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Suppression of TRIF-dependent signaling pathway of toll-like receptors by (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine

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ABSTRACT

Toll-like receptors (TLRs) play an important role in the recognition of microbial pathogens and induce innate immune responses. The recognition of microbial components by TLRs triggers the activation of myeloid differential factor 88 (MyD88)- and toll-interleukin-1 receptor domain-containing adapter inducing interferon-β (TRIF)-dependent downstream signaling pathways. Previously, we synthesized (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine (NVPP), which contains a nitrovinyl-phenyl and pyrrolidine. To evaluate the therapeutic potential of NVPP, its effect on signal transduction via the TRIF-dependent pathway of TLRs induced by lipopolysaccharide (LPS) or polyinosinic-polycytidylic acid (poly[I:C]) was examined. NVPP inhibited LPS or poly[I:C]-induced activation of nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3), and the phosphorylation of IRF3, as well as inhibiting the activation of interferon-inducible genes such as interferon inducible protein-10 (IP-10). These results suggest that NVPP can modulate TRIF-dependent signaling pathways of TLRs, potentially resulting in effective therapeutics for chronic inflammatory diseases.

1. Introduction

Innate immunity is considered to be essential for host defense against invading microbial pathogens. Pattern recognition receptors (PRRs), which are germ-line encoded receptors, play an important role in the innate immune reaction and adaptive immune responses by recognizing pathogen associated-molecular patterns (PAMPs) (Bjorkbacka et al., 2004; Medzhitov et al., 1997). The best studied PRRs are Toll-like receptors (TLRs). Activation of a TLR by its PAMP induces several intracellular signaling cascades, resulting in the production of cytokines, chemokines and the transcription of other genes important for host defense.

Broadly speaking, signaling through TLRs can be divided into two pathways leading to the recruitment of one or more Toll/interleukin (IL)-1 Receptor (TIR) domain-containing adapter molecules, namely myeloid differentiation factor 88 (MyD88) and Toll/IL-1R domain containing adapter inducing ineterferon-β (TRIF) (O'Neill and Bowie, 2007). These adapters facilitate the activation of the MyD88- and TRIF-dependent pathways. While the activation of TLR1, TLR2, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR11 with their ligands recruits MyD88, the activation of TLR3 with its ligand recruits TRIF. The activation of TLR4 recruits both MyD88 and TRIF (Takeda and Akira, 2005). This recruitment of adapter molecules triggers the cascade of a signaling pathway and the activation of transcription factor such as nuclear factor-κB (NF-κB) and IFN regulatory factor 3 (IRF3), leading to the induction of inflammatory cytokines, chemokines, and type I IFNs (Takeda and Akira, 2005).

All members of the TLR family, except for TLR3, signal inflammation via a conserved canonical pathway (Takeda and Akira, 2005). This pathway is initiated by a conserved cytosolic protein sequence termed the TIR domain, which activates signaling mediators including IL-1-associated kinase (IRAK)-1 and IRAK-4, TRAF-6, mitogen-activated protein kinases (MAPK) and IκB kinases, and leads to activation of the prototypic inflammatory transcription factor, NF-κB. This signaling pathway induces the expression of pro-inflammatory gene products, including cytokines (Akira et al., 2006).

In addition to the proinflammatory signals, some TLRs recruit Mal, TRAM and TRIF, giving rise to specificity in signaling (Creagh and O'Neill, 2006). TRIF has been shown to be critical for signaling by TLR4 and TLR3, while TRAM was shown to be required for signaling by TLR4 only. TRIF induces to the activation of IRF3 and the expression of IFN-inducible genes such as IFN-β and regulated on activation normal T-cell expressed and secreted (RANTES) (Fitzgerald et al., 2003; Gao et al., 1998; Kawai et al., 2001).

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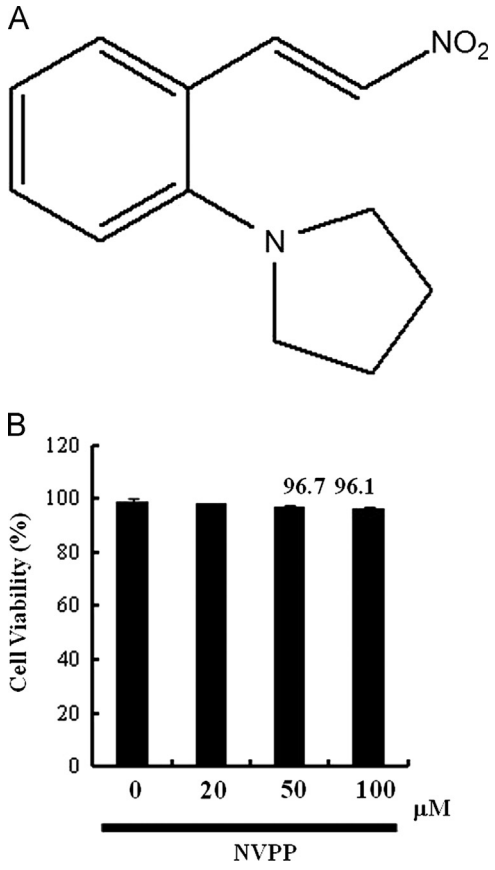

