Downregulation by lipopolysaccharide of Notch signaling, via nitric oxide

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Summary

The Notch signaling pathway appears to perform an important function in inflammation. Here, we present evidence to suggest that lipopolysaccharide (LPS) suppresses Notch signaling via the direct modification of Notch by the nitration of tyrosine residues in macrophages. In the RAW264.7 macrophage cell line and in rat primary alveolar macrophages, LPS was found to inhibit Notch1 intracellular domain (Notch1-IC) transcription activity, which could then be rescued by treatment with N(G)-nitro-l-arginine, a nitric oxide synthase (NOS) inhibitor. Nitric oxide (NO), which was produced in cells that stably express endothelial NOS (eNOS) and brain NOS (bNOS), also induced the inhibition of Notch1 signaling. The NO-induced inhibition of Notch1 signaling remained unchanged after treatment with 1H-[1,2,4]oxadiazolo[4,3-alpha]quinoxalin-1-one (ODQ), a guanylyl-cyclase inhibitor, and was not found to be mimicked by 8-bromo-cyclic GMP in the primary alveolar macrophages. With regards to the control of Notch signaling, NO appears to have a significant negative influence, via the nitration of Notch1-IC, on the binding that occurs between Notch1-IC and RBP-Jk, both in vitro and in vivo. By intrinsic fluorescence, we also determined that nitration could mediate conformational changes of Notch1-IC. The substitution of phenylalanine for tyrosine at residue 1905 in Notch1-IC abolished the nitration of Notch1-IC by LPS. Overall, our data suggest that an important relationship exists between LPS-mediated inflammation and the Notch1 signaling pathway, and that this relationship intimately involves the nitration of Notch1-IC tyrosine residues.

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Key words: LPS, Nitric oxide, Notch

Introduction

The Notch signaling pathway is an evolutionarily conserved, intracellular signaling mechanism that plays a key role in the determination of cell fate, differentiation, adult cell self-renewal, cancer, neurodegenerative disease, wound healing and inflammation (Bray, 2006; Hurlbut et al., 2007). Upon receipt of extracellular signals mediated via the binding of specific ligands – Jagged and Delta – the intracellular domain of Notch (Notch-IC) is released by proteolytic cleavage (Brou et al., 2000; Lieber et al., 2002; Mumm et al., 2000). This process triggers the γ-secretase-dependent proteolytic release of the Notch-IC from the membrane, and induces the nuclear translocation of Notch-IC, resulting in the formation of a complex with the CSL family [CBF1/RBP-Jk/KBF2 in mammals, Su(H) in *Drosophila* and *Xenopus*, and Lag2 in *Caenorhabditis elegans*] (De Strooper et al., 1999; Gordadze et al., 2001; Iso et al., 2002; Iso et al., 2001; Kao et al., 1998). In the absence of Notch-IC, CSL binds to co-repressors such as SKIP, SMRT, CoR and HDAC, thus inhibiting transcription of target genes. Notch-IC displaces the co-repressors, recruits co-activator complexes – including Lag3 and/or Mastermind, p300 and/or CBP, and P/CAF and/or GCN5 – and activates CSL-dependent transcription (Mumm and Kopan, 2000). Notch1-IC interacts with RBP-Jk/Su(H) primarily through the RAM domain, a sequence that is located N-terminally to the ankyrin repeats, resulting in the activation of target gene transcription (Tamura et al., 1995). Several downstream targets of Notch signaling have also been identified, including the Enhancer of split [E(spl)] complex genes, and the mammalian homologs of the *Hairy* and *E(spl)* genes, *Hes1* and *Hes5* (de la Pompa et al., 1997; Jarriault et al., 1995). After the transcriptional regulation of the target genes, Notch1-IC is degraded in the nucleus by the ubiquitin-proteasome system, with the aid of Fbw7, an E3 ligase for the ubiquitylation of Notch1-IC (Gupta-Rossi et al., 2001; Hubbard et al., 1997; Oberg et al., 2001; Wu et al., 2001).

Bacterial LPS is a major constituent of the cell walls of Gram-negative bacteria. The release of LPS from the bacterial cell walls into the blood circulation of the host causes the activation of immune cells, resulting in the production of cytokines such as TNFα and interleukins, as well as nitric oxide (NO) (Gross et al., 1993; Nakano et al., 1993). NO is a crucial molecular signal that exerts both physiological and pathological functions (Benhar et al., 2006; Bredt and Snyder, 1992; Gaston et al., 2006; Lipton et al., 2007; Lowenstein and Snyder, 1992; Nakamura et al., 2007; Nakamura

and Lipton, 2007; Satoh and Lipton, 2007; Snyder, 1992). One of the most concentrated recent studies regarding NO was undertaken in order to identify the functions of nitration of the sulfhydryl group(s) of cysteine and/or tyrosine residue(s), with regards to the modulation of protein function (Broillet, 1999; Lane et al., 2001; Martinez-Ruiz and Lamas, 2004). The evaluation of nitrated proteins on cysteine revealed a degenerate consensus sequence x[K/R/H]*C*[D/E], but the nitration of tyrosine is a phenomenon that has yet to be fully elucidated (Stamler et al., 1997). The nitration of tyrosine residues has been identified in several proteins, including Mn superoxide dismutase (MnSOD), low-density lipoprotein (LDL), prostacyclin synthase, SERCa2a, succinyl coA:3-oxoacid CoA transferase, actin, tyrosine hydroxylase and surfactant protein A (Chantler and Gratzer, 1975; Greis et al., 1996; Ischiropoulos et al., 1992; Leeuwenburgh et al., 1997; MacMillan-Crow and Thompson, 1999; Marcondes et al., 2001; Park et al., 2003; Viner et al., 1996; Zou et al., 1997). A recent report raised the possibility that the Notch signaling cascade might be regulated by NO via the nitration of tyrosine residues (Kanski et al., 2005). Despite these observations, the precise mechanisms underlying the connection between these two signaling pathways remain to be accurately delineated.

