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Role of transient receptor potential vanilloid 2 in LPS-induced cytokine production in macrophages

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ABSTRACT

There is considerable evidence indicating that intracellular Ca^{2+} participates as a second messenger in TLR4-dependent signaling. However, how intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is increased in response to LPS and how they affect cytokine production are poorly understood. Here we examined the role of transient receptor potential (TRP), a major Ca^{2+} permeation pathway in non-excitable cells, in the LPS-induced cytokine production in macrophages. Pharmacologic experiments suggested that TRPV family members, but neither TRPC nor TRPM family members, are involved in the LPS-induced TNFα and IL-6 production in RAW264 macrophages. RT-PCR and immunoblot analyses showed that TRPV2 is the sole member of TRPV family expressed in macrophages. ShRNA against TRPV2 inhibited the LPS-induced TNFα and IL-6 production as well as IκBα degradation. Experiments using BAPTA/AM and EGTA, and Ca^{2+} imaging suggested that the LPS-induced increase in $[Ca^{2+}]_i$ involves both the TRPV2-mediated intracellular and extracellular Ca^{2+} mobilizations. BAPTA/AM abolished LPS-induced TNFα and IL-6 production, while EGTA only partially suppressed LPS-induced IL-6 production, but not TNFα production. These data indicate that TRPV2 is involved in the LPS-induced Ca^{2+} mobilization from intracellular Ca^{2+} store and extracellular Ca^{2+}. In addition to Ca^{2+} mobilization through the IP_3-receptor, TRPV2-mediated intracellular Ca^{2+} mobilization is involved in NFκB-dependent TNFα and IL-6 expression, while extracellular Ca^{2+} entry is involved in NFκB-independent IL-6 production.

1. Introduction

Macrophages are a central player of innate immunity and inflammation. They are capable of producing a variety of proinflammatory cytokines such as TNFα and IL-6 following Lps stimulation [1,2]. Intracellular signaling pathways downstream of TLR4 stimulation by LPS have been extensively studied [1,2]. Upon LPS stimulation, TLR4 initiates a series of NFκB- and MAPK-associated intracellular signaling events, thereby inducing the expression of an array of proinflammatory cytokine genes.

Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) or Ca^{2+} fluxes are important for cellular responses to extracellular stimuli [3,4]. There is considerable evidence that intracellular Ca^{2+} participates as a second messenger in TLR4-dependent signaling [5]. Indeed, treatment with LPS causes a transient increase in $[Ca^{2+}]_i$, which is required for increased TNFα production in macrophages [6]. However, the molecular mechanisms by which LPS increases $[Ca^{2+}]_i$ and how the LPS-induced Ca^{2+} fluxes modulates proinflammatory cytokine production are still poorly understood.

Transient receptor potential (TRP) channels are a group of Ca^{2+}-permeable channels with diverse activation properties including temperature, pH changes, ADP-ribose, and diacylglycerol [7]. TRP channels are suggested to be an important Ca^{2+} entry pathway in non-excitable cells [7]. For instance, TRP vanilloid 4 (TRPV4)- and TRPV5-mediated Ca^{2+} influx is essential for terminal differentiation of osteoclasts and osteoclastic bone resorption [8,9]. Activation of the TRP melastatin 8 (TRPM8) variant in human lung epithelial cells also leads to increased expression of several cytokine and chemokine genes [10]. TRPV1 is expressed primarily in sensory nerves

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and protects against the onset of LPS-induced sepsis [11]. Reactive oxygen species-induced chemokine production has been shown to be mediated through TRPM2-mediated Ca^{2+} influx in monocytes/macrophages [12]. However, whether TRP channels are involved in the LPS-induced Ca^{2+} mobilization in macrophages has not been addressed.

TRPV2 is a Ca^{2+} permeable channel originally identified in rat brain and human myeloid cell line, CCRF-CEM [13]. Here we provide *in vitro* evidence that TRPV2 is involved in LPS-induced Ca^{2+} mobilization and induction of cytokines in RAW macrophages. Our data will also help elucidate the molecular mechanisms underlying the LPS-induced cytokine production in macrophages and thus identify the therapeutic targets that may prevent or treat a variety of acute and chronic inflammatory diseases.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) or Nacalai Tesque (Kyoto, Japan). Rabbit mAbs against IκBα, and phospho-IκBα (Ser32) (p-IκBα) were purchased from Cell Signaling (Beverly, MA), and a rabbit polyclonal Ab against mouse TRPV2 (mTRPV2) from Abcam Inc. (Cambridge, MA).