Fig. 1. (A) The structure of (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine (NVPP). (B) RAW264.7 cells were treated with NVPP (20, 50, 100 μM) for 4 h. Twenty microliters of the CellTiter 96 AQueous One Solution Reagent was added directly to culture wells. The plate was incubated at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. The absorbance was recorded at 490 nm with a 96-well plate reader. Veh, vehicle; NVPP, (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine.

A (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine (NVPP) (Fig. 1A), which contains a nitrovinyl-phenyl and pyrrolidine, is synthesized in our laboratory to use as a fundamental building block for preparation of biologically valuable molecules. Previously, we reported that NVPP inhibits the MyD88-dependent signaling pathway of TLRs (Eom et al., 2013). NVPP also suppressed NF-κB activation and inducible nitric oxide synthase (iNOS) expression induced by lipopolysaccharide (LPS; an agonist of TLR4), and macrophage-activating lipopeptide 2-kDa (MALP-2; an agonist of TLR2 and TLR6). However, whether or not NVPP inhibits the TRIF-dependent signaling pathway of TLRs remains unknown. Therefore, the present study attempted to identify the anti-inflammatory target of NVPP in the TRIF-dependent signaling pathway of TLRs.

2. Materials and methods

2.1. Chemical synthesis

NVPP was synthesized by a modification of an established procedure (Rabong et al., 2008). An oven dried 100 mL flask containing a stirring bar, had added to it 2-(pyrrolidin-1-yl)benzaldehyde (0.875 g, 5 mmol), KF (0.192 g, 0.327 mmol), Me_2NH_2Cl (0.815 g, 10 mmol), nitromethane (11.2 mL, 203 mmol) and toluene (11 mL). The flask was equipped with Dean-Stark apparatus and was refluxed for 5 h. The solvent was removed under a reduced pressure, and the residue was diluted with CH_2Cl_2 (30 mL) and washed with H_2O (20 mL). The organic layer was dried over anhydrous Na_2SO_4, and evaporated. The residue was purified by flash chromatography (ethyl acetate/hexane = 1/20) to afford NVPP (0.9 g, 60%) as a dark-red solid. Mp 175 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.33 (d, *J* = 13.4 Hz, 1H), 7.37 (d, *J* = 13.4 Hz, 1H), 7.30–7.17 (m, 2H), 6.83–6.66 (m, 2H), 3.30–3.23 (m, 4H), 1.94-1.85 (m, 4H). ^{13}C NMR (50 MHz) δ 148.7, 137.1, 132.3, 130.1, 127.2, 116.7, 116.2, 113.5, 50.4, and 23.2.

2.2. Reagents

Purified LPS was purchased from List Biologicals (San Jose, CA, USA) and polyinosinic-polycytidylic acid (poly[I:C]) was purchased from Amersham Biosciences (Piscataway, NJ, USA). LPS and poly [I:C] were dissolved in endotoxin-free water. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.3. Cell culture

RAW 264.7 cells (a murine monocytic cell line; ATCC TIB71) and 293T human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units/ml Penicillin (Invitrogen, Carlsbad, CA, USA), and 100 μg/ml Streptomycin (Invitrogen). Cells were maintained at 37 °C in a 5% CO_2/95% air environment.

2.4. Plasmids

A NF-κB (2 ×)-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). An IFNβ PRDIII-I-luciferase reporter plasmid was a gift of Kate Fitzgerald (University of Massachusetts Medical School, Worcester, MA). Heat shock protein 70 (HSP70)-β-galactosidase (β-gal) reporter plasmid was obtained from Robert Modlin (University of California, Los Angeles, CA). IP-10-luciferase reporter construct was obtained from Daniel Hwang (University of California, Davis, CA). All DNA constructs were prepared on a large scale using EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA, USA) for transfection.

