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Journal of Research and Practice in Dentistry
Vol. 2015 (2015), Article ID 290259, 46 minipages.
DOI:10.5171/2015.290259
www.ibimapublishing.com

Research Article

Expression and LPS-Induced Elevation of Nod2 and Calprotectin in the Submandibular Gland of Wild-Type and TLR4-Knockout Male Mice

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Received date: 10 January 2014
Accepted date: 18 April 2014
Published date: 10 August 2015

Academic Editor: Chiaki Kitamura

Cite this Article as: Purevjav Javkhlan, Guangfei Xu, Gang Chen, Chenjuan Yao, Yuka Hiroshima, Hiroshi Yoshimura, Toshihiko Nagata and Kazuo Hosoi (2015), “Expression and LPS-Induced Elevation of Nod2 and Calprotectin in the Submandibular Gland of Wild-Type and TLR4-Knockout Male Mice”, Journal of Research and Practice in Dentistry, Vol. 2015 (2015), Article ID 290259, DOI: 10.5171/2015.290259

Abstract

The salivary gland produces a number of inflammation-related cytokines and at least some of them including S100A8 and S100A9 (calprotectin subunits) in the gland are induced by endotoxins. The signaling pathway to induce these cytokines is complex and has not been thoroughly elucidated. Thus, we examined effects of lipopolysaccharide (LPS) on the level of salivary gland S100A8/A9 and investigated whether any signaling molecule is participated in elevation of these cytokines in this tissue. Toll-like receptor-4 (TLR4)-knockout C57BL/6 ($TLR4^{-/-}$) mice and their wild-type (WT), C57BL/6, counterpart ($TLR4^{+/+}$) were used in the experiment. Quantitative real-time RT-PCR, RT-PCR, and Western blotting were employed for analyses of mRNA and protein levels of S100A8/A9, nucleotide-

binding oligomerization domain-containing protein (Nod) 1, and Nod2. Following the injection of *E. coli* LPS, the mRNA levels of S100A8/A9 were strongly elevated in the submandibular gland (SMG) of WT mice and at small degree but clearly so even in the same tissue of TLR4-knockout counterparts; this suggests the existence of a signaling pathway other than TLR4 to induce S100A8/A9. In the SMG of WT mice but not TLR4-knockout counterparts, both mRNA and protein levels of Nod2 were elevated by LPS, these increases were completely blocked by cycloheximide (CHX). CHX simultaneously suppressed LPS-induced elevation of S100A8/A9 mRNAs. These results, although more experiment using Nod2 specific inhibitors is required, imply the possibility that S100A8/A9 mRNA levels were increased by LPS *via* both TLR4 and Nod2 signaling pathways in the mouse SMG.

Keywords: Nod2, calprotectin, S100A8/A9, LPS, submandibular gland

Introduction

S100A8 and S100A9 belong to the S100 protein family, whose members contain 2 Ca^{2+} binding sites of the EF-hand type (Donato, 2001). S100A8 and S100A9 in this family make a heterodimeric complex called calprotectin, which is a pleiotropic protein associated with inflammation. Namely, calprotectin binds divalent cations, such as zinc ions and shows a bacteriostatic effect (Stříž and Trebichavský, 2004). Secondly, calprotectin is a mediator in the neutrophil recruitment in response to lipopolysaccharide (LPS) (Pouliot et al., 2008), and thereby the neutrophils migrate to the sites of inflammation via the

circulation (Ryckman et al., 2003). Lastly, calprotectin has pro-inflammatory functions and exerts cytokine-like activities (Cuida et al., 1997) including activation of intracellular signaling pathways such as MAP-kinase and/or NF-κB (Hofmann et al., 1999; Arumugam et al., 2004; Zenz et al., 2005).

Calprotectin is produced abundantly and constitutively in the neutrophils and monocytes. This pro-inflammatory protein is expressed also in the squamous mucosal keratinocytes and innate immune cells present at the surface of mucosal tissue (Hsu et al., 2009). In the patient suffering from inflammatory diseases, the calprotectin level in the plasma, feces, dental calculus, gingival crevicular fluid, and saliva changes (Kido, et al., 2003). Especially in rheumatoid arthritis, the calprotectin level in the synovial fluid is increased (Hammer, et al. 2011).

