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Cutting Edge: Nitric Oxide Inhibits the NLRP3 Inflammasome

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Although the NLRP3 inflammasome plays a pivotal role in host defense, its uncontrolled activation is associated with inflammatory disorders, suggesting that regulation of the inflammasome is important to prevent detrimental effects. Type I IFNs and long-term LPS stimulation were shown to negatively regulate NLRP3 activation. In this study, we found that endogenous NO is involved in the regulation of NLRP3 inflammasome activation by either IFN-β pretreatment or long-term LPS stimulation. Furthermore, S-nitroso-N-acetylpenicillamine (SNAP), an NO donor, markedly inhibited NLRP3 inflammasome activation, whereas the AIM2 and NLRC4 inflammasomes were only partially inhibited by SNAP. An increase in mitochondrial reactive oxygen species induced by ATP was only modestly affected by SNAP treatment. Interestingly, S-nitrosylation of NLRP3 was detected in macrophages treated with SNAP, and this modification may account for the NO-mediated mechanism controlling inflammasome activation. Taken together, these results revealed a novel role for NO in regulating the NLRP3 inflammasome. The Journal of Immunology, 2012, 189: 5113–5117.

The inflammasome is a multiprotein complex that mediates the activation of caspase-1, which then processes pro–IL-1β and pro–IL-18 into mature IL-1β and IL-18. Among a number of sensor proteins reported to be involved in inflammasome activation, NLRP3, NLRP1b, NLRC4, and AIM2, have been established as the major sensors for the recognition of various pathogens or damage-associated molecular patterns.

NLRP3 activation is triggered by different types of stimuli (e.g., whole pathogens, uric acid crystals, nigericin, ATP) (1). The precise mechanism of NLRP3 inflammasome activation has not been determined; however, NLRP3 agonist-induced generation of mitochondrial reactive oxygen species (ROS) is likely to play a major role in NLRP3 activation (2–5). Despite the fact that NLRP3 inflammasome contributes to host defense against microbial pathogens, excessive activation due to mutations in the NLRP3 gene has been associated with a spectrum of autoimmune inflammatory disorders collectively known as “cryopyrin-associated periodic syndromes” (1). Additionally, NLRP3 has been implicated in obesity-induced inflammation and insulin resistance (6). Thus, appropriate regulation of inflammasome activation appears to be important to avoid detrimental effects.

It was demonstrated that activation of the inflammasome is regulated by several mechanisms. For instance, the protein level of NLRP3 is relatively low in resting macrophages, so that NLRP3 inflammasome formation is hardly induced until the expression level is increased by exogenous and endogenous factors, including TLR agonists and proinflammatory cytokines. This suggests that activation of the NLRP3 inflammasome is limited under normal conditions (7). In contrast, long-term priming with the TLR4 agonist LPS results in the suppression of NLRP3 inflammasome activity, reportedly owing to the induction of TRIM30 (8). It was also demonstrated that type I IFNs negatively regulate the activation of caspase-1 induced by NLRP3 agonists but not that induced by AIM2 or NLRC4 agonists, although the mechanism remains unclear (9). In a report published before the establishment of the inflammasome concept, it was suggested that NO directly inhibits caspase-1 activity, thereby preventing the release of mature IL-1β and IL-18 in response to LPS and IFN-γ (10). Because type I IFNs also induce the expression of IFN regulatory factor 1, a transcription factor required for the expression of inducible NO synthase (iNOS) (11), we addressed whether NO is involved in the inhibition of the NLRP3 inflammasome by type I IFNs. In the current study, we analyzed the role of NO in the regulation of NLRP3 inflammasome activation.

Materials and Methods

Mice

Female C57BL/6 (wild-type [WT]) mice were purchased from Japan SLC. IFN-α/β receptor 1 (IFNAR1) knockout (KO) mice were kindly provided by Masao Mitsuyama (Kyoto University, Kyoto, Japan). The online version of this article contains supplemental material.

Abbreviations used in this article: IFNAR1, IFN-α/β receptor 1; iNOS, inducible NO synthase; KO, knockout; L-NMMA, Nω-monomethyl-L-arginine; mROS, mitochondrial reactive oxygen species; poly(dA:dT), poly(desoxyadenylic-thymidylic) acid; rIFN-β, recombinant mouse IFN-β; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetylpenicillamine; WT, wild-type.

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by Prof. Michel Aguet (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland). KO mice for iNOS were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free conditions and used at 7–9 wk of age. All animal experimental procedures were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine.