2.5. Transfection and luciferase assay

The assays were performed as described previously (Ahn et al., 2009; Youn et al., 2009). Briefly, RAW264.7 cells were co-transfected with a luciferase plasmid and HSP70-β-gal plasmid as an internal control using SuperFect transfection reagent (Qiagen) according to the manufacturer's instructions. Luciferase and β-gal enzyme activities were determined using commercial luciferase assay and β-gal enzyme systems (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized by β-gal activity.

2.6. Western blotting

The procedure was performed as previously described (Youn et al., 2006b; Yun et al., 2009). Equal amounts of extracts were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the separated proteins were electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked with phosphate-buffered saline containing 0.1% Tween 20 and 3% nonfat dry milk, and was blotted with the indicated antibodies and secondary antibodies conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, USA). The reactive bands were visualized with an enhanced chemiluminescence system (Intron, Seongnam, Korea). To reprobe with different antibodies, the membrane was stripped in 0.2 N of NaOH at room temperature for 10 min.

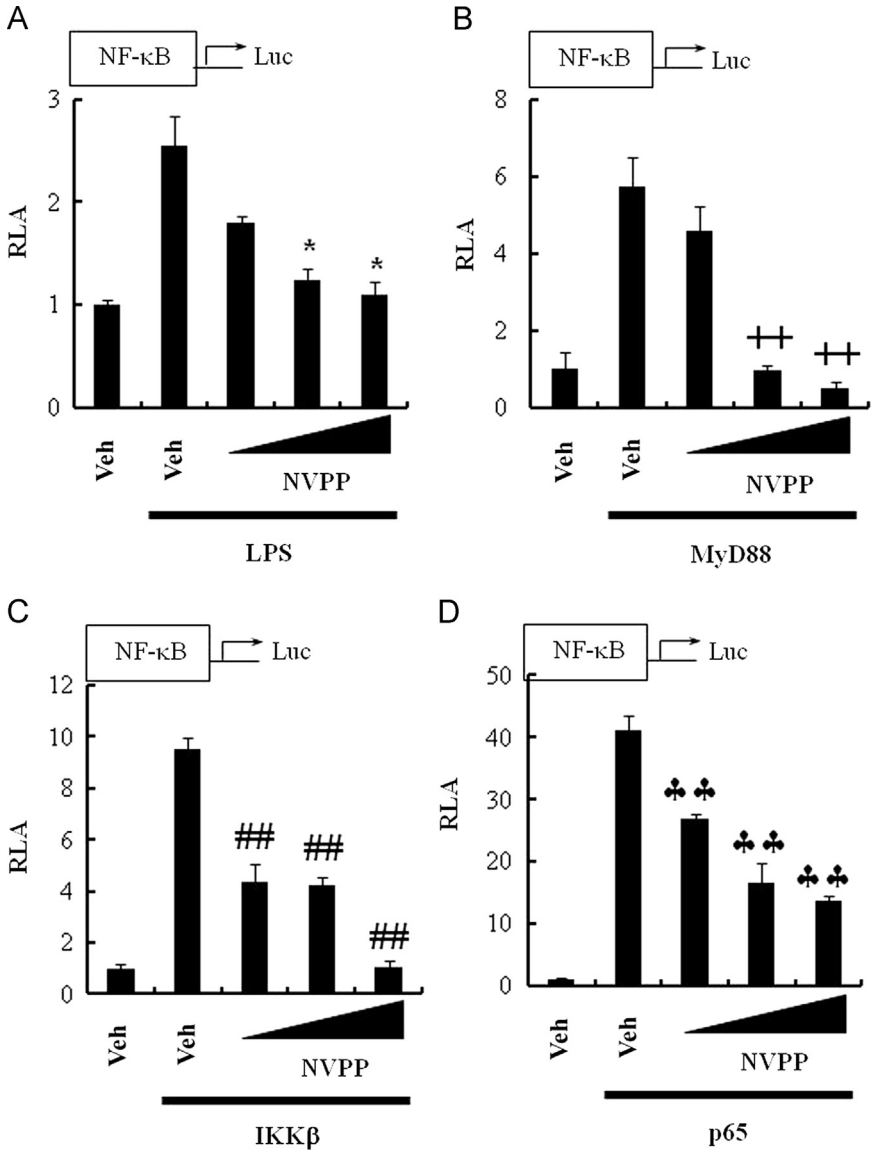

Fig. 2. NVPP suppresses MyD88-dependent signaling pathway of TLR4. (A) RAW264.7 cells were transfected with NF-κB luciferase reporter plasmid and pre-treated with NVPP (20, 50 or 100 μM) for 1 h and then treated with LPS (10 ng/ml) for an additional 8 h. Cell lysates were prepared and luciferase and β-gal enzyme activities were measured. Relative luciferase activity was normalized with β-gal activity. Values are mean ± S.E.M. ($n=3$). ∗ denotes a result that is significantly different from LPS alone (A), $P<0.05$ (#). (B–D) 293 T cells were transfected with NF-κB luciferase reporter plasmid and the expression plasmid of MyD88 (B), IKKβ (C) or p65 (D). Cells were further treated with NVPP (20, 50 or 100 μM) for 18 h. Relative luciferase activity was normalized with β-gal activity. Values represent mean ± S.E.M. ($n=3$). + denotes a result that is significantly different from MyD88 alone, $P<0.01$ (++) (B). # denotes a result that is significantly different from IKKβ alone, $P<0.01$ (##) (C). ♣ denotes a result that is significantly different from p65 alone, $P<0.01$ (♣♣) (D). Veh, vehicle; NVPP, (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine.

2.7. Cell viability test

Cell viability was assessed by using a 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) based colorimetric assay. Viability tests were performed by adding a small amount of the CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) directly to culture wells, incubating for 4 h, and then recording the absorbance at 490 nm with a 96-well plate reader.