In our current study, we determined that the activation of Notch1 can be suppressed by LPS via the nitration of tyrosine residues in rat alveolar macrophages. In particular, the NO-mediated nitration of Notch1-protein tyrosine residues is linked to conformational changes. To our knowledge, this is the first study to pinpoint the relationship that exists between Notch1 and the LPS signaling pathway in inflammation.

Results

LPS, via NO, suppresses Notch1-IC transcriptional activity in macrophage cells

In order to characterize the effects of LPS on the activity of Notch1, we assessed the degree to which the active form of Notch1 (Notch1-IC) induced transcriptional activity (Saxena et al., 2001). When cells were treated with LPS, the transcriptional activity of Notch1-IC was suppressed, and this effect occurred in a dose-dependent manner (Fig. 1A). The expression of Notch1-IC was discovered to significantly induce the activation of the 4×CSL-Luc reporter system in the RAW264.7 macrophage cell line (Fig. 1B). When we treated cells with 5 μM of LPS, the Notch1-IC-mediated 4×CSL transcriptional activity was suppressed to a substantial degree (Fig. 1B). The basic helix-loop-helix (bHLH) proteins Hes1 and Hes5, the genes of which both harbor multiple RBP-Jk-binding DNA sequences on their promoter, were identified as essential targets of Notch (Ohtsuka et al., 1999). Therefore, we confirmed the effects of LPS on the Notch1 signaling pathway using the *Hes1* and *Hes5* reporter systems, respectively. The expression of Notch1-IC significantly induced the activation of the *Hes1* and *Hes5* reporter systems (Fig. 1C,D). Treatment with LPS was observed to inhibit Notch1-IC-induced natural *Hes1* and *Hes5* promoter transcriptional activity (Fig. 1C,D) and protein expression (Fig. 1E). We also confirmed the protein expression level with other proteins regulated by NO. Coinciding with previous reports (Asayama et al., 1985; Faris et al., 1998; Kasibhatla et al., 1998; Masuda et al., 1988; Shiki et al., 1987), the protein induction of Mn-SOD and CD95L were effectively suppressed by NO (Fig. 1E).

In order to determine the actual role in which inducible nitric oxide synthase (iNOS) is involved in the suppression of Notch1, RAW264.7 or alveolar macrophage cells were pre-exposed to nitro-

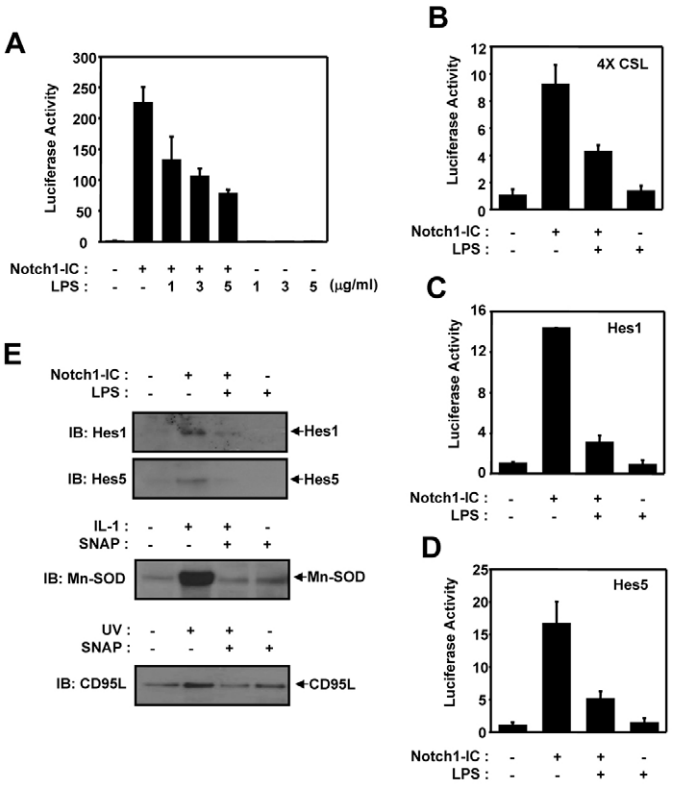
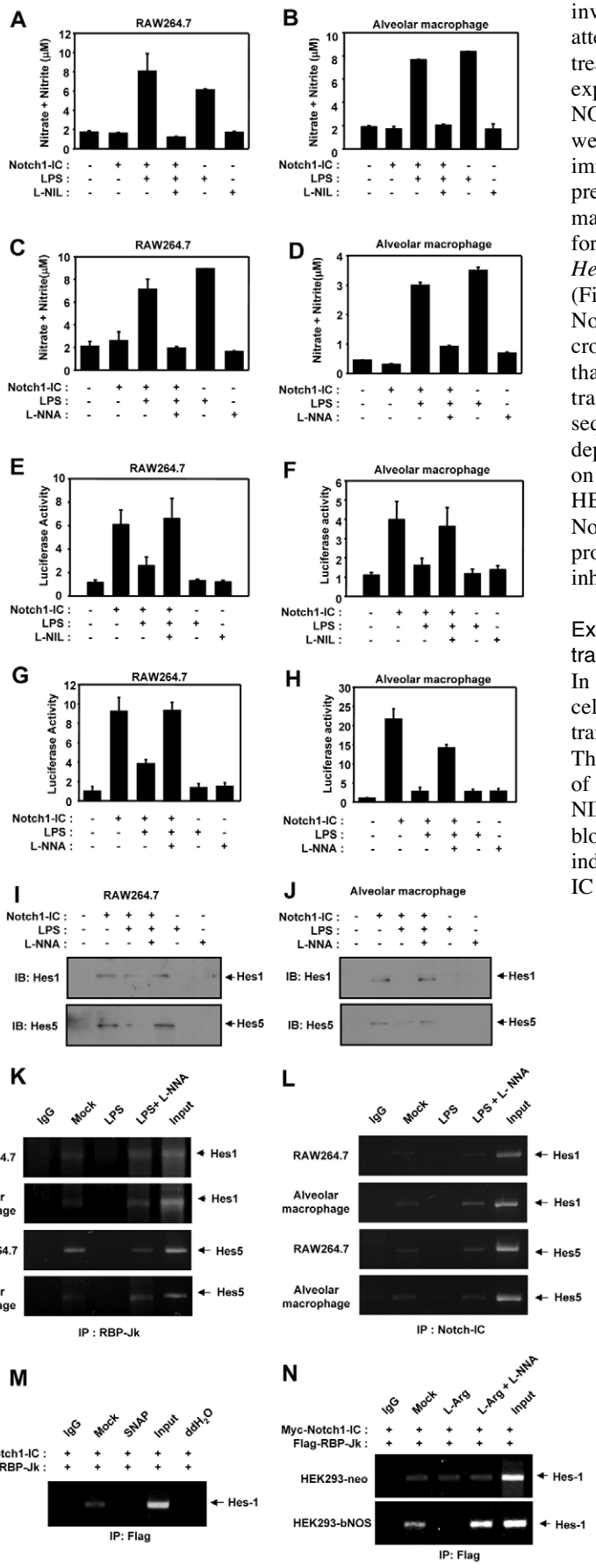