2.2. Cell culture and transfection

A macrophages cell line RAW264 (RIKEN BioResource Center, Tsukuba, Japan), and HEK293T were maintained in DMEM (Nacalai Tesque, Kyoto, Japan) containing 10% FBS (BioWest, Miami, FL). To produce HEK293T cells expressing TRPV2, pcMV6-Kan/Neo-mTRPV2 (OriGene Technologies, RockVille, MD) was transfected by Lipofectamine™2000 (Invitrogen Life Technologies, CA). Murine bone marrow-derived macrophages were prepared as described [14].

2.3. Lentivirus short hairpin RNA (shRNA) vector construction and gene transduction

The construction of the shRNA for TRPV2 (NM_011706), and gene transduction were carried out as previously reported [15]. The sense and antisense oligonucleotides used are shown in Supplementary Table 1. Lentivirus-infected cells were detected as GFP-positive. After 4 weeks of selection, over 90% of cells were GFP-positive and used for further analysis.

2.4. Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR was performed with SYBR Green PCR Master MixReagent Kit (Applied Biosystems, Foster City, CA) [16]. The primer sets used to detect each of TRPV members are shown in Supplementary Table 2.

2.5. Measurement of TNFα and IL-6 protein levels in culture media

The TNFα and IL-6 protein levels were determined by the commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN).

2.6. Immunoblot analysis

Immunoblot analysis was performed as described [16]. Membranes were immunoblotted with one of following primary Abs; p-IκBα (1:1000), IκBα (1:2000), α-tubulin (1:16,000), or mTRPV2 (1:500).

2.7. Measurement of $[Ca^{2+}]_i$ concentration

Measurement of $[Ca^{2+}]_i$ was performed with epifluorescent microscopy (Hamamatsu Photonics, Hamamatsu, Japan) as previously described [17]. Calibration between fluorescence ratio and Ca^{2+} concentration was performed *in situ* as described [18].

2.8. Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effect of TRP channel blockers on the LPS-induced cytokine production in RAW264 and murine macrophages

Macrophages express several TRP channels including TRPC, TRPM, and TRPV family members [12,19]. To explore if TRP family members play a role in the LPS-induced inflammatory response, and if so, which TRP channel(s) are involved, we first used ruthenium red (RR), a non-selective TRP channel blocker [20], Gadolinium (Gd), a TRPC channel blocker at a low concentration (30 μM) and a TRPC and a TRPV channel blocker at a high concentration (1 mM) [20,21], and flufenamic acid (FFA), a TRPC and TRPM channel blocker [21,22]. Treatment with LPS at a dose of 5 ng/ml significantly increased TNFα and IL-6 mRNA expression ($P < 0.01$), which was markedly inhibited by RR (10 μM) (Fig. 1A). Treatment with Gd at a dose of 30 μM did not affect the LPS-induced TNFα and IL-6 mRNA expression (Fig. 1B), but, at a dose of 1 mM, it significantly inhibited the LPS-induced IL-6 mRNA expression ($P < 0.01$), but not TNFα mRNA expression (Fig. 1C). FFA (100 μM) did not affect the LPS-induced TNFα and IL-6 mRNA expression (Fig. 1D). Similarly, treatment with RR (10 μM) significantly suppressed the LPS-induced TNFα and IL-6 secretion ($P < 0.01$), while treatment with Gd at 1 mM inhibited the LPS-induced IL-6 secretion ($P < 0.01$), but not TNFα secretion (Supplementary Fig. 1).

We also examined effects of RR and Gd on the LPS-induced cytokine secretion from murine bone marrow-derived macrophages. RR (10 μM) significantly suppressed the TNFα and IL-6 secretion stimulated by LPS (10 ng/ml) for 6 h and 24 h ($P < 0.01$) (Fig. 1E). Gd (1 mM) suppressed IL-6 ($P < 0.05$), but not TNFα, secretion stimulated by LPS for 24 h (Fig. 1F).