2.8. Statistical analysis

Data were obtained from triplicate experiments. Values are expressed as the mean ± standard error of the mean (S.E.M.). Differences in the data were evaluated using Student's *t* test. A *P*-value less than 0.05 was taken as a statistically significant difference.

3. Results

3.1. NVPP suppresses MyD88-dependent signaling pathway of TLRs

To evaluate the cytotoxic nature of NVPP in RAW 264.7 cells, toxicity was determined, using a 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) based colorimetric assay. Treatment with 100 μM NVPP reduced population viability by only 4% (Fig. 1B), and therefore, all subsequent experiments utilized 100 μM NVPP.

Broadly, TLRs signaling pathways consist of MyD88- and TRIF-dependent pathways. Since both MyD88- and TRIF-dependent pathways can lead to NF-κB activation, NF-κB is the common downstream signaling component of TLRs. TLR4 triggers the activation of both MyD88- and TRIF-dependent pathways. Therefore, NF-κB activation induced by LPS (TLR4 agonist) was used as a readout for the activation of TLRs. NVPP suppressed the LPS-induced activation of NF-κB in

a dose-dependent manner, as determined by the luciferase reporter gene assay (Fig. 2A).

To further investigate the regulation of MyD88-dependent signaling pathways by NVPP, NF-κB activation was induced by the overexpression of MyD88, IKKβ, or p65 in 293T cells. NVPP suppressed the agonist-independent activation of NF-κB induced by MyD88 (Fig. 2B), IKKβ (Fig. 2C) or p65 (Fig. 2D), demonstrating that NVPP suppresses MyD88-dependent signaling pathways.

3.2. NVPP suppresses TRIF-dependent signaling pathway of TLR4

We further investigated whether NVPP could inhibit the TRIF-dependent signaling pathway of TLR4. Since TRIF-dependent signaling pathway induces the activation of transcription factor IRF3 (Fitzgerald et al., 2003), IRF3 activation was used as the readout for the TRIF-dependent pathway. NVPP inhibited LPS-induced IRF3 activation, as determined by a reporter gene assay using the IFNβ promoter domain containing the IRF3 binding site (IFNβ PRDIII-I) (Fig. 3A). NVPP also inhibited the phosphorylation of IRF3, as determined by Western blotting (Fig. 3B).

To further investigate if NVPP could modulate the TRIF-dependent pathway, the expression of genes associated with the TRIF-dependent pathways, such as IP-10, was measured by a luciferase reporter gene assay and Western blotting. NVPP suppressed LPS-induced IP-10 expression, as determined by the luciferase reporter gene assay (Fig. 3C) and by Western blotting (Fig. 3D). These results suggested that NVPP inhibits the TRIF-dependent signaling pathway derived from TLR4 activation.

