science, Kanazawa University, Kanazawa, Japan

Received 11 September 2011; received in revised form 13 February 2012; accepted 9 March 2012

## KEYWORDS

Cyclooxygenase-2;  
Glial cells;  
Kawasaki disease;  
*Lactobacillus casei*;  
Toll-like receptor  
family

Kawasaki disease is an acute illness of early childhood that is characterized by prolonged fever and vasculitis of unknown pathogenesis. *Lactobacillus casei* cell wall extract (LCWE)-induced vasculitis in mice is a well-validated model of Kawasaki disease. In the nervous system, glial cells play an important role in fever development. This study investigated whether LCWE directly stimulates glial cells, resulting in the induction of cyclooxygenase-2 (COX2), which is required for prostaglandin synthesis and fever development. We found that LCWE induced COX2 expression and activated the nuclear factor- $\kappa$ B signaling pathway in rat B92 glial cells, but Toll-like receptor-2, which is one of the receptors for LCWE, could not be detected in the cells. These results suggest that LCWE activates the nuclear factor- $\kappa$ B signaling pathway and induces COX2 in rat B92 glial cells through another LCWE receptor other than Toll-like receptor-2.

Copyright © 2012, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. All rights reserved.

\* Corresponding author. Department of Physiology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8640, Japan.

E-mail address: [ns@med.kanazawa-u.ac.jp](mailto:ns@med.kanazawa-u.ac.jp) (N. Sugimoto).

## Introduction

Kawasaki disease, which is the most common cause of multisystem vasculitis with prolonged fever in childhood in eastern Asia, is always considered in the differential diagnosis of fever of unknown origin.<sup>1</sup> After a single intraperitoneal injection of *Lactobacillus casei* cell wall extract (LCWE), mice develop a focal and localized coronary arteritis that is histopathologically similar to the coronary artery lesions found in human Kawasaki disease.<sup>2</sup> Recent studies concerning signaling through Toll-like receptors (TLRs) in knockout mice have suggested that TLR2 signaling may play a role in LCWE-induced mouse Kawasaki disease.<sup>2</sup> However, the mechanisms of the pathogenesis and prolonged fever in Kawasaki disease have not yet been elucidated. Fever occurs due to the action of prostaglandin E2 (PGE2) mediated by cyclooxygenase-2 (COX2). Glial cells may play a crucial role in the production of PGE2 for developing fever in the brain.<sup>3</sup> This study investigated whether LCWE directly stimulates glial cells, resulting in the induction of COX2.

## Methods and materials

LCWE fragments were obtained from *L. casei* (ATCC 11578) as previously described.<sup>4</sup> Briefly, the bacterial cells were disrupted by overnight incubation in 4% sodium dodecyl sulfate in twice their packed volume. Cell wall fragment preparations were sonicated for 2 h. During sonication, the cell wall fragments were maintained at 4°C. After sonication, the cell wall fragments were centrifuged for 1 h at 20,000 × *g* at 4°C, and the supernatant was retained. B92 rat glial cells, Chinese hamster ovary (CHO) cells, COS7 monkey kidney cells, and NIH3T3 mouse fibroblast cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37°C in 5% CO<sub>2</sub>. Proteins were extracted from the cells, and the protein concentrations were determined with protein assay reagents. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene fluoride membranes, which were incubated with primary antibodies (1:1000), followed by incubation with a horseradish peroxidase (HRP)-linked secondary antibody (1:2000). The blots were developed with the Immobilon Western Chemiluminescence HRP Substrate (Millipore, Billerica, MA, USA).

Anti-phospho-specific nuclear factor (NF)-κB p65 (Ser536), anti-IκB-α, anti-phospho-specific p44/p42 MAPK (Thr202/Tyr204), anti-β-actin, and HRP-linked anti-rabbit IgG were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-COX2 and anti-NOD2 antibodies were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-TLR2 antibody was obtained from Abnova Corporation (Taipei, Taiwan). TLR2 agonist (lipoteichoic acid) and NOD2 agonist (insoluble peptidoglycan) were purchased from InvivoGen (San Diego, CA, USA).