Reagents

Ultra-pure LPS, Pam3CSK4, and Salmonella flagellin were purchased from InvivoGen (San Diego, CA); nigericin, poly(deoxyadenylic-thymidylic) acid (poly(dA:dT)), 5-nitroso-N-acyetylpenicillamine (SNAP), 8-homoguanosine 5′, 5′-cyclic monophosphate sodium salt, S-methyl methanethiosulfonate, and sodium t-ascorbate were purchased from Sigma-Aldrich (St. Louis, MO); N9-mono-methyl-t-arginine (L-NMMA) was purchased from Dojindo (Kumamoto, Japan); EZ-Link Biotin-HDPP was purchased from Pierce (Rockford, IL); recombinant mouse IFN-β, carrier-free (rIFN-β) was purchased from PBL Interferon Source (Piscataway, NJ); Griess reagent kit was purchased from Invitrogen (Grand Island, NY); anti-NLRP3 mAb (Cyo-2) and anti-ASC pAb (AL177) were purchased from Enzo Life Sciences (Exeter, U.K.); anti-caspase-1 p10 (M-20) pAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse IL-1β biotinylated Ab was purchased from R&D Systems (Minneapolis, MN); anti-iNOS mAb was purchased from Transduction Laboratories (Lexington, KY); ELISA kit for mouse IL-1β was purchased from eBioscience (San Diego, CA); and recombinant mouse IL-1β biotinylated Ab was purchased from Medical & Biological Laboratories (Nagoya, Japan). The following primers were used: TRIM30 sense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′, antisense: 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, anti-SNAP 9nt antisense: 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′, anti-caspase-1 p10 (M-20) pAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse IL-1β biotinylated Ab was purchased from R&D Systems (Minneapolis, MN); anti-iNOS mAb was purchased from Transduction Laboratories (Lexington, KY); ELISA kit for mouse IL-1β was purchased from eBioscience (San Diego, CA); and recombinant mouse IL-1β biotinylated Ab was purchased from Medical & Biological Laboratories (Nagoya, Japan). The following primers were used: TRIM30 sense: 5′-GCGGACAGTCTTTGTCTGCAGGTGTCG-3′, antisense: 5′-GGCAGTGGCCCTTTCCGCCGTCCG-3′ and β-actin sense: 5′-TGGAGGACTCTGGGACCATGAAAC-3′, antisense: 5′-TAAAGCCGACGTCAGAAGCTCGG-3′.

Cells

Peritoneal exudate cells collected 4 d after i.p. injection of thioglycollate medium were incubated for 3 h in RPMI 1640 medium supplemented with 10% FCS at 37°C in 5% CO2. Nonadherent cells were washed out, and adherent macrophages were primed with LPS or Pam3CSK4 at a final concentration of 50 ng/ml in Opti-MEM I (Invitrogen). Then, the cells were treated with 5 μM nigericin for 60 min, 5 mM ATP for 30 min, 600 ng poly(dA:dT) using Lipofectamine LTX and Plus Reagent (Invitrogen) for 60 min, or 175 ng flagellin using GenomeONE-NEO Transfection Reagent HVJ Envelope vector Kit (Ishihara Sangyo, Osaka, Japan) for 60 min. rIFN-β and L-NMMA were used at final concentrations of 100 U/ml and 500 μM, respectively.

Results and Discussion

NO is involved in the inhibitory effect of IFN-β on caspase-1 activation via the NLRP3 inflammasome

IFN-β was shown to inhibit the expression of pro–IL-1β and caspase-1 activation via NLRP3 inflammasome, but the mechanism remains unknown (9). To analyze whether NO generated upon IFN stimulation is responsible for the inhibitory effect of type I IFNs on NLRP3 inflammasome activation, peritoneal macrophages were primed with Pam3CSK4, a TLR2 ligand, in the presence or absence of IFN-β and/or L-NMMA, an NO synthase inhibitor, before being stimulated with nigericin. In agreement with the previous study, we observed clear decreases in nigericin-induced caspase-1 activation, the protein level of pro–IL-1β, and the secretion of mature IL-1β when priming was done in the presence of IFN-β (Fig. 1A, 1B). The specificity of the inhibitory effect of IFN-β was confirmed using IFNAR1-deficient macrophages (Fig. 1A, 1B). As expected, both the expression of iNOS and NO production were strongly induced in WT macrophages primed with Pam3CSK4 and rIFN-β (Fig. 1A, 1C). Interestingly, the addition of L-NMMA, which inhibited the production of NO, canceled the inhibitory effect of IFN-β on caspase-1 activation (Fig. 1A, 1C). In contrast, L-NMMA only modestly restored the level of pro–IL-1β in rIFN-β–treated macrophages primed with Pam3CSK4, resulting in a partial recovery of the secretion of mature IL-1β induced by nigericin (Fig. 1A, 1B).