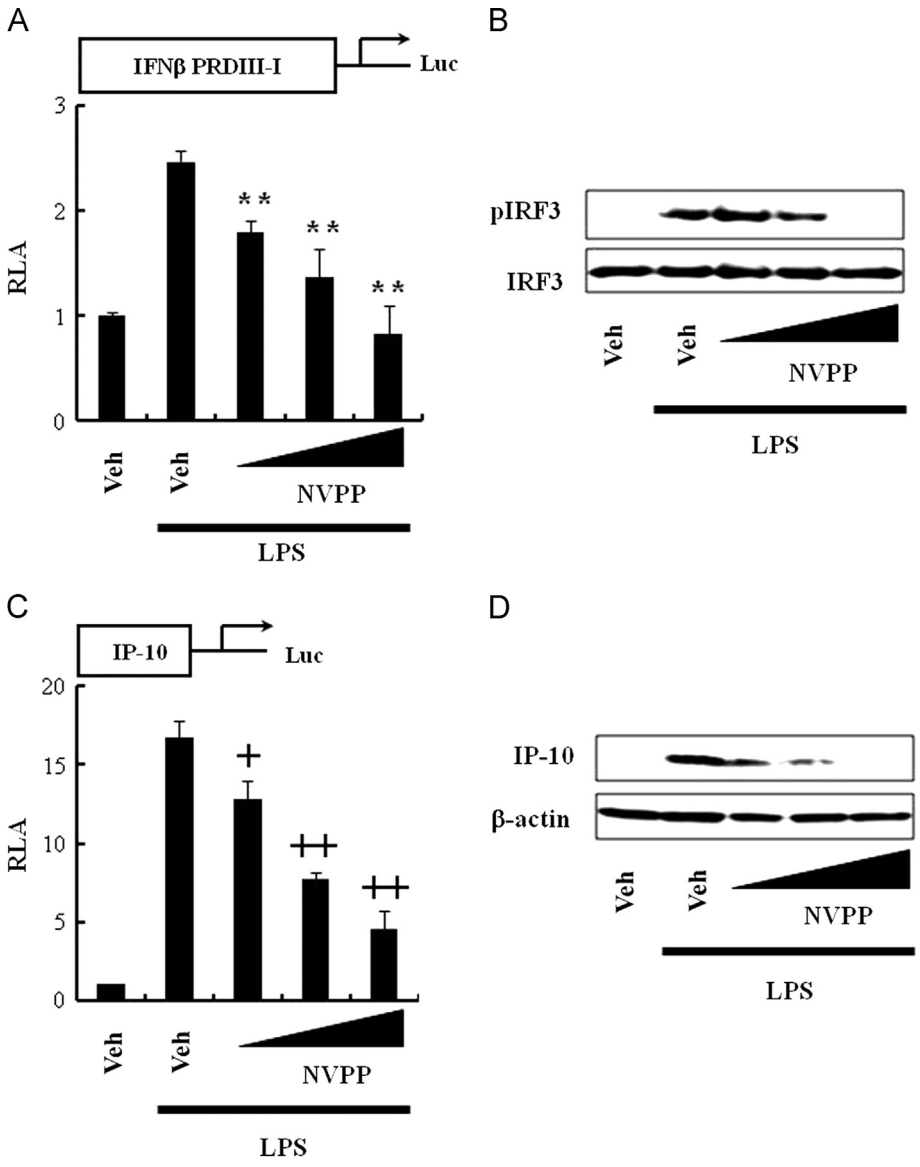

Fig. 3. NVPP suppresses IRF3 activation induced by LPS. (A) RAW264.7 cells were transfected with IRF3 binding site (IFNβ PRDIII-I) luciferase reporter plasmid and pretreated with NVPP (20, 50 or 100 μM) for 1 h and then treated with LPS (10 ng/ml) for an additional 8 h. Cell lysates were prepared and luciferase and β-gal enzyme activities were measured. Relative luciferase activity was normalized with β-galactosidase activity. Values are mean ± S.E.M. ($n=3$). ∗ denotes a result that is significantly different from LPS alone, $P<0.01$ (∗∗). (B) RAW264.7 cells were pretreated with NVPP (20, 50 or 100 μM) for 1 h, and were then further stimulated with LPS (10 ng/ml) for a further 90 min. Cell lysates were analyzed for pIRF3 and IRF3 proteins by Western blotting. (C) RAW264.7 cells were transfected with IP-10-luciferase reporter plasmid and pretreated with NVPP (20, 50 or 100 μM) for 1 h and then treated with LPS (10 ng/ml) for an additional 8 h. Cell lysates were prepared and luciferase and β-gal enzyme activities were measured. Relative luciferase activity was normalized with β-gal activity. Values represent the mean ± S.E.M. ($n=3$). + denotes a result that is significantly different from LPS alone, $P<0.05$ (+), $P<0.01$ (++). (D) RAW264.7 cells were pretreated with NVPP (20, 50 or 100 μM) for 1 h, and were then further stimulated with LPS (10 ng/ml) for a further 8 h. Cell lysates were analyzed for IP-10 and β-actin proteins by Western blotting. Veh, vehicle; NVPP, (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine.