Fig. 1. LPS inhibits Notch1 transcriptional activity. (A) RAW264.7 cells were transfected for 24 hours with the expression vector for *4×CSL-Luc* and *Notch1-IC*, along with *lacZ*, and then exposed to the indicated amount of LPS for 24 hours. (B-D) RAW264.7 cells transfected for 24 hours with the indicated combinations of expression vector for *4×CSL-Luc* (B), *Hes1-Luc* (C) or *Hes5-Luc* (D) and *Notch1-IC* along with *lacZ*, and then exposed to 5 μg/ml LPS for 24 hours. The cells were then lysed and assayed for luciferase activity. The activity of the luciferase reporter in each of the samples was then normalized according to the β-galactosidase activity measured in the same sample. (E) The cell lysates were also subjected to immunoblotting analysis with the anti-Hes1 or anti-Hes5 antibody (top panels). THP-1 cells were treated with IL-1 (5 ng/ml) for 16 hours (middle panel). Jurkat T cells were exposed to UV light (60 J/m^2) (bottom panel). The cells were then treated with 200 μM SNAP for 8 hours. The cell lysates were subjected to immunoblotting analysis with the anti-Mn-SOD or anti-CD95L antibody. These results represent the means ± average deviation of triplicates from one of three independent experiments.

L-arginine (L-NNA; a general inhibitor of NOS) or L-N6-(1-Iminoethyl)-lysine (L-NIL; a specific inhibitor of iNOS) prior to LPS treatment, and we then measured the quantity of NO (Fig. 2A-D) and of Notch1-IC-mediated transcriptional activity (Fig. 2E-H) in those cells.

Both inhibitors completely suppressed increases in NO production in the RAW264.7 and alveolar macrophage cells stimulated by LPS (Fig. 2A-D). Upon treatment with the NOS inhibitor, Notch1-IC transcriptional activity was restored, suggesting that iNOS is directly involved in the generation of NO and the inhibition of Notch1 signaling (Fig. 2E,G). Furthermore, we attempted to determine the role of iNOS in the suppression of Notch1-IC activity, using primary cultured alveolar macrophages from rats. We found a similar result, in which NOS-inhibitor treatment rescued the LPS-induced suppression of Notch1-IC activity (Fig. 2F,H). In addition, western blot analysis revealed that Notch1-IC transfection increased the steady-state levels of Hes1 and Hes5 proteins, which are the products of the genes



involved in signaling downstream of Notch; however, LPS attenuated their expression (Fig. 2I,J). Furthermore, NOS-inhibitor treatment restored the LPS-induced suppression of *Hes1* and *Hes5* expression (Fig. 2I,J). In order to investigate the possible role of NO in the suppression of endogenous Notch1 transcription activity, we conducted a series of formaldehyde-crosslinked chromatin immunoprecipitation (ChIP) experiments. First, we measured the presence of endogenous NO in the RAW264.7 and alveolar macrophage cells. Our data indicated the efficient and selective formaldehyde crosslinking of Notch1-IC with RBP-Jk and the *Hes1* or *Hes5* promoter regions, to activate their gene expression (Fig. 2K,L). Intriguingly, we observed the dissociation of the Notch1-IC and RBP-Jk complex from the formaldehyde crosslinking when in the presence of NO, thereby demonstrating that NO inhibits the binding of Notch1-IC and RBP-Jk transcriptional complexes to a specific target chromosomal sequence, thus affecting the regulation of Notch1-IC–RBP-Jk-dependent transcription. Next, we investigated the effects of NO on ectopically expressed Notch1-IC and RBP-Jk in HEK293, HEK293-neo and HEK293-bNOS cells. Transduction with Notch1-IC and RBP-Jk significantly increased binding on the *Hes1* promoter; however, exogenous and endogenous NO significantly inhibited their binding (Fig. 2M,N).

Exogenous and endogenous NO prevents Notch1-IC transcriptional activity

In order to explore the effects of NO on Notch activity in another cell line, we attempted to characterize Notch1-IC-induced transcriptional activity in the presence of an exogenous NO donor. The expression of Notch1-IC significantly induced the activation of the *4×CSL-Luc*, *Hes1-Luc* and *Hes5-Luc* reporter systems in NIH3T3 cells. Coinciding with Fig. 1, the exogenous NO generators blocked Notch1-IC transcriptional activity, and *Hes1* and *Hes5* induction (Fig. 3A). Furthermore, NAP had no effect on Notch1-IC transcriptional activity or *Hes1* and *Hes5* induction (Fig. 3B),