These observations suggest that TRPV family members, but neither TRPC nor TRPM, are involved in the LPS-induced TNFα and IL-6 production in macrophages. Since the effects of RR and Gd are more prominent in RAW264, we used RAW264 in the following experiments.

3.2. Expression of TRPV2 in RAW264 macrophages

We examined expression of mRNAs for TRPV family members (TRPV1∼6) in RAW264 macrophages. RT-PCR analysis revealed that TRPV2 mRNA is expressed in RAW264 macrophages (Fig. 2A). There were no appreciable amounts of mRNAs for TRPV1, 3, 4, 5, and 6. We examined TRPV2 protein expression in RAW264 macrophages. Immunoblot analysis identified two immunoreactive bands at ∼110 and at ∼85 kD (Fig. 2B), which may be the products of post-translational modification such as glycosylation and phosphorylation [23]. Expression of TRPV2 protein in RAW264 macrophages was further confirmed by the positive immune-staining with an anti-TRPV2 antibody in immune-cytochemical studies (Supplementary Fig. 2).

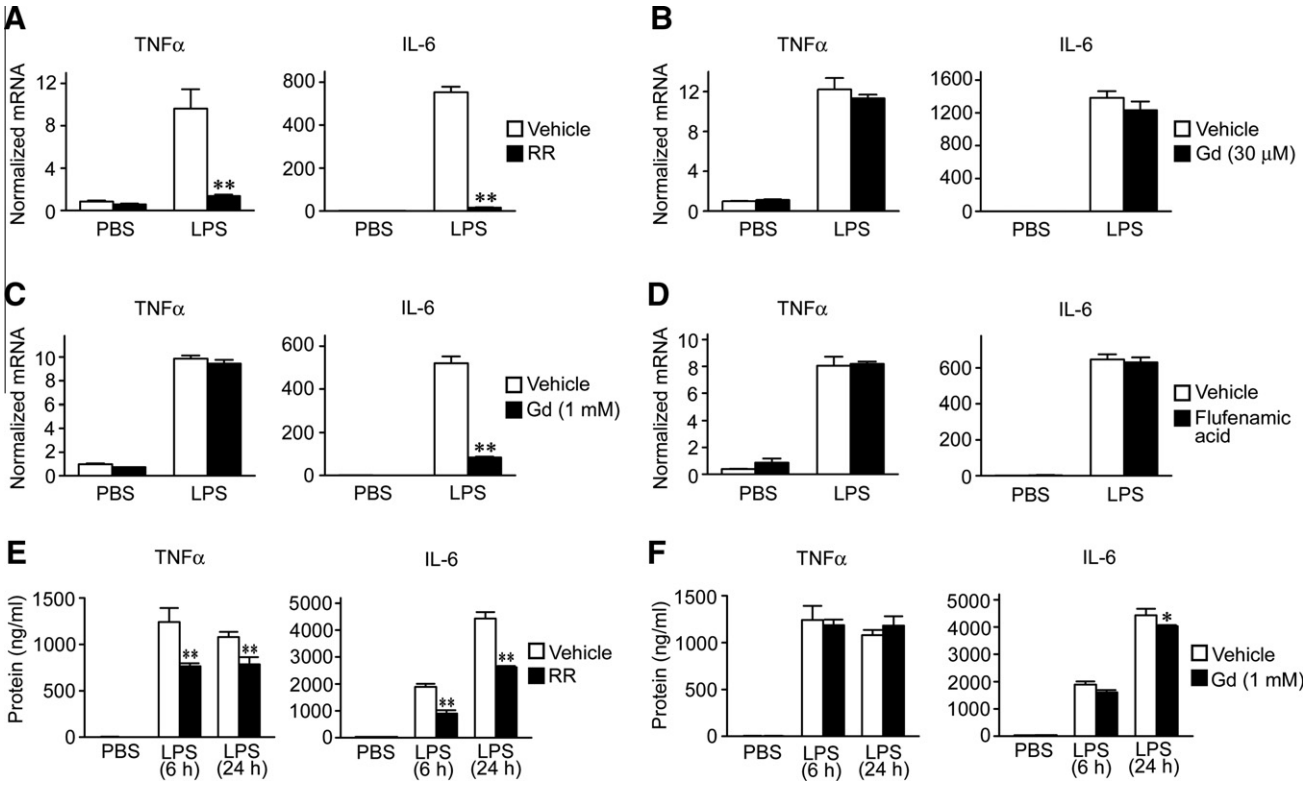

Fig. 1. Effect of TRP channel blockers on the LPS-stimulated cytokine mRNA expression in RAW264 macrophages and cytokine protein secretion from murine bone marrow macrophages. (A–D) Effect of pretreatments with RR at 10 μM (panel A), Gd at 30 μM (panel B), Gd at 1 mM (panel C), and flufenamic acid at 100 μM (panel D) on the LPS-induced TNFα (left panel) and IL-6 (right panel) mRNA expression. In this and following figures, data for mRNA expression were normalized to the value for 36B4 as an internal control, and expressed relative to the control value (vehicle application and no LPS stimulation). LPS; 5 ng/ml for 4 h. Data are mean of 4 experiments. *: $P < 0.05$, **: $P < 0.01$. E, F, Effect of pretreatments with RR at 10 μM (panel F) and Gd at 1 mM (panel G) on the LPS-induced TNFα (left panel) and IL-6 (right panel) protein secretion. LPS; 10 ng/ml for 6 h and 24 h. Data are mean of 4 experiments. *: $P < 0.05$, **: $P < 0.01$.

3.3. Effect of TRPV2 knock-down on the LPS-induced cytokine mRNA expression