3.3. NVPP suppresses TRIF-dependent signaling pathway of TLR3

Although the TLR4 signaling pathway can trigger the activation of transcription factors mediated through both the MyD88- and TRIF-dependent pathways, the TLR3 signaling pathway triggers the activation of transcription factors only through the TRIF-dependent pathway. Therefore, induction of NF-κB and IRF3 activation by poly[I:C] can be used as a readout for the TRIF-dependent pathway. NVPP inhibited poly[I:C]-induced NF-κB and IRF3 activation, as determined by a luciferase reporter gene assay (Fig. 4A and B). NVPP also suppressed poly[I:C]-induced IP-10 expression, as determined by the luciferase reporter gene assay (Fig. 4C) and by Western blotting (Fig. 4D). These results suggested that NVPP inhibited the TRIF-dependent signaling pathway derived from TLR3 activation.

3.4. NVPP does not suppress the activation of IRF3 induced by downstream signaling components of the TRIF-dependent pathway of TLRs

Presently, to further identify the molecular targets of NVPP for the inhibition of the TRIF-dependent pathway, the downstream component (TRIF, TBK1 or IRF3) of the pathway was transfected into 293T cells. The TRIF-dependent pathway of TLRs led to IRF3 activation mediated through TRIF and TBK1. NVPP did not suppress IRF3 activation induced by TRIF, TBK1 or IRF3 5D (constitutively active form of IRF3), as determined by an IRF3 binding site (IFNβ PRDIII-I) reporter gene assay (Fig. 5A–C). These results suggest that the target of NVPP is not TRIF-dependent downstream signaling components, including adapter molecules themselves. The target