During inflammation, calprotectin expression is induced in the epidermal keratinocytes, gastrointestinal epithelial cells and fibroblasts (Hsu et al., 2009). Since the expression or induction of calprotectin in the salivary gland is important in terms of the oral defense system, the regulation of its expression was studied previously (Javkhlan et al., 2011). In this previous study, we showed that mRNAs and proteins for both S100A8 and S100A9 are induced by LPS via Toll-like receptor 4 (TLR4) in the mouse submandibular and parotid glands (SMG and PG, respectively). We also noticed that S100A8 and S100A9 were elevated to a small degree by LPS in the same glands in LPS hyposensitive TLR4-mutant C3H/HeJ mice, implying the possibility of presence of LPS-signaling(s) other than the one via TLR4 in the salivary glands (Javkhlan et al., 2011).

The family of nucleotide-binding oligomerization domain-containing protein-like receptors (NLRs) contains modules of the nucleotide-binding oligomerization domain (NOD) and belongs to a group of intracellular pattern-recognition receptors (Inohara et al., 2005). NOD-containing protein (Nod) 1 and Nod2 are the most studied members of the NLR family, and it is well known that mutations in the Nod2 gene are associated with Crohn's disease (Ogura et al., 2001a; Hugot et al., 2001). In contrast, its up-regulation is associated with Blau's syndrome (Miceli-Richard et al., 2001). The ligands for Nod1 and Nod2 are bacterial molecules containing the D-glutamyl-meso-diaminopimelic acid moiety and muramyl dipeptide (MDP), respectively. The major responses upon challenges of these ligand via cytosolic Nod1 and Nod2 are activation of NF-κB through IKK (Strober et al., 2006).

In macrophages, one of these molecules, Nod2, is reported to respond to LPS as well as to MDP (Pauleau and Murra, 2003).

In the present study, therefore, we examined if the expression of salivary gland S100A8 and S100A9 is also regulated by Nod2 or by other members of the NLR family, since these subunits (S100A8 and S100A9) of the inflammatory protein calprotectin, though at low level, respond to LPS in the salivary glands lacking normal TLR4 expression (Javkhlan et al., 2011).

Materials and Methods

Reagents

LPS (*Escherichia coli* serotype 0111:B4), Tri-Reagent™, cycloheximide (CHX), mouse monoclonal anti-β-actin-peroxidase, and sets of primers for RT-PCR and quantitative real-time RT-PCR were obtained from Sigma-Aldrich (St. Louis, MO, USA). Superscript™ One Step RT-PCR with the Platinum *Taq* System was obtained from Invitrogen (Carlsbad, CA, USA), whereas SYBR Green Primescript™ RT-PCR kit (Perfect Real Time) was purchased from TaKaRa (Shiga, Japan).

Complete EDTA-free protease inhibitor cocktail tablets were obtained from Boehringer Mannheim (Mannheim, Germany).

Goat anti-Nod1 antibody came from Santa Cruz (Santa Cruz, CA, USA); and goat anti-Nod2 antibody, from IMGENEX (San Diego, CA, USA). Rabbit anti-goat IgG horseradish peroxidase conjugate and Immobilon-P transfer membranes were purchased from Beckman Coulter (Brea, CA, USA) and Millipore Co. (Billerica, MA, USA), respectively. Can Get Signal® was obtained from Toyobo Co. Ltd. (Osaka, Japan), whereas an Enhanced Chemical Luminescence (ECL) Detection Kit was purchased from GE Healthcare (Buckinghamshire, UK).