## Results

First, we investigated whether LCWE induced COX2 expression in B92 glial cells. B92 cells were incubated in

DMEM without FBS for 24 h and then treated with LCWE (10 μg/mL) for 16 h. Cell extracts were immunoblotted with anti-COX2 or anti-β-actin antibodies. LCWE treatment of B92 cells increased the levels of COX2 expression (Fig. 1A). Next, to determine the effects of LCWE on the NF-κB signaling pathway, we investigated the levels of IκB-α and phospho-NF-κB after LCWE stimulation. B92 cells were incubated in DMEM without FBS for 24 h and then treated with LCWE (10 μg/mL) for 1 h. Cell extracts were immunoblotted with anti-IκB-α or phospho-NF-κB antibodies. LCWE decreased the levels of IκB-α expression and increased the levels of phospho-NF-κB expression (Fig. 1B), indicating activation of the NF-κB signaling pathway in B92 glial cells.

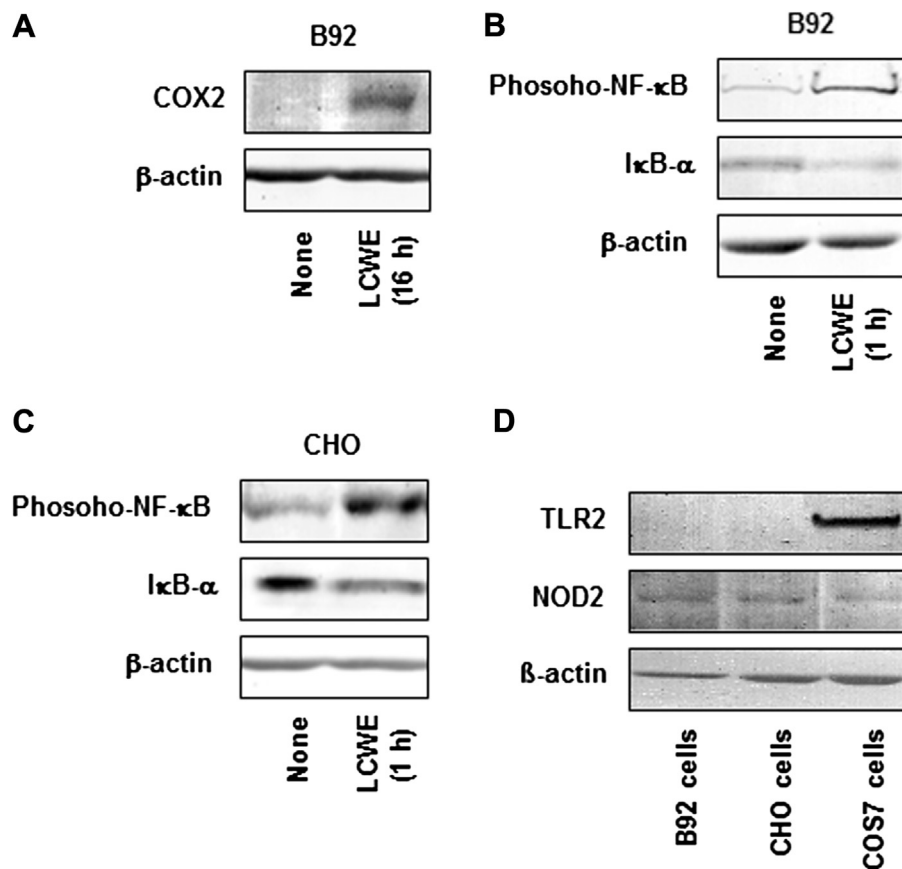
Similarly to B92 cells, LCWE activated the NF-κB signaling pathway in CHO cells (Fig. 1C) and COS7 cells (data not shown), but failed to induce the expression of COX2 in these cells (data not shown). Next, we investigated the levels of TLR2, which is thought to be the receptor of LCWE,<sup>2</sup> by immunoblotting with an anti-TLR2 antibody. Interestingly, TLR2 expression was detectable in COS7 cells, but the expression in B92 and CHO cells was below the limit of detection (Fig. 1D). Our recent study demonstrated that LCWE activated NF-κB and p44/p42 MAPK in NIH3T3 mouse fibroblast cells,<sup>5</sup> in which TLR2 and COX2 expression was detectable (Figs. 2A and 2B). Although LCWE and the TLR2 agonist induced the activation of NF-κB and p44/p42 MAPK in this study (data not shown), LCWE and the TLR2 agonist failed to induce an upregulation of COX2 expression in NIH3T3 cells (Fig. 2B).

The nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family is a major form of innate immune sensors, as is the TLR family.<sup>6</sup> NOD2 is critical for host defense against bacterial infection.<sup>6,7</sup> NOD2 acts as a cytosolic sensor of distinct peptidoglycan fragments from both gram-negative and gram-positive bacteria.<sup>6,7</sup> Interestingly, NOD expression was detectable in COS7, B92, and CHO cells (Fig. 1D). Thus, we investigated whether a NOD2 agonist induced COX2 expression in B92 glial cells. Treatment with a NOD2 agonist, but not a TLR2 agonist, increased the levels of COX2 expression dose-dependently (Figs. 2C and 2D). Similar to COX2 expression, a NOD2 agonist, but not a TLR2 agonist, activated p44/p42 MAPK in B92 cells (Fig. 2E).

## Discussion

Prostaglandins are crucial fever mediators in the central nervous system that act through the activation of prostaglandin receptors.<sup>8</sup> A recent study suggested that the induction of COX2 in glial cells plays an important role in producing PGE2 and developing fever.<sup>3</sup> LCWE induced the expression of COX2 in glial cells in this study (Fig. 1A). However, the significance and mechanism of LCWE-induced COX2 expression is still unknown. Further studies are required to determine the role of LCWE in the induction of COX2 and developing fever *in vivo*.

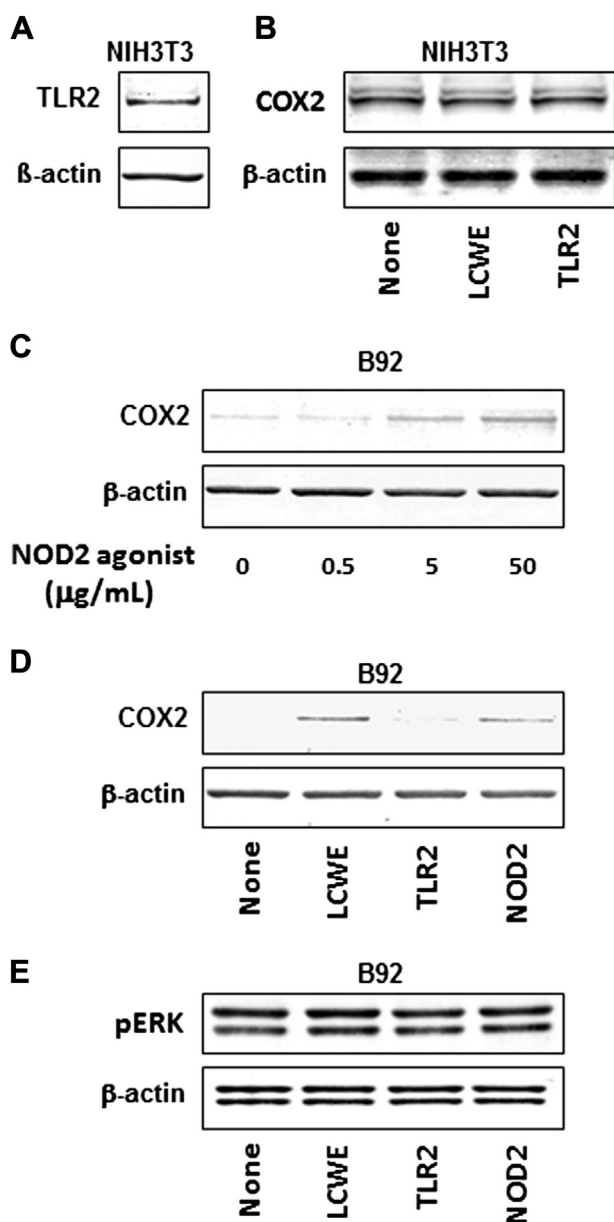
Rat COX2 is encoded by a 5.7-kb genomic DNA segment with 10 exons. The 5' end of the flanking promoter region of rat COX2 contains multiple regulatory elements, including a putative NF-κB binding site, a CCAAT/enhancer binding protein-binding site, and a cAMP-response element.<sup>9,10</sup> The NF-κB signaling pathway plays a crucial role in a variety of physiological and pathological events, including