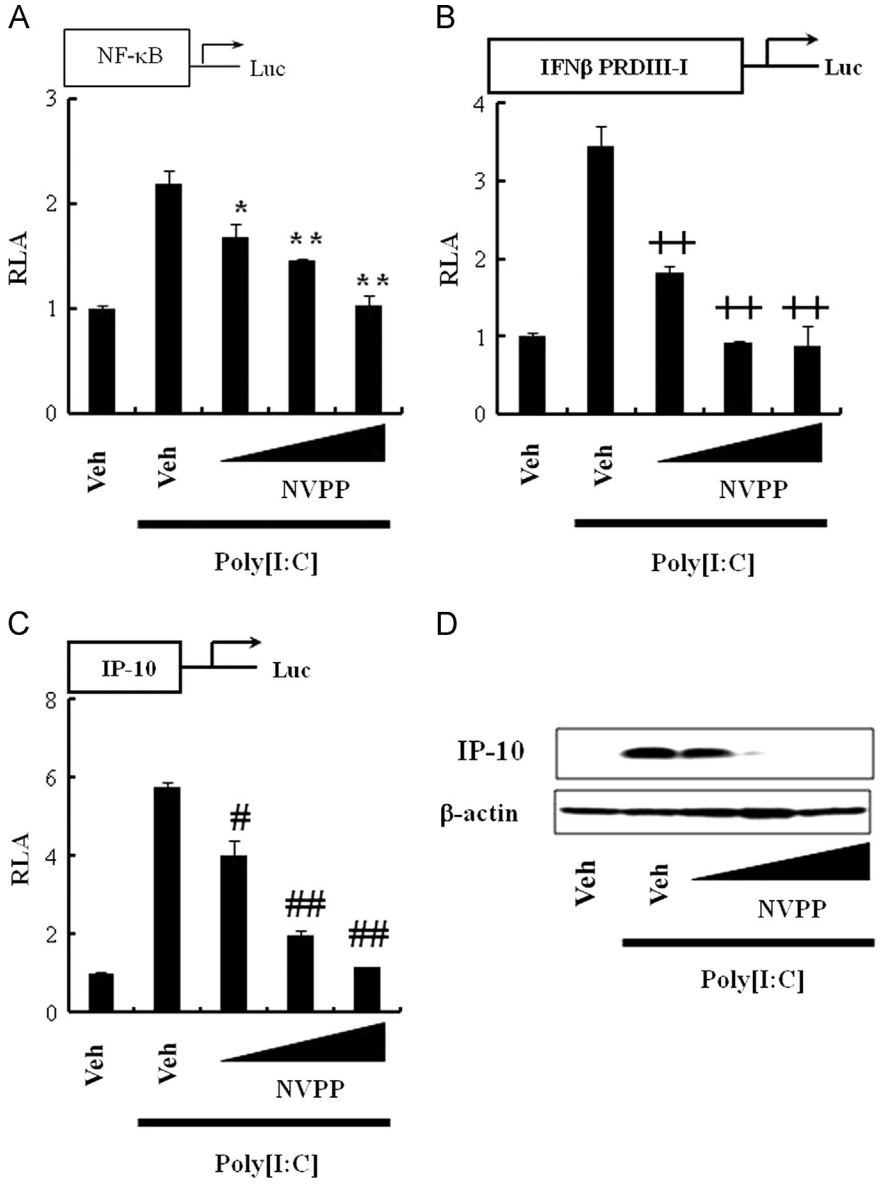

Fig. 4. NVPP suppresses TRIF-dependent signaling pathway of TLR3. (A-C) RAW264.7 cells were transfected with NF-κB (A), IRF3 binding site (IFNβ PRDIII-I) (B) and IP-10 (C) luciferase reporter plasmid and pre-treated with NVPP (20, 50 or 100 μM) for 1 h and then treated with poly[I:C] (10 μg/ml) for an additional 8 h. Cell lysates were prepared and luciferase and β-gal enzyme activities were measured. Relative luciferase activity was normalized with β-gal activity. Values represent the mean ± S.E.M. ($n=3$). ∗ denotes a result that is significantly different from poly[I:C] alone, $P<0.05$ (∗), $P<0.01$ (∗∗) (A). + denotes a result that is significantly different from poly[I:C] alone, $P<0.01$ (++) (B). # denotes a result that is significantly different from poly[I:C] alone, $P<0.05$ (#), $P<0.01$ (##) (C). (D) RAW264.7 cells were pretreated with NVPP (20, 50 or 100 μM) for 1 h, and were then further stimulated with poly[I:C] (10 μg/ml) for a further 8 h. Cell lysates were analyzed for IP-10 and β-actin proteins by Western blotting. Veh, vehicle; NVPP, (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine.