Animals and LPS/CHX Treatments

Three pairs of TLR4-knockout, C57BL/6 ($TLR4^{-/-}$) mice were obtained from OrientalBioService, Inc. (Kyoto, Japan); they were mated in our animal facility to obtain off-springs. The wild-type,

C57BL/6NCrSn ($TLR4^{+/+}$) male mice, aged 7-8 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan). They will be simply designated as TLR4-knockout (TLR4-KO) and wild-type (WT) mice in this paper. All animals were housed under standard conditions (12-h light/12-h darkness cycle at 22-25°C) with free access to food and water. They were used for experiments at the age of 8 to 9 weeks.

In the RT-PCR experiment to estimate the salivary gland mRNA levels of S100A8, S100A9, IL-1β, Nod1/Nod2, TLR4, and β-actin, mice were injected *i.p.* with LPS dissolved in saline at a dose of 0.4 mg/kg body weight (Yao et al., 2005a); and control animals, with saline. The animals were euthanized by cervical dislocation at 3 h after the saline- or LPS-treatment. In the quantitative real-time RT-PCR experiment to examine the effects of LPS and CHX

on the mRNA levels of S100A8 and S100A9, animals were divided into 4 groups and injected with CHX and LPS either singly or in combination. CHX or control saline was injected into mice *i.p.*, 30 min prior to LPS injection; and the animals were euthanized by cervical dislocation at 3 h after the LPS injection. Dosages of CHX and LPS were 50 mg (Tait et al., 2005) and 0.4 mg/kg body weight, respectively.

In the Western blotting experiment to examine the effects of CHX and LPS on the Nod1 and Nod2 protein levels, animals were divided into 4 groups, each given these 2 reagents singly or in combination similarly as described above.

In all of the above experiments, each group consisted of 4-5 mice for quantitative analyses; whereas 2-3 mice were used for other

assays. The protocols used in the present animal experiments were approved by the Institutional Review Board of the Animal Committee of The University of Tokushima.

Preparation of Total RNA, RT-PCR, and Quantitative Real-Time RT-PCR

The mice were euthanized by cervical dislocation and the SMG and PG were carefully removed from each mouse. Special care was taken when the SMG were dissected from sublingual gland. The removed SMG and PG were used for isolation of total RNA with Tri-reagent by the standard procedure. RT-PCR amplification of mRNAs for S100A8, S100A9, IL-1β, Nod1, Nod2, and β-actin was performed as described earlier (Javkhlan et al 2011; Yao et al., 2010). Briefly, RT-PCR amplification of S100A8

and S100A9 mRNAs was mostly performed by using the SuperScript™ One Step RT-PCR system in a thermal cycler (TaKaRa Thermal Cycler MP, model TP 3000, Shiga, Japan). The primers used were prepared according to the published sequences (Javkhlan et al 2011; Yao et al., 2005b; Takahashi et al., 2006; Yao et al., 2005a; Table 1).

Please See Table 1 in the PDF Version

Quantitative real-time RT-PCR analysis was performed to measure the expression level of S100A8, S100A9, and β-actin transcripts. The sequential reactions were performed by using a One Step SYBR Green Primescript™ RT-PCR kit according to the manufacturer's instruction, and using primers as reported previously (Javkhlan et al 2011; Table 1). Amounts of S100A8

and S100A9 mRNAs were normalized by the amount of β-actin mRNA measured as an internal standard.

Preparation of Tissue Extracts and Western Blotting

The SMG, spleen, and adipose tissue were homogenized in 5 mM HEPES buffer (pH 7.5, containing 50 mM mannitol, 10mM $MgCl_2$, 1mM PMSF and 1×Protease inhibitor cocktail) and centrifuged at 14,000 rpm for 15 min at 4°C to obtain their supernatants, the protein concentrations of which were determined by the Bradford method (Bradford et al., 1976). The tissue extract prepared was denatured under reducing conditions (95°C for 5 min). Sixty micrograms of protein were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the separated proteins were transferred onto Immobilon-P membranes, which were next

blocked with PVDF blocking reagent at room temperature for 3 h. The membranes were then incubated at 4°C overnight with 0.5 μg/ml of anti-Nod1 antibody and 0.1 μg/ml of anti-Nod2 antibody in “Solution 1” provided in Can Get Signal® kit. They were subsequently washed and incubated at room temperature for 1 h with 8,000 times-diluted rabbit anti-goat IgG horse radish peroxidase-conjugate in Can Get Signal®, “Solution 2.” For the internal standard, β-actin was visualized by reacting the membrane with 50,000-times diluted mouse monoclonal anti-β-actin-peroxidase at room temperature for 1 h. All membranes were subjected to the ECL reaction and exposed to X-ray films.