These data suggested that NO is the molecule contributing to the inhibitory effect of IFN-β on NLRP3 inflammasome activation. In iNOS-deficient macrophages, NO production in response to Pam3CSK4 and rIFN-β was not observed, and rIFN-β treatment did not reduce the nigericin-induced caspase-1 activation (Fig. 1C, 1D). Thus, we confirmed that NO plays a critical role in the inhibition of the NLRP3 inflammasome by IFN-β. Recently, a study showed that IFN-β induces the
expression of SOCS1, which mediates the inhibition of ROS production (12). Therefore, our findings suggest a novel mechanism of inhibition of inflammasome activation by type I IFNs in addition to the SOCS1-mediated one.

**NO donor SNAP inhibits the NLRP3 inflammasome, whereas the AIM2 and NLRC4 inflammasomes are only partially affected**

To further confirm the inhibitory effect of NO on the activation of NLRP3 inflammasome, macrophages primed with Pam$_3$CSK$_4$ were incubated in the presence of SNAP, an NO donor, before stimulation with inflammasome activators. Substantial levels of NO were generated in the cultures when various concentrations (125–500 μM) of SNAP were added (Fig. 2A). Under this experimental condition, caspase-1 activation and the secretion of mature forms of IL-1β and IL-18 induced by nigericin or ATP were significantly decreased by SNAP in a dose-dependent manner (Fig. 2B–E). This result substantiated the NO-dependent inhibition of the NLRP3 inflammasome. In contrast, SNAP treatment partially diminished these cellular responses induced by poly(dA:dT) or flagellin, agonists for AIM2 and NLRC4, respectively (Fig. 2B–E). Judging from these results, it appeared that the NLRP3 inflammasome is more susceptible to NO than are the AIM2 and NLRC4 inflammasomes and that the inhibitory effect of NO on the NLRP3 inflammasome is not simply due to direct inhibition of caspase-1 enzymatic activity by NO, as proposed in a previous report (10).

**Endogenous NO induced by long-time LPS priming serves as a negative regulator of NLRP3 inflammasome activation**

It was shown that, after long-term priming with LPS, macrophages become refractory to the activation of NLRP3. Actually, macrophages primed with LPS for 12 h secreted markedly lower levels of IL-18 after stimulation with nigericin or ATP compared with those primed for 4 h (Fig. 3A). A possible explanation for this inhibitory effect is that LPS induces the expression of TRIM30, which negatively regulates the NLRP3 inflammasome (8). However, we observed that long-term priming with Pam$_3$CSK$_4$ did not lead to suppression of NLRP3 inflammasome activation, even though TRIM30 expression was upregulated to some extent (Fig. 3B, 3C). LPS, but not Pam$_3$CSK$_4$, induced type I IFN production, iNOS expression, and NO generation in macrophages (Fig. 1C, Supplemental Fig. 1A, 1B) (11, 13). Therefore, we hypothesized that NO induced by long-term LPS priming may mediate the inhibitory effect. To test this possibility, iNOS-deficient macrophages were stimulated with nigericin or ATP after being primed with LPS for 4 or 12 h. We found that these NLRP3 agonists induced higher levels of IL-18 secretion from iNOS-deficient macrophages than from WT macrophages if the macrophages were primed for 12 h (Fig. 3D). Similar results were obtained in experiments using L-NMMA (Supplemental Fig. 1C, 1D). In contrast, there was no significant difference between iNOS-deficient macrophages and WT macrophages in the secretion of IL-18 in response to NLRP3 agonists when the cells were primed with LPS for 4 h, at which time iNOS was not expressed (Fig. 3D, Supplemental Fig. 1E). Accordingly, our results suggest that endogenous NO induced by long-time LPS priming inhibits NLRP3 inflammasome activation. Secretion of IL-18 induced by flagellin or poly(dA:dT) after long-term priming with LPS was even greater than that was after a 4-h LPS priming (Fig. 3E), supporting that NLRC4 and AIM2 inflammasomes are more resistant to NO than is the NLRP3 inflammasome.