Fig. 2. LPS suppresses Notch1-IC transcriptional activity through NO in macrophage cells. (A-H) RAW264.7 (A,C,E,G) or alveolar macrophage (B,D,F,H) cells were transfected for 24 hours with the indicated combinations of expression vector for *4×CSL-Luc* with *Notch1-IC* along with *lacZ*. (A-J) The cells were pre-treated with 100 μM L-NIL (A,B,E,F) or 2 mM L-NNA (C,D,G,H,I,J) for 30 minutes and then exposed to 5 μg/ml LPS for 24 hours. (A-D) NO released into the culture medium was then determined by the Griess method and represents nitrate+nitrite formation per 1×10^6 cells. (E-H) Cells were lysed and assayed for luciferase activity. The activity of the luciferase reporter in each of the samples was then normalized according to the β-galactosidase activity measured in the same sample. These results represent the means ± average deviation of triplicates from one of three independent experiments. (I,J) The cell lysates were also subjected to immunoblotting analysis with anti-Hes1 or anti-Hes5 antibody. (K,L) RAW264.7 or alveolar macrophage cells were pre-treated with 2 mM L-NNA for 30 minutes and then exposed to 5 μg/ml LPS for 24 hours. (M) HEK293 cells were transfected with expression vector for Notch1-IC and RBP-Jk. The cells were then treated with 200 μM SNAP for 8 hours. (N) HEK293-neo or HEK293-bNOS cells were transfected with expression vector for *Notch1-IC* and *RBP-Jk*. The cells were pre-treated with 2 mM L-NNA for 30 minutes and then exposed to 20 mM L-Arg for 16 hours. The cells were crosslinked with formaldehyde and DNA was immunoprecipitated with the indicated antibodies. The immunoprecipitated DNA was analyzed by PCR using primers recognizing the *Hes1* or *Hes5* promoters. As a negative control, we also tested a sample with vehicle only and pre-immune IgG, and included an input sample.

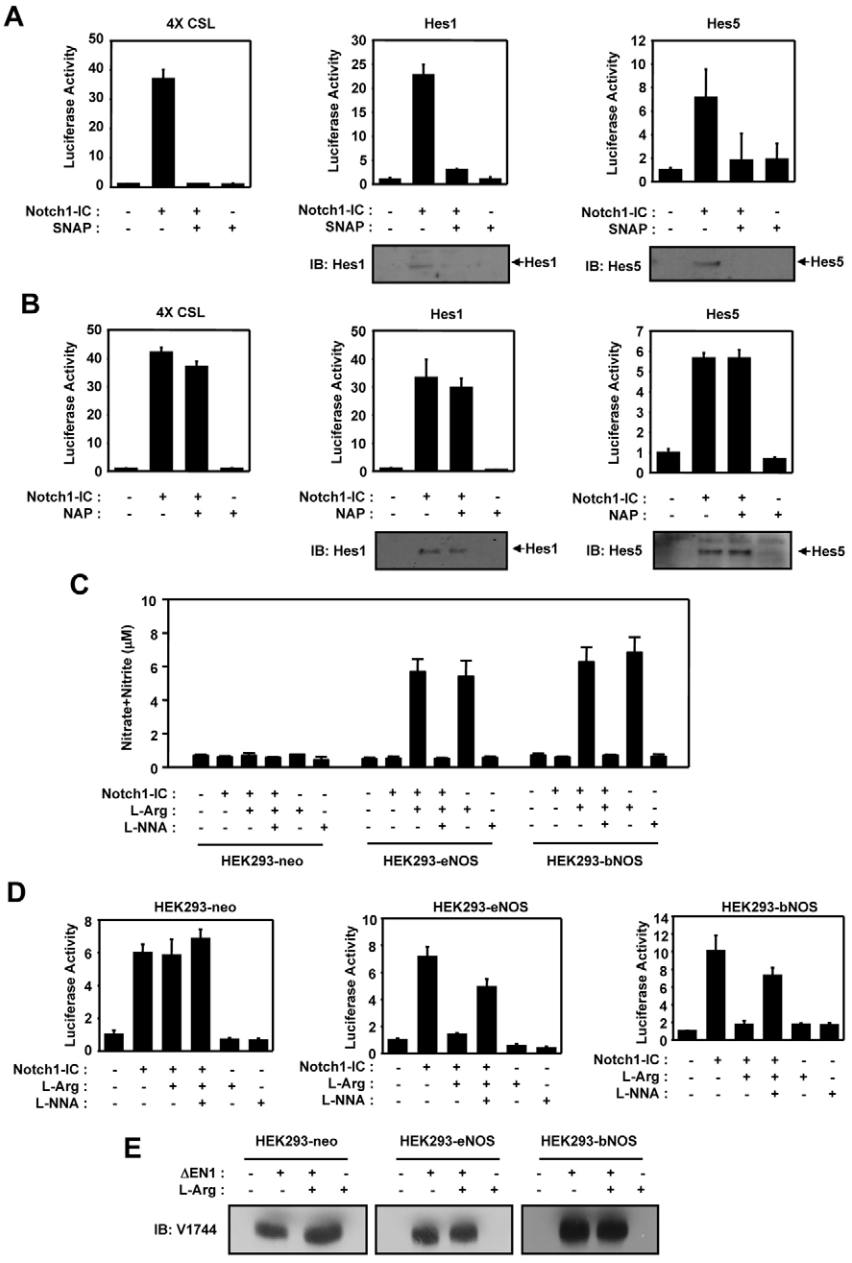


Fig. 3. NO modulates Notch transcriptional activity. (A,B) NIH3T3 cells were transfected for 40 hours with the indicated combinations of expression vector for *4×CSL-Luc*, *Hes1-Luc* or *Hes5-Luc* and *Notch1-IC* along with *lacZ*. The cells were then treated with 200 μM SNAP (A) or 200 μM NAP (B) for 8 hours. The cell lysates were subjected to immunoblotting analysis with the anti-Hes1 or anti-Hes5 antibody. (C,D) HEK293-neo, -eNOS or -bNOS cells were transfected for 32 hours with *4×CSL-Luc* and *Notch1-IC* along with *lacZ*. The cells were pre-treated with 2 mM L-NNA for 1 hour and then exposed to 20 mM L-Arg for 16 hours. (C) NO released into the culture medium was determined by the Griess method and represents nitrate+nitrite formation per 1×10^6 cells. (D) Cells were lysed and assayed for luciferase activity. The activity of the luciferase reporter in each of the samples was then normalized according to the β-galactosidase activity measured in the same sample. These results represent the means ± average deviation of triplicates from one of three independent experiments. (E) HEK293-neo, HEK293-eNOS and HEK293-bNOS cells were transfected with expression vector for *ΔEN1* and then exposed to 20 mM L-Arg for 16 hours. The cell lysates were subjected to immunoblotting analysis with the anti-V1744 antibody.

thereby suggesting that NO acts as a negative regulator of the Notch signaling pathway.