To specifically address the role of TRPV2 in the LPS-induced TNFα and IL-6 production, we used the knock-down strategy with shRNA against TRPV2. RT-PCR and immunoblot analysis confirmed that both TRPV2 mRNA and protein levels are reduced in RAW264 macrophages transfected with shRNA for TRPV2 (V2KD-RAW macrophages), but not in those with scramble RNA (Scr-RAW macrophages) (Fig. 2C and D). Similar to wild-type RAW264 macrophages, LPS treatment (10 ng/ml) significantly increased TNFα and IL-6 mRNA expression in Scr-RAW macrophages ($P < 0.01$). In V2KD-RAW macrophages, the LPS-induced TNFα and IL-6 expression was significantly reduced (Fig. 2E). Collectively, these observations suggest that TRPV2 is crucially involved in the LPS-induced TNFα and IL-6 production in macrophages.

3.4. Role of TRPV2 in NFκB signaling pathway

We next examined the role of TRPV2 in NFκB signaling pathway, since it is the major pathway for LPS-induced cytokine production. Treatment with LPS-induced degradation of IκBα, which persisted for 60 min. Knock-down of TRPV2 significantly inhibited IκBα degradation (Fig. 3A and B). Treatment with RR (10 μM) abolished IκBα degradation, whereas Gd (1 mM) had no effect (Fig. 3A and B).

3.5. Role of TRPV2 on the LPS-induced $[Ca^{2+}]_i$ mobilization

We measured changes in $[Ca^{2+}]_i$ with a Ca^{2+} indicator Fura-2 to examine if TRPV2 is indeed involved in the LPS-induced intracellular Ca^{2+} mobilization. Treatment of RAW264 macrophages with LPS (5 ng/ml) induced a significant increase in $[Ca^{2+}]_i$ from 168 ± 26 nM to 661 ± 60 nM in 40 min ($n = 19$, $P < 0.01$). In V2KD-RAW macrophages, but not in Scr-RAW macrophages, the LPS-induced increase in $[Ca^{2+}]_i$ was suppressed (Fig. 4A). We also examined the effects of a TRPV2 channel blockers. RR (10 μM) and Gd (1 mM) significantly suppressed the LPS-induced increase in $[Ca^{2+}]_i$: from 142 ± 16 nM to 287 ± 23 nM for RR ($n = 18$, $P < 0.01$), and 135 ± 12 nM to 225 ± 14 nM for Gd ($n = 19$, $P < 0.01$) (Fig. 4A).