**Figure 1.** *Lactobacillus casei* cell wall extract (LCWE) induced (A) cyclooxygenase-2 (COX2) expression and the (B) activation of the nuclear factor (NF)- $\kappa$ B signaling pathway in B92 cells. (C) LCWE activated the NF- $\kappa$ B signaling pathway in CHO cells. The LCWE-induced activation of the NF- $\kappa$ B signaling pathway was independent of the Toll-like receptor-2 (TLR2) (D) in B92 and CHO cells. (A) The levels of COX2 protein expression were evaluated by Western blotting in total cell lysates prepared from B92 cells treated with LCWE for 16 h. (B) The levels of phospho-NF- $\kappa$ B and I $\kappa$ B- $\alpha$  protein expression were evaluated by Western blotting in total cell lysates prepared from B92 cells treated with LCWE for 1 h. (C) The levels of phospho-NF- $\kappa$ B and I $\kappa$ B- $\alpha$  protein expression were evaluated by Western blotting in whole-cell lysates prepared from CHO cells treated with LCWE for 1 h. (D) The levels of TLR2 and NOD2 expression were evaluated by Western blotting in whole-cell lysates prepared from B92, CHO, and COS7 cells.

inflammation, immune responses, and apoptosis. In the canonical pathway, NF- $\kappa$ B proteins are bound to inhibitory molecules (I $\kappa$ Bs) and sequestered in the cytoplasm in an inactive state. When cells are stimulated by appropriate factors, the I $\kappa$ B kinase (IKK) complex containing catalytically active IKK $\alpha$  and IKK $\beta$  and the regulatory scaffold protein IKK $\gamma$ /NEMO phosphorylates I $\kappa$ B, leading to its ubiquitination and proteasomal destruction. NF- $\kappa$ B is subsequently released from inhibition to enter the nucleus where it can either repress or activate gene transcription. In human glial cells, the NF- $\kappa$ B element strongly alters COX2 promoter activity.<sup>11</sup> Our results in rat glial cells may be consistent with those in humans because LCWE activated the NF- $\kappa$ B signaling pathway and induced COX2 expression (Figs. 1A and 1B). However, in other cells, the activation of the NF- $\kappa$ B signaling pathway was not sufficient for the induction of COX2. Although an LCWE agonist activated NF- $\kappa$ B signaling in CHO cells (Fig. 1C), COS7 cells, and NIH3T3 cells (data not shown), LCWE failed to enhance the expression of COX2 in these cells in this study. These results indicated that the NF- $\kappa$ B signaling pathway might be necessary but not sufficient for COX2 induction under LCWE signaling.

The deletion of TLR2 failed to generate mouse Kawasaki disease by LCWE in TLR2 knockout mice,<sup>2</sup> suggesting that TLR2 is a receptor of LCWE. However, LCWE induced the activation of NF- $\kappa$ B signaling and the induction of COX2 in B92 cells in which TLR2 expression was not detected in this study (Fig. 1D). Moreover, MAPK was activated by LCWE but not by a TLR2 agonist in B92 cells (Fig. 2E). These results indicate the presence of other LCWE receptors other than TLR2 in B92 rat glial cells. NOD2, which is another innate immunoreceptor, was detected in both B92 and CHO cells (Fig. 2B). NOD2 acts as a cytosolic sensor of distinct peptidoglycan fragments from both gram-negative and gram-positive bacteria.<sup>6,7</sup> Once activated, NOD2 oligomerizes and recruits the NF- $\kappa$ B activating kinase RICK through homotypic CARD-CARD interactions involving their amino-terminal CARD motifs. RICK interacts with the regulatory scaffold protein IKK $\gamma$ /NEMO, triggering I $\kappa$ B phosphorylation and NF- $\kappa$ B activation. The cell walls of *L. casei* are composed of peptidoglycan and associated uncharged polysaccharides,<sup>12</sup> and thus, LCWE are crude peptidoglycan preparations. In our study, a NOD2 agonist stimulated MAPK (Fig. 3 2E) and the expression of COX2 dose-dependently (Fig. 2C), suggesting