The next key issue involved whether endogenous NO, by eNOS and bNOS, was sufficient for the suppression of Notch1 activity. In order to address the effects of endogenous NO on Notch1, we used three cell lines that stably express vector: control (HEK293-neo), eNOS (HEK293-eNOS), and bNOS (HEK293-bNOS) (Fig. 3C) (Kim et al., 1997). The HEK293-neo cells did not generate endogenous NO when they were exposed to N(G)-nitro-l-arginine (L-Arg) (Fig. 3C) (Kim et al., 1997). The HEK293-eNOS and HEK293-bNOS cells were also exposed to L-Arg to induce an elevation in NO production (~5.5-7 μM/10^6 cells). The elevated level of NO in the stable cell line was quite similar to that of iNOS-induced NO generation in inflammation (7.5 μM/10^6 cells) (Fig. 3C) (Kim et al., 1997).

In the vector-only stable cell line, Notch1-IC-mediated transcriptional activity was not suppressed as a result of the endogenous NO induced by L-Arg treatment (Fig. 3D). However, in HEK293-eNOS and HEK293-bNOS cells, endogenously generated NO inhibited the Notch1-IC transcription activities of both cell lines (Fig. 3D). These data strongly indicate that endogenous NO is crucial in the suppression of Notch1 signaling. In order to determine the actual manner in which NOS is involved in the suppression of Notch1, the cells were pre-exposed to L-NNA (an L-Arg structural analog and specific inhibitor of NOS) prior to L-Arg treatment, and we then measured the transcriptional activity in those cells (Fig. 3D). Upon treatment with the NOS inhibitor, Notch1-IC transcriptional activity was restored, suggesting that NOS is directly involved in the generation of NO and the inhibition of Notch1 signaling (Fig. 3D). We also conducted the processing of the constitutive active form of Notch1 (ΔEN1) in the presence of endogenous NO. ΔEN1 cleavage and the steady-state level of Notch1-IC protein did not appear to be influenced by NO, suggesting that endogenously generated NO does not regulate Notch1 proteolytic processing (Fig. 3E).

NO-mediated cyclic GMP signaling is not involved in the suppression of Notch signaling

NO signaling is converted into cyclic guanosine-3′,5′-monophosphate (cGMP) generation via binding to the soluble form of guanylyl cyclase (sGC). Guanylyl cyclases are enzymes that convert guanosine-5′-triphosphate (GTP) to cGMP, which functions as a second messenger and appears to participate in the regulation of a variety of signaling cascades (Bellamy and Garthwaite, 2002). Thus, the next key issue concerned whether or not cGMP was indeed necessary for the facilitation of Notch signaling suppression by LPS. The transcriptional activity induced by Notch1-IC is not recovered as a result of ODQ (a blocker of soluble guanylyl cyclase) (Hussain et al., 1997) treatment, thereby suggesting that guanylyl cyclase did

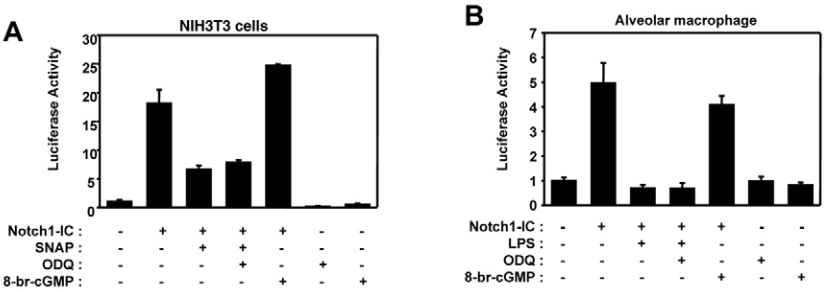


Fig. 4. NO-mediated cyclic GMP signaling is not involved in the suppression of Notch signaling. (A) NIH3T3 cells were transfected with the *4×CSL-Luc* promoter, plus/minus *Notch1-IC*, along with *lacZ*. The cells were pre-treated with 100 μM ODQ for 1 hour prior to treatment with 200 μM SNAP, or were exposed to 100 μM 8-Bromo-cGMP for 8 hours. (B) Rat primary alveolar macrophage cells were transfected for 24 hours with the indicated combinations of expression vector for *4×CSL-Luc* and *Notch1-IC* along with *lacZ*. The cells were pre-treated with 100 μM ODQ for 1 hour prior to treatment with exposure to 5 μg/ml LPS for 24 hours, or were exposed to 100 μM 8-Bromo-cGMP for 24 hours. The cells were lysed and assayed for luciferase activity. The activity of the luciferase reporter in each of the samples was then normalized according to the β-galactosidase activity measured in the same sample. These results represent the means ± average deviation of triplicates from one of three independent experiments.

not play an important role in the suppression of Notch1 signaling in the alveolar macrophages (Fig. 4A). The alveolar macrophage cells that expressed Notch1-IC and the luciferase reporter system were also exposed to 8-Bromo-cGMP. In this case, we determined that there were no significant differences in the control of Notch1-IC transcription activity (Fig. 4A). These results indicate that cGMP, which is generated by guanylyl cyclase, does not play any significant role in the suppression of Notch1 signaling in macrophages. In NIH3T3 cells, which are exogenous NO generators, S-nitro-N-acetyl-penicillamine (SNAP)-induced suppression of Notch activity was not restored as a result of ODQ treatment. Also, 8-Bromo-cGMP did not influence Notch activity (Fig. 4B), suggesting that guanylyl cyclase was not involved in the suppression of Notch signaling by LPS.