3.6. Role of Ca^{2+} mobilization in cytokine induction and IκBα degradation

To explore the role of Ca^{2+} mobilization in cytokine production, we first used BAPTA/AM and EGTA. When BAPTA/AM permeates into the cell, it is converted to BAPTA by intracellular esterase, and BAPTA chelates intracellular Ca^{2+}, while EGTA cannot permeate through the plasma membrane, thereby chelating extracellular Ca^{2+}. Intracellular Ca^{2+} removal by BAPTA suppressed the LPS-induced TNFα and IL-6 mRNA expression (Fig. 4B). On the other hand, pre-incubation with EGTA had no effect on TNFα mRNA expression, but partially suppressed IL-6 mRNA expression ($P < 0.01$) (Fig. 4C). We also examined the involvement of intracellular Ca^{2+} mobilization through ryanodine receptor and IP_3 receptor in the LPS-induced cytokine mRNA expression. In this study, dantrolene (30 μM), a ryanodine receptor inhibitor, did not inhibit the LPS-induced TNFα or IL-6 mRNA expression (Fig. 4D). On the other hand, Xestospondin C (20 μM), an IP_3 receptor inhibitor, partially inhibited the TNFα mRNA induction, and almost completely inhibited the IL-6 mRNA induction (Fig. 4E).

BAPTA/AM treatment markedly suppressed LPS-induced IκBα degradation, while EGTA had no effect (Supplementary Fig. 3).

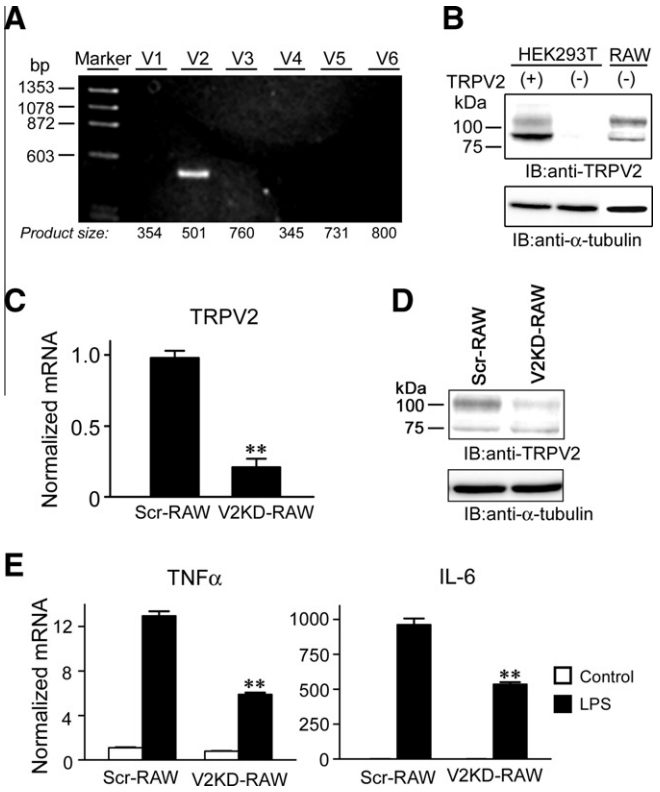

Fig. 2. Expression of TRPV channels and effects of TRPV2 knock-down on the LPS-stimulated cytokine mRNA production in RAW264. (A) Expression of mRNA for TRPV1∼6 channels in RAW264 macrophages was analyzed with RT-PCR. (B) Expression of TRPV2 protein in RAW264 macrophages was examined with Western blot analysis. Left 2 lanes are data for HEK293T cells transfected with or without mTRPV2. (C) Effect of infection with lentivirus-scramble RNA (Scr-RAW) or lentivirus-shTRPV2 (V2KD-RAW) on TRPV2 mRNA expression in RAW264 macrophages. **: $P < 0.01$. (D) Effect of infection of lentivirus-scramble RNA (Scr-RAW) or lentivirus-shTRPV2 (V2KD-RAW) on TRPV2 protein expression in RAW264 macrophages. (E) Effect of TRPV2 knock-down on the LPS-induced cytokine mRNA expression. LPS; 10 ng/ml for 4 h. Data are mean of 4 experiments. *: $P < 0.05$, **: $P < 0.01$.