Statistics

ANOVA and Fisher's PLSD (Protected Least Significant Difference) were applied for statistical analysis of the data obtained by quantitative real-time RT-PCR.

Results

Effects of LPS on expression of S100A, S100A9, Nod1, and Nod2 mRNAs in the salivary glands of WT and TLR4-KO mice

The effects of the endotoxin LPS on mRNA expression levels of S100A8 and S100A9 (the subunits of calprotectin) in the SMG and PG of WT and TLR4-KO mice were first examined (Fig. 1a, b). In the SMG, LPS elevated the mRNA levels of S100A8 and S100A9

in not only the WT mice but also in the TRL4-KO ones (Fig. 1a). This mRNA elevation in the TLR4-KO mice was not limited to S100A8 and S100A9; for the Fig. 1a, bmRNA level of the cytokine, IL-1β was also increased by LPS in these mice. Similar results were obtained when total RNA from the PG was analyzed (Fig.1b).

Please See Figure 1a and 1b in the PDF Version

Since Nod1 and Nod2 are cytosolic components of the innate immune system capable of binding with LPS to activate NF-κB (Inohara et al., 2001; Pauleau and Murra, 2003), we examined if these components were involved in the induction of the S100A8 and S100A9 mRNAs. RT-PCR experiments were first conducted to detect the Nod1 and Nod2 mRNAs in the SMG from WT and TLR4-KO mice (Fig. 2a). Both Nod1 and Nod2 mRNAs were

expressed in this tissue; and the mRNA for Nod2 in the WT mice, but not that in the TLR4-KO mice, was strongly increased by LPS. Also, the Nod2 mRNA level in untreated TLR4-KO mice was higher than that in WT mice. On the contrary, different from the effects on Nod2, Nod1 mRNA levels, though distinctly expressed in the SMG, did not change upon LPS stimulation. The expression of TLR4 mRNA and its absence were confirmed in WT and TLR4-KO mice, respectively (Fig. 2). Data comparable to those for the SMG were obtained when the PG from the same mice was analyzed (Fig. 2b).

Please See Figure 2a and 2b in the PDF Version

Effects of CHX on LPS-induced changes in S100A8, S100A9, Nod1, and Nod2 mRNA/protein levels in the SMG of WT and TLR4-KO mice

If Nod2 signaling, in addition to TLR4 signaling, was involved in the elevation of S100A8 and S100A9 mRNA levels, inhibition of Nod2 synthesis by a protein synthesis inhibitor would be expected to reduce the LPS-induced elevation of the S100A8 and S100A9 mRNA levels in WT mice. Thus, we focused on the SMG and examined the effect of CHX on these elevated levels of S100A8 and S100A9 mRNA by performing quantitative real-time RT-PCR (Fig. 3). The results showed that the injection of LPS into the WT mice markedly increased the S100A8 and S100A9 mRNA levels in the Fig. 3a, b

Please See Figure 3a and 3b in the PDF Version

SMG; and such elevation was strongly inhibited in mice pre-treated with CHX, implying that Nod2 having been increased in expression by LPS potentiated induction of the S100A8 and S100A9 mRNA levels. This idea was supported by the Nod2 Western blotting experiment (see Fig. 4), in which the Nod2 protein level in the SMG of WT mice was shown to be increased by LPS and that such increase was blocked by CXH.

In WT mice, the S100A8 and S100A9 mRNA levels were increased to some extent by the injection of CHX alone, and the levels in CHX + LPS group were not higher than those in CHX group (Fig. 3a). Although the increase in S100A8 and S100A9 mRNA levels by CHX is unknown at present, we hypothesized

the presence of putative suppressor for transcription of these mRNAs, whose synthesis is inhibited by CHX (see Discussion).