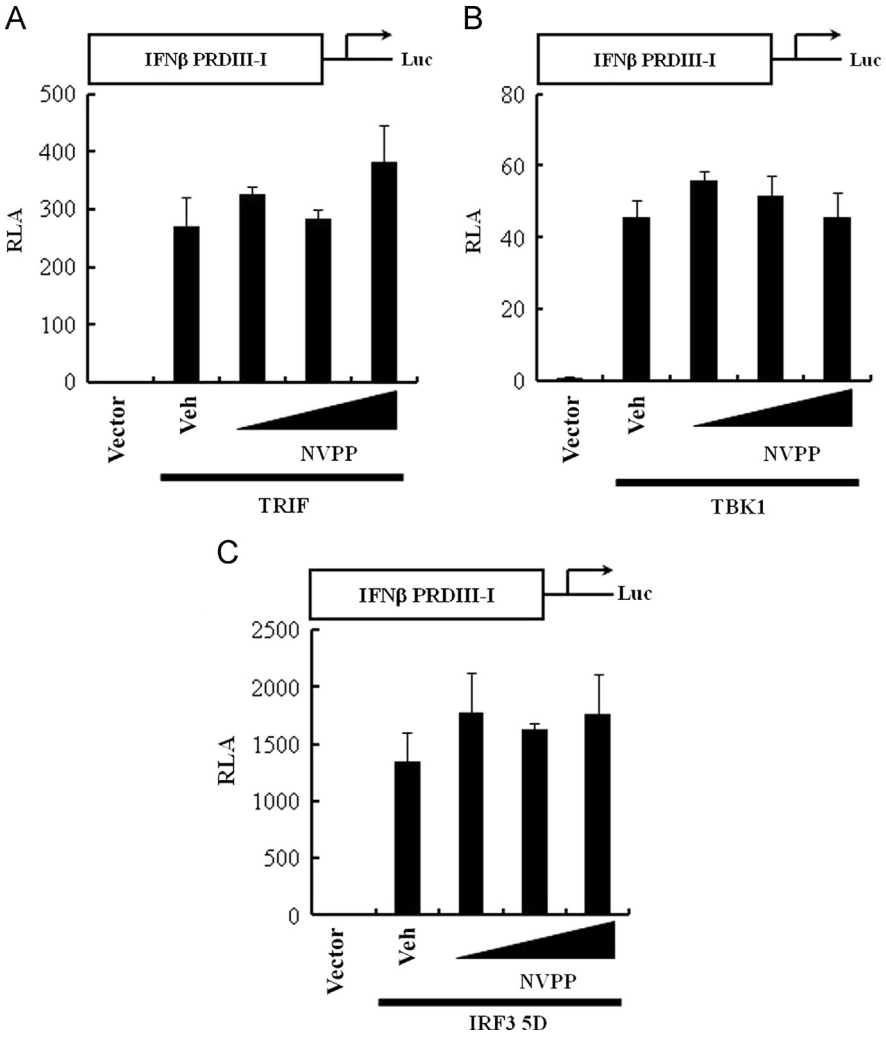

Fig. 5. NVPP suppresses IRF3 activation induced by downstream signaling components. (A) 293T cells were transfected with IRF3 binding site (IFNβ PRDIII-I)-luciferase reporter plasmid and the expression plasmid of TRIF (A), TBK1 (B) or IRF3 5D (C). Cells were further treated with NVPP (20, 50 or 100 μM) for 18 h. Relative luciferase activity was normalized with β-gal activity. Values represent mean ± SEM ($n=3$). Veh, vehicle; NVPP, (*E*)-1-(2-(2-nitrovinyl)phenyl)pyrrolidine.

may be components upstream of adapter molecules, including TLRs, or events leading to the activation of TLRs by agonists.

4. Discussion

The MyD88- and TRIF-dependent signaling pathways are two major downstream signaling pathways of TLRs which activate transcription factor NF-κB and IRFs. Both the NF-κB and IRFs are simultaneously activated in response to microbial infection, but the target genes induced by the activation of NF-κB and IRFs are different. The activation of NF-κB induces proinflammatory cytokines, but the activation of IRFs induces type I IFN genes (Honda and Taniguchi, 2006; Stetson and Medzhitov, 2006; Wietek and O'Neill, 2007). The type I IFN gene, a central gene in establishing the innate antiviral response, is activated by the cooperative activation of NF-κB and IRF3. TLR3 and TLR4 activation induce the expression of genes encoding IFN and chemokines (Servant et al., 2002). The signaling pathways leading to NF-κB and IRF3 are triggered by the binding of pathogen-specific products to TLRs in macrophages and dendritic cells (Uematsu and Akira, 2007). TLRs are vital component of host's immune system for sensing all the major classes of microorganisms, including bacteria, viruses, and fungi.

Deregulated TLR-mediated cellular responses can lead to chronic inflammation, which, in turn, contribute to the development and progress of many inflammatory diseases. Growing evidence suggests that TRIF-dependent signaling pathway of TLRs is important in inflammatory responses and in the development of certain chronic diseases. The expression of the majority (> 70%) of LPS-induced genes is regulated through the TRIF-dependent signaling pathway (Schafer et al., 1998). Therefore, the modulation of the TRIF-dependent pathway of TLRs is important for anti-inflammatory strategies. These facts suggest that the suppression of the TRIF dependent signaling pathway and the consequent down-regulation of IRF3 activation by NVPP can significantly suppress the target gene expression of TLR signaling pathways.

TRIF is the critical adapter molecule which facilitates the activation of TLR3 and TLR4 signaling pathways (O'Neill, 2008). The results from our previous reports have shown that the TRIF signaling pathway is negatively regulated by a number of molecules; however, in most cases, they do not target TRIF specifically, but affect downstream components of the TRIF pathway such as TBK1 (Park and Youn, 2010; Youn et al., 2005; Youn et al., 2006a). We also previously demonstrated that many phytochemicals with the α, β-unsaturated carbonyl group structural motif which confers Michael addition inhibits TLR4 dimerization (Ahn et al., 2009; Youn et al., 2008; Youn et al., 2006b). It is well documented that molecules with such a structural motif are highly reactive with the sulfhydryl group of cysteine by Michael addition. TLRs have several cysteine residues in extracellular and cytoplasmic domains which

may be involved in disulfide bond formation for receptor dimerization. NVPP also has the α, β-unsaturated nitro group structural motif, which confers the possibility of Michael addition. It is conceivable that NVPP will may interact with the cysteine residue in TLR4 leading to the inhibition of TLR4 dimerization. In future research, the exact molecular target of NVPP in TLR signaling pathways will be identified.

In the present study, we demonstrated that NVPP suppresses TRIF-dependent pathways of TLR3 and TLR4. The suppression of TRIF pathway of TLR3 and TLR4 by NVPP is accompanied by the down-regulation of the activation of NF-κB and IRF3, and of their target genes, including IFNβ and IP-10. All the present results raise the important possibility that TLR-mediated inflammatory responses and consequent risks for many chronic inflammatory diseases can be regulated by the nitrovinyl derivate, NVPP.

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