**Figure 2.** (A) TLR2 is present in NIH3T3 cells. (B) LCWE and a TLR2-specific agonist (TLR2) failed to enhance COX2 expression in NIH3T3 cells. (C) A NOD2-specific agonist induced COX2 expression dose-dependently in B92 cells. A NOD2 agonist, but not a TLR2 agonist, (D) induced COX2 expression and (E) activated MAPK in B92 cells. (A) The levels of TLR2 expression were evaluated by Western blotting in whole-cell lysates prepared from NIH3T3 cells. (B) The levels of COX2 protein expression were evaluated by Western blotting in total cell lysates prepared from NIH3T3 cells treated with LCWE or TLR2 agonist for 16 h. (C) The levels of COX2 protein expression were evaluated by Western blotting in total cell lysates prepared from B92 cells treated with several doses of NOD2 agonist for 16 h. (D) The levels of COX2 protein expression were evaluated by Western blotting in total cell lysates prepared from B92 cells treated with LCWE, TLR2 agonist, or NOD2 agonist for 16 h. (E) The levels of phospho-MAPK protein expression were evaluated by Western blotting in whole-cell lysates prepared from B92 cells treated with LCWE, TLR2 agonist, or NOD2 agonist for 1 h.

that the actions of LCWE might be mimicked by a NOD2 agonist. Our results raise the possibility that NOD2 receptors are involved in the LCWE signaling pathway in B92 glial cells.

We showed here that LCWE activated NF- $\kappa$ B signaling independent of TLR2, but little is known about the physiological and pathological mechanisms of LCWE pathogenesis. Further studies are required to determine the role of the NLR family, including NOD1/2, in LCWE-mediated NF- $\kappa$ B signal activation.

## Acknowledgments

The authors thank Dr Takako Ohno-Shosaku for providing the B92 rat glial cells. This work was supported by Grants-in-Aid for Science and Culture from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

- Muise A, Tallett SE, Silverman ED. Are children with Kawasaki disease and prolonged fever at risk for macrophage activation syndrome? *Pediatric* 2003;112:e495–7.
- Rosenkranz ME, Schulte DJ, Agle LMA, Wong MH, Zhang W, Ivashkiv L, et al. TLR2 and MyD88 contribute to *Lactobacillus casei* extract-induced focal coronary arteritis in a mouse model of Kawasaki disease. *Circulation* 2005;112:2966–73.
- Hanada R, Leibbrandt A, Hanada T, Kitaoka S, Furuyashiki T, Fujihara H. Central control of fever and female body temperature by RANKL/RANK. *Nature* 2009;462:505–9.
- Lehman TJ, Walker SM, Mahnovski V, McCurdy D. Coronary arteritis in mice following the systemic injection of group B *Lactobacillus casei* cell walls in aqueous suspension. *Arthritis Rheum* 1985;28:652–9.
- Saito T, Sugimoto N, Ohta K, Shimizu T, Ohtani K, Nakayama Y, et al. Phosphodiesterase inhibitors suppress *Lactobacillus casei* cell wall-induced NF- $\kappa$ B and MAPK activations and cell proliferation through protein kinase A- or exchange protein activated by cAMP-dependent signal pathway. *Sci World J*, doi:10.1100/2012/748572.
- Fukata M, Vamadevan AS, Abreu MT. Toll-like receptors (TLRs) and Nod-like receptors (NLRs) in inflammatory disorders. *Sem Immunol* 2009;21:242–53.
- Shaw PJ, Lamkanfi M, Kanneganti TD. NOD-like receptor (NLR) signaling beyond the inflammasome. *Eur J Immunol* 2010;40:624–7.
- Ushikubi F, Segi E, Sugimoto Y, Murata T, Matsuoka T, Kobayashi T. Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature* 1998;395:281–4.
- Sirois J, Levy LO, Simmons DL, Richards JS. Characterization and hormonal regulation of promoter of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. *J Biol Chem* 1993;268:12199–206.
- Morris JK, Richards JS. An E-box region within the prostaglandin endoperoxide synthase-2 (PGS-2) promoter is required for transcription in rat ovarian granulosa cells. *J Biol Chem* 1996;271:16633–43.
- Avarez S, Blanco A, Fresno M, Murioz-Fernandez MA. Nuclear factor- $\kappa$ B activation regulates cyclooxygenase-2 induction in human astrocytes in response to CXCL12: role in neuronal toxicity. *J Neurochem* 2010;113:772–83.
- Shida K, Kiyoshima-Shibata J, Kaji R, Nagaoka M, Nanno M. Peptidoglycan from lactobacilli inhibits interleukin-12 production by macrophages induced by *Lactobacillus casei* through Toll-like receptor 2-dependent and independent mechanisms. *Immunology* 2009;128:e858–69.