**Analysis of the mechanism by which NO regulates NLRP3 inflammasome activation**

To elucidate the mechanism by which NO exerts its inhibitory effect, particularly on the NLRP3 inflammasome, we measured mitochondrial ROS (mtROS), which are known to promote...
NLRP3 activation (3–5). SNAP treatment only modestly affected the increase in mtROS induced by ATP (Fig. 4A), and it was difficult to conclude whether the inhibition of NLRP3 by NO depends solely on the decrease in mtROS generation. NO induces the upregulation of antioxidant genes, including thioredoxin reductase-1, which may disturb NLRP3 inflammasome activation (2) (Supplemental Fig. 2A). However, inhibition of NLRP3 inflammasome by just a 1-h treatment with SNAP was also observed in the presence of cycloheximide (Supplemental Fig. 2B, 2C). Thus, the de novo synthesis of antioxidant proteins did not appear to account for the inhibitory effect of NO on NLRP3 inflammasome, at least at an early time after exposure to SNAP. NO is known to increase the synthesis of the second messenger cyclic GMP, but IL-1β secretion was not affected by 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt, an analog of cyclic GMP (Supplemental Fig. 2D). S-nitrosylation is a covalent addition of an NO group onto protein cysteine thiols to form S-nitroso-proteins, and it has been increasingly recognized as a post-translational modification regulating the protein functions (14). We used the biotin-switch technique, as previously described, to determine whether the NLRP3 protein can be a target of S-nitrosylation (15). Biotinylated proteins were detected in cell lysates from macrophages treated with SNAP for 1 h, only when the lysates were reduced with ascorbate before labeling with protein cysteine thiols with biotin (Fig. 4B), indicating that S-nitroso-proteins in the lysates were successfully biotinylated. Interestingly, caspase-1 and NLRP3 were detected in S-nitrosylated proteins enriched from the labeled lysates using an avidin resin (Fig. 4C). We also found that the C-terminal region of NLRP3 is more susceptible to S-nitrosylation than is the N-terminal region (Supplemental Fig. 2E). From these results, we propose that a direct modification of the NLRP3 protein by S-nitrosylation is the mechanism responsible for the early inhibition of NLRP3 inflammasome activation by NO. A direct S-nitrosylation of caspase-1 itself may explain the partial inhibition of the AIM2 and NLRC4 inflammasomes by NO. Further analysis is necessary to confirm the relevance of S-nitrosylation as a regulatory mechanism of the NLRP3 inflammasome. Another possibility to be addressed is that NO interferes with other proteins involved in NLRP3 inflammasome activation or with mitochondrial DNA release, thereby inhibiting the NLRP3 inflammasome (4, 5).