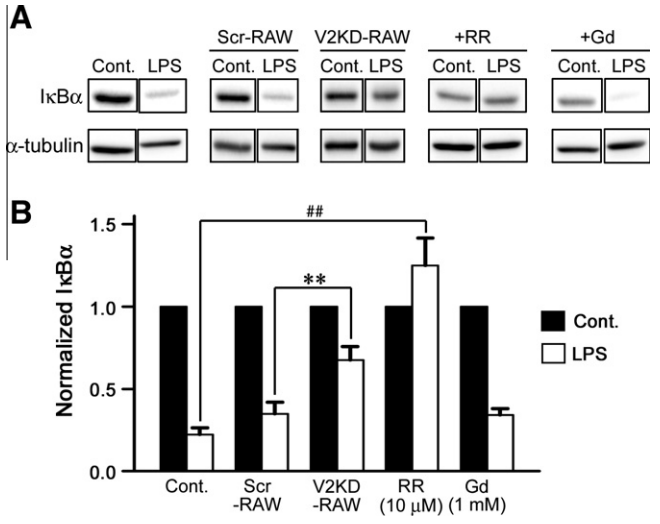

Fig. 3. Effect of TRPV2 knock-down and blockers on the LPS-induced IκBα degradation. (A) Representative data of Western blot analysis against IκBα, and α-tubulin as an internal control. Data were obtained just before (0 min) and 60 min after LPS treatment (5 ng/ml). (B) Densitometric analysis of 4–5 experiments. Density values for IκBα were normalized by that for α-tubulin, and expressed as a relative to the value just before LPS treatment (0 min). **: $P < 0.01$ vs. Scr-RAW; ##: $P < 0.01$ vs. control.

4. Discussion

Macrophages are capable of producing a variety of proinflammatory cytokines such as TNFα and IL-6 [2]. Upon Lps stimulation, TLR4 initiates a series of intracellular signaling pathways involving NFκB, thereby inducing an array of cytokine production [3,4]. Because LPS increases $[Ca^{2+}]_i$, which is shown to be required for increased cytokine production in macrophages [6,24], it is likely that intracellular Ca^{2+} participates as a second messenger in TLR4-dependent signaling [5,6]. However, how LPS increases $[Ca^{2+}]_i$ in macrophages are poorly understood. There is considerable evidence that TRP channels are an important Ca^{2+} entry pathway in non-excitable cells [7]. This study was, therefore, designed to elucidate the potential role of TRP channels in the LPS-induced Ca^{2+} fluxes and cytokine production in macrophages.

Pharmacologic experiments suggested that the LPS-induced TNFα and IL-6 production is markedly inhibited by RR, a non-selective TRP channel blocker. Moreover, treatment with Gd at the higher dose sufficient to block TRPV channels efficiently suppressed the LPS-induced IL-6 mRNA expression, which was unaffected at the lower dose. It is, therefore, likely that TRPV family members are involved in the LPS-induced TNFα and IL-6 production in macrophages. RT-PCR and immunoblot analyses revealed that TRPV2 mRNA and protein are expressed in RAW264 macrophages, where no appreciable amounts of mRNA for other TRPV family members are detected, which is consistent with previous reports [19,25]. Importantly, the LPS-induced TNFα expression and IL-6 expression were significantly reduced in V2KD-RAW246 macrophages relative to Scr-RAW macrophages. These observations suggest that TRPV2 is crucially involved in the LPS-induced TNFα and IL-6 production in macrophages.

Intracellular Ca^{2+} participates as a second messenger in TLR4-dependent signaling [5]; the LPS-induced transient increase in $[Ca^{2+}]_i$ is required for cytokine increase in macrophages [6]. Knock-down experiments suggest that TRPV2 is involved in LPS-induced $[Ca^{2+}]_i$ increase in RAW macrophages. BAPTA/AM chelates intracellular Ca^{2+}, while EGTA chelates extracellular Ca^{2+}. Treatment with BAPTA/AM abolishes both the LPS-induced TNFα and IL-6 production, while EGTA only inhibits the LPS-induced IL-6 production. BAPTA/AM inhibited LPS-induced IκBα degradation, EGTA had no effect on the LPS-induced IκBα degradation. A lipophilic compound RR can enter into the cell, and can inhibit TRPV2 localized intracellularly, while a cationic blocker Gd cannot permeate cell membrane and can inhibit TRPV2 localized on surface membrane. Thus, the data on BAPTA/AM and EGTA appear to be consistent with the finding that RR inhibits both TNFα and IL-6 production, while Gd only inhibits IL-6 production. Since RR, but not Gd, inhibited LPS-induced IκBα degradation, NFκB- and IκBα-dependent pathway may require intracellular Ca^{2+} mobilization, but not extracellular Ca^{2+} flux. Collectively, intracellular Ca^{2+} mobilization is crucial for NFκB-dependent TNFα and IL-6 expression, while extracellular Ca^{2+} entry is important in NFκB-independent IL-6 expression, and for both pathways, TRPV2 plays a role.