In the TLR4-KO mice, though it is low-level, the LPS injection increased the S100A8 and S100A9 mRNA levels in the SMG (Fig. 3b), whose results were consistent with those shown in Fig. 1b, and confirming the presence of a signaling pathway besides TLR4. SMG S100A8 and S100A9 mRNA levels were increased by LPS even in the presence of CHX. It is possible from these data that the pathway uninvolving TLR4 was independent of protein synthesis; *i.e.*, LPS is supposed to have triggered directly the signaling system to activate the transcription of mRNAs for S100A8 and S100A9. Injection of CHX alone induced an elevation of mRNAs for S100A8 and S100A9 similarly as WT mice.

Elevations of S100A8 and S100A9 mRNAs by LPS in the control knockout mice were moderate compared to those in the WT ones. Thus, the TLR4 signaling pathways in the WT mice may have been potentiated by a putative pathway linked to the activation of transcription of S100A8 and S100A9 mRNAs. The signaling molecule(s) in the NLR family could be a candidate(s) of such a pathway.

Thus, in addition to the mRNA analysis shown in Fig. 2, the expression of 2 NLR proteins, Nod1 and Nod2, in the SMG of WT and TLR4-KO mice was examined by Western blotting (Fig. 4a, b).

Please See Figure 4a and 4b in the PDF Version

The specificity of antibodies was first confirmed by the Fig. 4a, b experiment shown in Fig. 4a, where Nod1 and Nod2 proteins at

approximate molecular masses of 96-kDa and 115-kDa, respectively, were specifically detected. These sizes well agreed with the ones reported previously (Inohara et al., 1999; Ogura et al., 2011b). The effects of CHX on LPS-induced changes in Nod1 and Nod2 protein levels are shown in Fig. 4b. In both WT and TLR4-KO mice, the Nod1 protein level in the SMG did not change in response to the single or combined injection of LPS and CHX. However, the Nod2 protein level in the WT mice was markedly increased by the injection of LPS; and this increase was completely inhibited by the pre-treatment of the animals with CHX (Fig. 4b). On the other hand, in the TLR4-KO mice, the profile of changes in the Nod2 protein level was completely different from that of the WT mice: First, the level was not increased by the LPS injection, either in the presence or absence of CHX. Secondly, the Nod2 protein level was higher in the TLR4-KO control than in

the WT one. The results in Fig. 4 are well consistent with the Nod1/Nod2 mRNA data shown in Fig. 2a.

Discussion

Using LPS-hyporesponsive TLR4-mutant C3H/HeJ and their WT counterpart, it was previously shown that mRNA and protein levels of S100A8 and S100A9, *i.e.*, subunits of calprotectin, in the SMG and PG are increased by treatment with LPS (Javkhlan et al., 2011). This increase is mostly mediated via TLR4, but a small increase was also noticed in S100A8 and S100A9 mRNA levels in response to LPS in the salivary glands of even C3H/HeJ mice. It is well known that C3H/HeJ mice bear a point mutation in their TLR4 gene, leading to the replacement of a proline with a histidine at position 712 in the TLR4 protein molecule (Poltorak

et al., 1998). This mutation then causes dysfunction of TLR4. Yet, it cannot be ruled out whether genes other than TLR4 are not also affected in C3H/HeJ mice. In the present study, we employed TLR4-KO and their WT mice in order to avoid unnecessary confusion due to unanticipated gene mutation that might occur in C3H/HeJ mice. As shown by the TLR4 amplification experiment, the lack of TLR4 mRNA in the TLR4-KO mice and its expression in the WT ones (C57BL/6 strain) were confirmed (Fig. 2); thus the differences observed between WT and TLR4-KO mice were obviously mediated via TLR4. In the SMG and PG of TLR4-KO and control WT mice, LPS consistently increased the mRNA and protein levels of S100A8 and S100A9, supporting the previous data (Javkhlan et al., 2011).