NO has no effect on the subcellular localization of Notch1-IC and RBP-Jk

We investigated the subcellular location of endogenous Notch1 and RBP-Jk within the RAW264.7 cells via immunofluorescence. In a previous report, EDTA treatment facilitated Notch1 processing, thereby increasing Notch1-IC activity (Rand et al., 2000). As shown in supplementary material Fig. S1, Notch1-IC immunoreactivity was localized mainly in the nuclei in the absence of LPS treatment. However, when the cells were exposed to exogenous LPS and/or L-NNA, Notch1-IC subcellular localization did not change substantially, suggesting that NO does not significantly regulate the Notch signaling pathway via the disruption of the cellular distribution of Notch1-IC. Similarly, we determined that the cellular localization of RBP-Jk also remained largely unchanged. This would appear to suggest that NO has no significant effect on Notch1-IC and RBP-Jk cellular localization.

NO prevents Notch1-IC–RBP-Jk physical interaction both in vitro and in vivo

Next, we demonstrated the effects of NO on the binding of Notch1-IC and RBP-Jk in intact cells. Here, HEK293 cells were transiently transfected with Myc–Notch1-IC and Flag–RBP-Jk. After 48 hours, we treated the cells with exogenous NO for 8 hours, and then assessed the affinity with which these two proteins bound to one another (Fig. 5A). We evaluated the physical association between Notch1-IC and RBP-Jk in immunocomplexes that were collected using anti-Flag antibody. In the presence of exogenous NO, the association between Notch1-IC and RBP-Jk was severely disrupted as compared with that observed in the vehicle-treated control, suggesting that NO might perform a crucial function in Notch1-IC–RBP-Jk interaction (Fig. 5A). Conversely, under conditions identical to those depicted in Fig. 5A, we analyzed the immunocomplexes that had been precipitated against anti-Flag antibody. The immunocomplexes from the NO-treated cells had lower degrees of Notch1-IC–RBP-Jk binding affinity than that witnessed in the vehicle-treated control (Fig. 5B). Our results indicate that NO, at least in part, is somehow involved in Notch signaling, via the disruption of the Notch1-IC–RBP-Jk transcription complex. In order to further ascertain whether NO plays a negative role in Notch1-IC–RBP-Jk binding in vitro, we added NO to the Notch1-IC–RBP-Jk immunocomplexes in vitro. In the presence of NO, the interaction between Notch1-IC and RBP-Jk was suppressed as compared with that observed in the absence of NO treatment (Fig. 5C,D). The interaction between glutathione-S-transferase (GST)–Notch1-IC and RBP-Jk was confirmed on bead complexes in the absence of exogenous NO. The formation of the GST–RBP-Jk and Notch1-IC complex was suppressed substantially as a result of NO treatment in vitro (Fig. 5E).

We then examined LPS-induced suppression of Notch1-IC–RBP-Jk interaction in RAW264.7 cells. The functional association between Notch1-IC and RBP-Jk was suppressed by LPS and restored by L-NNA treatment (Fig. 5F). Furthermore, we attempted to determine the in vivo inhibition of complex formation between Notch1-IC and RBP-Jk using rat alveolar macrophages. When rats are exposed to LPS through tail vein injection, NO generation is robustly increased in alveolar macrophages. Thus, we isolated the alveolar macrophages from LPS-injected rats (Park et al., 2000), and then performed in vivo coimmunoprecipitation using Notch1-IC recognition antibody. LPS treatment suppressed the functional association between Notch1-IC and RBP-Jk (Fig. 5G). Next, we evaluated the formation of the complex, in order to define more precisely the role of NO in the negative regulation of Notch1-IC–RBP-Jk–Mastermind-mediated signaling. Remarkably, the formation of the Notch1-IC–RBP-Jk–Mastermind complex was prevented in the presence of NO, suggesting that NO might play a crucial role with regards to the downregulation of Notch1-IC-mediated transcription activity, by disrupting the formation of the active complex (Fig. 5H).

In vivo nitration of Notch1-IC and nitration-induced conformational change

We then attempted to determine whether NO directly affects the Notch1–RBP-Jk complex via nitration. Therefore, we introduced anti-nitrotyrosine- or anti-nitrocystein-specific antibodies, in an attempt to characterize the modification of Notch1. Interestingly, nitrated Notch1-IC was detected by western blotting using the anti-nitrotyrosine antibody (Fig. 6A). Furthermore, to determine the mechanism of Notch1-IC inhibition by NO, we performed the titration of reactive thiol group(s) in the cysteine residues of Notch1-