We also examined the involvement of 2 major intracellular Ca^{2+} mobilization pathways, ryanodine receptor and IP_3 receptor. In this study, a ryanodine receptor inhibitor dantrolene does not affect the LPS-induced TNFα or IL-6 mRNA expression. Thus, although RR is a potent inhibitor of ryanodine receptor [26], it is unlikely that ryanodine receptor contributes to the inhibitory effect of RR on the LPS-induced cytokine mRNA expression. In contrast, an IP_3 receptor inhibitor Xestospongin C partially inhibits the LPS-induced TNFα mRNA expression, and almost completely inhibits the LPS-induced IL-6 mRNA expression. Thus, intracellular Ca^{2+} mobilization through both IP_3 receptor and TRPV2 appears to play a role in the LPS-induced TNFα mRNA expression. Relative contribution of

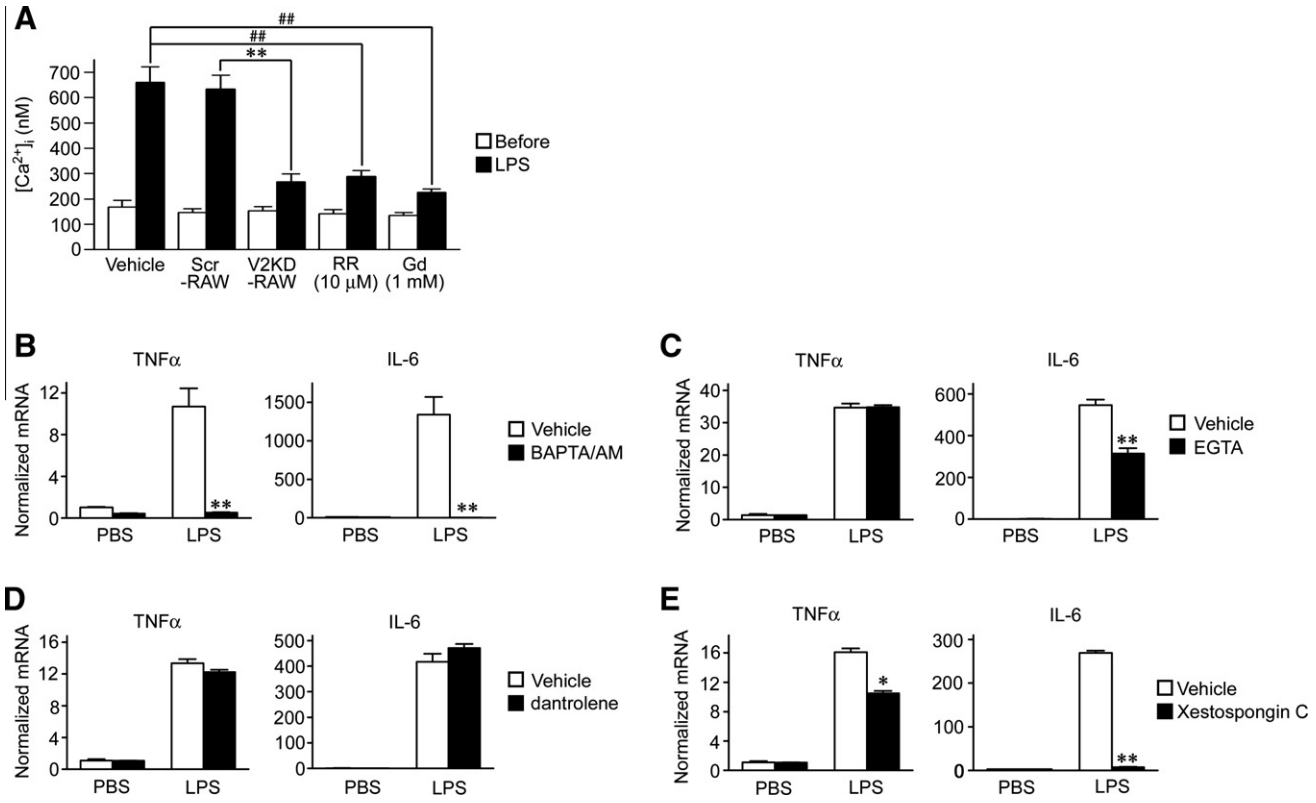

Fig. 4. Effect of shRNA and TRPV2 blockers on the LPS-induced in increase $[Ca^{2+}]_i$, and effect of Ca^{2+} removal and inhibition of intracellular Ca^{2+} mobilization on the LPS-induced cytokine mRNA expression A, $[Ca^{2+}]_i$ was measured with Ca^{2+} indicator Fura-2 before (filled bars) and 40 min (open bars) after LPS (5 ng/ml) application to RAW264 macrophages. Data are mean ± SEM of 18 or 19 experiments. **: P < 0.01 vs Scr-RAW; ##: P < 0.01 vs vehicle. B, C, D, E, Effects of removal of intracellular Ca^{2+} with BAPTA/AM (50 μM) (panel B), extracellular Ca^{2+} with EGTA (5 mM) (panel C), and inhibition of intracellular Ca^{2+} mobilization through a ryanodine receptor with dantrolene (30 μM) (panel D), and through an IP_3 receptor with Xestospongin C (20 μM) (panel E) on the LPS-induced TNFα (left panel) and IL-6 (right panel) mRNA expression. LPS; 5 ng/ml for 4 h. Data are mean of 4 experiments. *:P < 0.05, **:P < 0.01.

IP_3 receptor and TRPV2 to the LPS-induced TNFα mRNA expression and their relationship (additive or synergistic) remain unclear. In this study, BAPTA/AM and Xestospongin C completely inhibits the IL-6 mRNA induction, and EGTA and Gd partially inhibits it. These observations may imply that the LPS-induced IL-6 production is caused at least in part by Ca^{2+} mobilization solely from intracellular source and partly by