In this study, we found that NO regulates the activation of NLRP3 inflammasome. Because both NO and NLRP3 are known to play roles in a wide range of physiological responses, NO-mediated inhibition of the NLRP3 pathway might have significance not only in the beneficial host defense against microbial pathogens but also in the pathophysiology of NLRP3-associated detrimental diseases. For example, NLRP3 was implicated in the pathogenesis of ischemia–reperfusion injury (16), and the involvement of NO was demonstrated in preconditioning-induced tissue protection from ischemia–reperfusion injury (17). Therefore, it is worth considering whether inhibition of NLRP3 activation by NO is at least one of the mechanisms of tissue protection by ischemic preconditioning. Further studies are necessary to fully describe the role and mechanism of the inhibitory effect of NO on NLRP3, and a better understanding of the mechanism may shed new light on the activation process of the NLRP3 inflammasome.
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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1: LPS, but not Pam3CSK4, induces iNOS expression. (A) Macrophages were stimulated with LPS or Pam3CSK4 for the indicated time periods, and the protein level of iNOS in cell lysates was analyzed by Western blotting with an anti-iNOS Ab. (B, E) Macrophages from WT mice, IFNAR1-deficient mice, or iNOS-deficient mice were stimulated with LPS for 4 or 12 h. (B) The concentration of nitrite in the culture supernatants was measured. (E) Cell lysates were subjected to Western blotting with antibodies specific to the indicated proteins. (C, D) WT or NLRP3-deficient macrophages were primed with LPS for 12 h in the absence or presence of L-NMMA. The cells were then stimulated with nigericin, ATP, or Poly(dA:dT) (C), or infected with live Listeria monocytogenes EGD at a multiplicity of infection (MOI) of 50 for 2 h (D), and the level of IL-18 in the culture supernatants was determined by ELISA. L-NMMA treatment significantly increased the secretion of IL-18 in response to nigericin, ATP, or L. monocytogenes. Although the induction of caspase-1 activation by L. monocytogenes infection at low MOIs has been reported to depend on multiple receptors, such as AIM2, NLRP3, and NLRC4, NLRP3 is entirely responsible for caspase-1 activation induced by infection with L. monocytogenes at an MOI of 50 after LPS priming (Nature 2006 440:228, J. Immunol. 2010 185:1186, and Supplemental Fig. 1D). Thus, the result suggested that the inhibition of the NLRP3 inflammasome by NO can occur when activated macrophages producing NO are infected with high doses of L. monocytogenes. NLRP3-deficient mice were kindly provided by Prof. Jürg Tschopp (University of Lausanne).
Supplemental Figure 2: SNAP treatment resulted in the up-regulation of antioxidant genes, whereas de novo synthesis of proteins was not required for the early inhibition of the NLRP3 inflammasome by NO. (A) WT macrophages were incubated with or without SNAP for 3 or 6 h, and mRNAs of thioredoxin reductase-1 (Txnrd1), thioredoxin 1 (Txn1), thioredoxin-interacting protein (Txnip), and NAD(P)H dehydrogenase [quinone] 1 (Nqo1) were analyzed by RT-PCR. The primers used are as follows; Txnrd1 sense: 5'-TGCTGGCTCAGAGGCTGTAT, antisense: 5'-CTTCGACCTGCCACCTCCTA; Txn1 sense: 5'-ATCTGGTTCTGCTGAGACGC, antisense: 5'-TGGAAGGTCGGCATGCATTT; Txnip sense: 5'-TTGAACCCACTCGGCTCAATCATGGTG, antisense: 5'-GACTGAGTCTCTGAACACTGGAGTTC; Nqo1 sense: 5'-TTTCTCTGGCCGATTCAGAGT, antisense: 5'-GGCTGCTTGGAGCAAAATAG. (B) WT macrophages were left unprimed or primed with Pam3CSK4 for 3 h and treated with or without 50 ng/ml of cycloheximide for 30 min. SNAP was then added to some wells. The cells were further incubated for 1 h and stimulated with ATP or nigericin for 30 min. (D) Macrophages primed with Pam3CSK4 were treated for 1 h with increasing concentrations of 8-Br-cGMP before stimulation with ATP for 30 min. (B and D) The level of IL-18 in the culture supernatants was determined by ELISA. (C) Macrophages were treated with the indicated concentrations of cycloheximide for 30 min and then stimulated with Pam3CSK4 for 3 h. The protein level of pro-IL-1β in the cell lysates was analyzed by Western blotting with an anti-IL-1β antibody. We verified that these concentrations of cycloheximide were sufficient to inhibit protein synthesis. (E) HEK293 cells were transiently transfected with FLAG-tagged expression vectors for full-length mouse NLRP3 (NLRP3_full) or its N-terminal (NLRP3_N) or C-terminal (NLRP3_C) fragments. After 48 h, the cells were treated with or without SNAP for 1 h, and S-nitrosylated proteins were labeled by the biotin switch method as mentioned in Fig. 4. The labeled lysates and biotinylated protein fractions were subjected to Western blotting with an anti-FLAG antibody. Experiments were repeated at least three times with consistent results. The expression vectors for NLRP3_full, NLRP3_N, and NLRP3_C were constructed in this study. Briefly, cDNAs encoding full-length mouse NLRP3 and its truncated fragments were amplified from a mouse spleen cDNA preparation by PCR using KOD Plus polymerase (TOYOBO) and primer sets indicated below. The amplified fragments were digested with restriction enzymes and inserted into the pFLAG-CMV2 vector (Sigma-Aldrich). The primers used are as follows; NLRP3_full sense: 5'-CCTGCGGCCGCAACAGTCTCGGTTTCAAG, antisense: 5'-CCTGGTACCTACCAGGAAATCTCGAAGACTA; NLRP3_N sense: 5'-CCTGCGGCCGCAACAGTCTCGGTTTCAAG, antisense: 5'-TGGGAGTCGAGACTAAAGTCTTCCTCGTGCATTCG; NLRP3_C sense: 5'-AGGAGGCCGCCGGTGAGTGCAGCGTCGAGCTGAGCCACT, antisense: 5'-CCTGGTACCTACCAGGAAATCTCGAAGACTA.