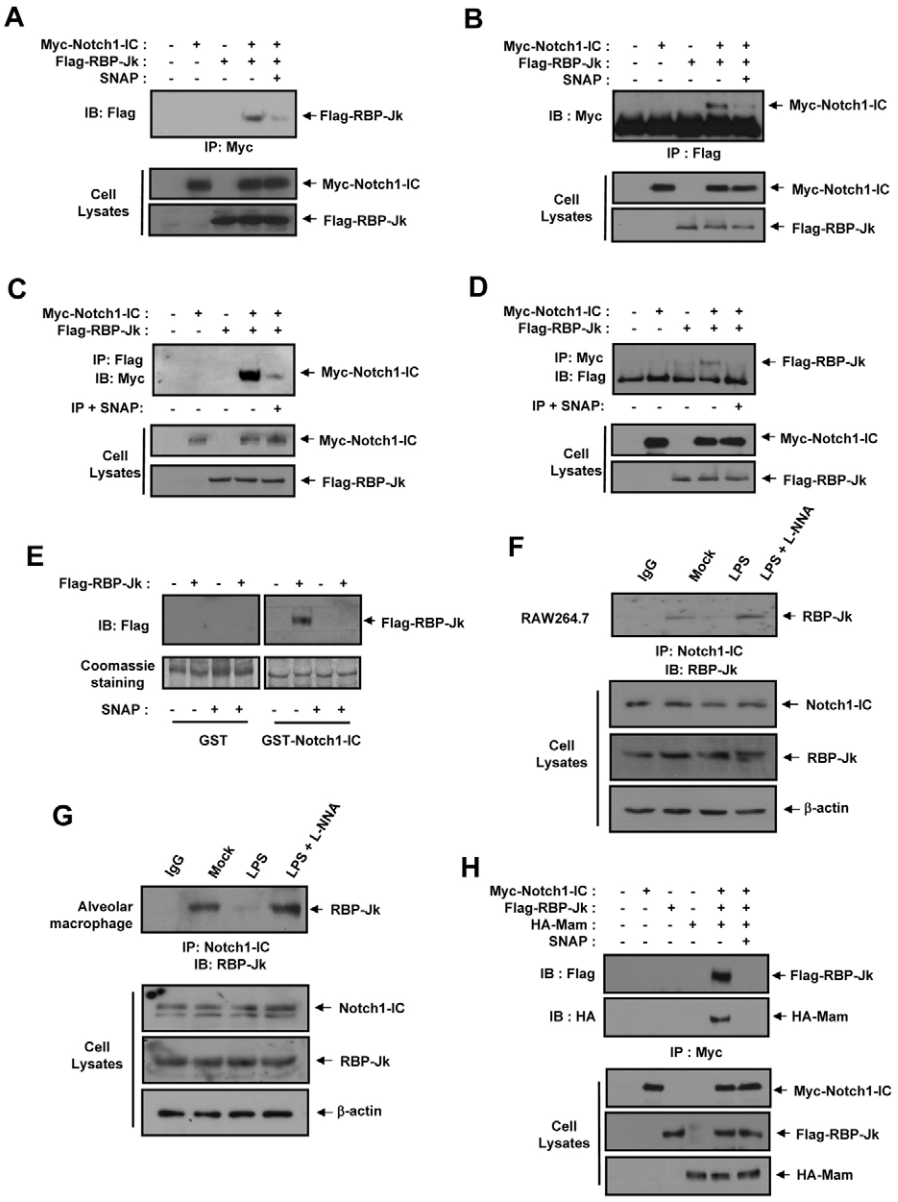


Fig. 5. NO prevents the physical interaction between Notch1-IC and RBP-Jk both in vitro and in vivo. (A,B) HEK293 cells were transfected with *Myc–Notch1-IC* and *Flag–RBP-Jk*, and then the cells were exposed to 200 μM SNAP for 8 hours. After 48 hours, the cells were lysed and immunoprecipitated against anti-Myc (A) or anti-Flag (B) monoclonal antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-Flag monoclonal antibody. (C,D) HEK293 cells were transfected with *Myc–Notch1-IC* and *Flag-RBP-Jk*. The cells were lysed and immunoprecipitated against anti-Flag (C) or anti-Myc (D) monoclonal antibody, and then exposed to 200 μM SNAP for 1 hour on ice. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-Myc monoclonal antibody. (A-D) The expression of Notch1-IC or RBP-Jk was analyzed via immunoblotting using anti-Myc or anti-Flag monoclonal antibody, respectively. (E) Recombinant GST and GST–Notch1-IC proteins, immobilized on glutathione-agarose beads, were exposed to 200 μM SNAP for 1 hour on ice, and extensively washed to remove remnant SNAP. HEK293 cells were transfected with *RBP-Jk*, and then the cells were lysed and added to the immobilized proteins. The beads were extensively washed, eluted and analyzed using SDS-PAGE immunoblotting against anti-Flag monoclonal antibody. Coomassie blue staining represents immobilized proteins. (F) RAW264.7 cells were pre-treated with 2 mM L-NNA for 30 minutes and then exposed to 5 μg/ml LPS for 24 hours. The cells were lysed and immunoprecipitated against anti-Notch1-IC antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-RBP-Jk antibody. (G) Primary alveolar macrophage cells from 5 μg/ml LPS-injected rats were lysed and immunoprecipitated against anti-Notch1-IC antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-RBP-Jk antibody. (F,G) The expression of Notch1-IC or RBP-Jk was analyzed via immunoblotting using anti-Notch1-IC or anti-RBP-Jk antibody, respectively. Probing with an antibody to β-actin was used as a loading control. (H) HEK293 cells were transfected for 40 hours with the indicated combinations of expression vector for *Myc–Notch1-IC*, *Flag–RBP-Jk* and *HA-Mastermind*, and then the cells were exposed to 200 μM SNAP for 8 hours. After 48 hours, the cells were lysed and immunoprecipitated against anti-Myc monoclonal antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-Flag or anti-HA monoclonal antibody. The expression of Notch1-IC, RBP-Jk or Mastermind (Mam) was analyzed via immunoblotting using anti-Myc, anti-Flag or anti-HA monoclonal antibody, respectively.

IC with 55′-dithiobis-(2-nitrobenzoic acid) (DTNB). We observed that the cysteine residues of Notch1-IC had no reactivity with NO (Fig. 6B). This provides us with the important fact that the nitration of Notch1 at the tyrosine residue is of great importance with regards to the regulation of Notch signaling. However, we could not determine the nitration of RBP-Jk (data not shown). Thus, we were able to conclude that the NO-mediated suppression of Notch signaling is controlled via nitration.