entry of extracellular Ca^{2+} through TRPV2, which is triggered by intracellular Ca^{2+} mobilization through IP_3 receptor, a mechanism similar to the store-operated (SOC) or the receptor-operated Ca^{2+} entry (ROC) in TRPC channels [27,28]. The preliminary immune-cytochemical experiments confirm the presence of immune-positive staining both inside the cell and on the plasma membrane (Supplementary Fig. 2). Immuno-positive staining is present mainly inside the cell before Lps stimulation, and on the plasma membrane after LPS stimulation (Supplementary Fig. 2); however, the mechanism, kinetics, and implication of the TRPV2 transport are not addressed in this study and require further examination.

Recent paper using TRPV2 KO mice showed that TRPV2 has a pivotal role in macrophage particle binding and phagocytosis, without a significant role in inflammatory cytokine induction [29]. This effect was dependent on Na^+ influx through TRPV2, while in our experiments Ca^{2+} mobilization through TRPV2 appears to be crucial for LPS-induced cytokine induction. The different finding between their study and ours might be attributable to different experimental conditions: they stimulated murine peritoneal macrophages with LPS at 0.1 or 0.5 μg/ml for 24 h, while we stimulated RAW264 with LPS at 5 or 10 ng/ml for 4 h. To make experimental protocol comparable, we stimulated murine bone marrow-derived macrophages with LPS (10 ng/ml) for 6 and 24 h. Consistent with our experiments in RAW264 cells, RR suppressed TNFα and IL-6 secretion, and Gd (1 mM) suppressed IL-6 secretion. Thus, LPS may have different actions at low (5–10 ng/ml) and high (0.1–0.5 μg/ml) concentrations. To test this possibility, further studies are required.

5. Conclusion

This study represents the first demonstration that TRPV2 constitutes Ca^{2+} permeation pathways involved in the LPS-induced cytokine production in macrophages. Given the pathophysiologic role of TLR4 signaling [14,30], our data will also help identify the therapeutic targets that may prevent or treat a variety of inflammatory diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.082.

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