Here, we focused on the NLRs, particularly, Nod1 and Nod2, in the salivary gland in relation to the S100A8/A9 induction, since one of them, Nod2, in macrophages is reported to respond to LPS and to activate NF-κB similarly as signaling via TLR4 (Pauleau and Murra, 2003). In the present study, we found for the first time that 1) Nod2 mRNA and protein were expressed in the SMG, one of the major salivary glands. 2) LPS strongly induced the Nod2 mRNA and protein in WT but not in TLR4-KO mice, indicating that this endotoxin activated Nod2 transcription via the TLR4 signaling in the salivary gland. CHX actually inhibited LPS-induced elevation of the Nod2 protein in the SMG in WT mice. Also, 3) levels of both mRNA and protein of Nod2 in this tissue were significantly higher in the TLR4-KO mice than those in WT mice.

In the SMG of WT mice, marked elevations of mRNA levels for S100A8 and S100A9 elicited by LPS were significantly blocked by the presence of CHX; these changes and changes in the levels of Nod2 mRNA/protein were in parallel. These results suggest that Nod2, increased in expression by LPS, may have activated the transcription of mRNAs of S100A8 and S100A9. The fact implies a possible involvement of Nod2 protein in the induction of S100A8/S100A9 mRNAs by LPS. Although this implication is still preliminary and needs more experiments using a specific inhibitor of Nod2 or using Nod2-KO animals, the present study tentatively proposes the hypothesis regarding the relation between Nod2 and S100A8/A9 in response to LPS stimulation.

In the SMG of TLR4-KO mice a higher level of Nod2 was expressed than that observed in WT mice. This fact is well supported by the finding that macrophages or mice made insensitive to TLRs potentially respond to Nod2 stimulation (Kim et al., 2008). Also, the Nod2 protein level was not altered by CXH treatment in the SMG of TLR4-KO mice, implying the constitutively high expression of Nod2 in these mice. Therefore, regardless of the presence or absence of CHX, the low level-elevation of the mRNAs for S100A8 and S100A9 caused by LPS may be elucidated to have occurred via Nod2. The hypothesis that LPS directly activates Nod2 needs to be verified using TLR4-KO mice.

Another issue that has arisen in the present study is the effects of CHX on the S100A8 and S100A9 mRNA levels in the SMG. In both

TLR4-KO and WT mice, CHX itself elevated the mRNA levels for S100A8 and S100A9. The reason for this increase is unclear at present; but we hypothesize the presence of some constitutively expressed putative suppressor of S100A8 and S100A9 transcription with short half life (as the effect was seen in 3 h), the synthesis of which was inhibited by CHX. This way, the elevation by CHX of S100A8/A9 mRNAs in the SMG in WT and TLR4-KO mice (Fig. 3) could be well elucidated.

Lastly, in TLR4-KO mice, a higher level of Nod2 is expressed, and though at small degree, the S100A8 and S100A9 mRNA are induced by LPS. Nod2 seems to play a pivotal role in the LPS signaling in the salivary gland as well in the case where the TLR4 pathway is insensitive. Thus, calprotectin hetero subunits, S100A8 and S100A9, in the SMG seemed to have increased in

expression by LPS via the Nod2 signaling pathway in addition to the TLR4 one. In WT mice, having both of these signaling pathways, the S100A8 and S100A9 mRNA inductions are potentiated in this tissue.

We conducted the present experiment by using CHX to block Nod2 protein synthesis. In our present study, CHX successfully inhibited Nod2 protein elevation in the WT mice, though the inhibitor would not be specific to Nod2 protein synthesis. Before drawing final conclusions, it would be important to confirm our present data by performing experiments using Nod2-specific inhibitors (Huang et al., 2008; Bielig et al., 2010) and such inhibitors are currently under investigation in our laboratory.

Acknowledgement

The authors are grateful to Dr. Makoto Fukui, Department of Preventive Dentistry, Institute of Health Biosciences, The University of Tokushima Graduate School, for his help in the statistical analysis.

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