At this point, we attempted to determine the reasons for the NO-mediated dissociation of Notch1-IC and RBP-Jk, and the suppression of Notch signaling. We postulated that nitration might trigger conformational changes, thereby preventing the association of Notch1-IC and RBP-Jk. In order to test this hypothesis, we assessed any conformational changes occurring to purified Notch1-IC as the result of nitration, using intrinsic fluorescence. Purified Notch1-IC proteins exhibited intrinsic fluorescence following excitation at a wavelength of 278 nm. Tryptophan fluorescence tended to be sensitive to the polarity of the surrounding environment, and typically shifted from a maximum emission of 350 nm in water to 310-340 nm in non-polar protein regions. The maximum emission of Notch1-IC protein was 343 nm, indicating that the tryptophan residues in Notch1-IC were distributed between non-polar and polar environments (Fig. 6C). When purified Notch1-IC was exposed to exogenous NO, the emission maximum underwent a blue-shift (5 nm) and the fluorescence intensity was reduced by approximately 50%, as compared with that recorded in conjunction with the native Notch1-IC in the absence of NO (Fig. 6C).

We then examined LPS-induced nitration of endogenous Notch1-IC in RAW264.7 cells. Here, we detected the nitrotyrosine modification of endogenous Notch1-IC in LPS-treated cells, but

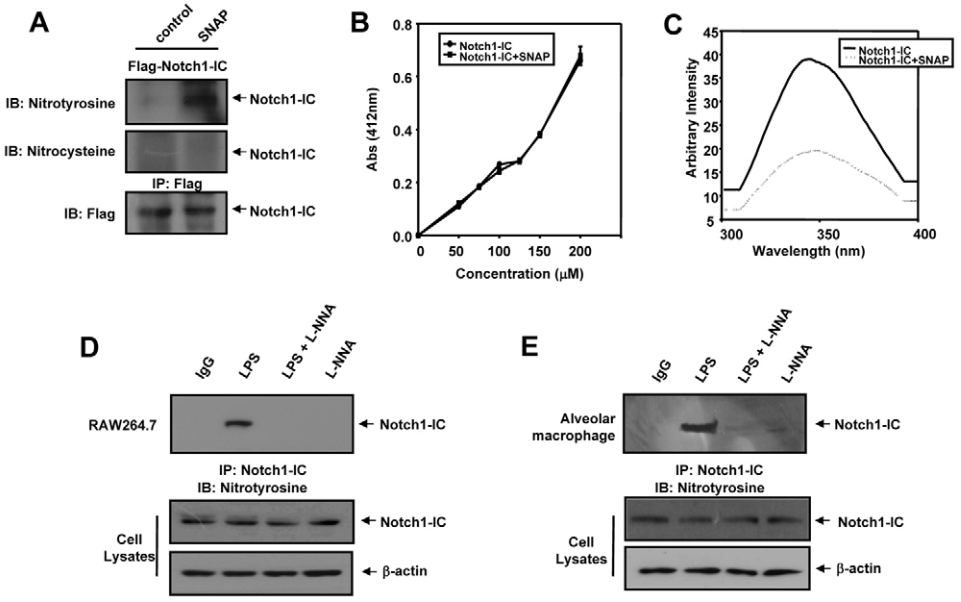


Fig. 6. Nitration of the Notch tyrosine residue 1905 mediated a conformational change. (A) HEK293 cells were transfected with *Myc–Notch1-IC*, after which the cells were exposed to 200 μM SNAP for 8 hours. The cells were lysed and immunoprecipitated against anti-Myc monoclonal antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-nitrocysteine or anti-nitrotyrosine antibodies. (B) Various concentrations of purified Notch1-IC (0, 50, 75, 100, 125, 150 or 200 μM) were pre-treated with 200 μM SNAP for 30 minutes on ice and then incubated with 0.20 mM DTNB. Maximum absorbance (Abs) was obtained at 412 nm. (C) The purified Notch1-IC proteins were exposed to 200 μM SNAP and dissolved in 50 mM Tris-HCl at a pH of 7.4. The intrinsic fluorescence spectra were acquired at an excitation wavelength of 278 nm, and excitation and emission slits of 5 nm. We conducted an emission wavelength scan from 300 nm to 400 nm. (D) RAW264.7 cells were pre-treated with 1 mM L-NNA for 30 minutes and then exposed to 5 μg/ml LPS for 24 hours. The cells were lysed and immunoprecipitated against anti-Notch1-IC antibody. (E) Primary alveolar macrophage cells from 1 mM L-NNA- and 5 μg/ml LPS-injected rats were lysed and immunoprecipitated against anti-Notch1-IC antibody. (D,E) The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-nitrotyrosine antibody. The expression of Notch1-IC or RBP-Jk was analyzed via immunoblotting using anti-Notch1-IC or anti-RBP-Jk antibody, respectively. Probing with an antibody to β-actin was used as a loading control.

not in L-NNA pre-treated cells (Fig. 6D). Furthermore, we tried to determine the in vivo modification of Notch1-IC using rat alveolar macrophages. After the rats were exposed to LPS through tail vein injection, we isolated the alveolar macrophages from rats injected with LPS, with or without L-NNA (Park et al., 2000), and then performed immunoprecipitation and immunoblotting using Notch1-IC and nitrotyrosine recognition antibodies. Ultimately, we determined the tyrosine modification of Notch1-IC in the LPS-treated rats in vivo (Fig. 6E).

Mapping of nitrated tyrosine residues in Notch1-IC

To study the mechanism of nitration of Notch1-IC, we first determined the domain(s) of Notch1-IC responsible for its nitration. We constructed a series of Notch1-IC deletion mutants and tested their effects on nitration by NO. HEK293 cells were transfected with Notch1-IC, Notch1-IC-N, Notch1-IC-ΔNΔC or Notch1-IC-C, and were then treated with the NO donor SNAP. We then performed immunoprecipitation and immunoblotting using anti-Flag and anti-nitrotyrosine antibodies. We determined the tyrosine nitration of Notch1-IC and Notch1-IC-N; NO had no effect on the tyrosine modification of Notch1-IC-ΔNΔC and Notch1-IC-C (Fig. 7A). The N-terminal of Notch1-IC contains three conserved tyrosine residues: Y1905, Y1928 and Y2064. To identify the tyrosine residue(s) targeted by NO, we constructed mutant proteins in which each of these three residues was replaced with phenylalanine (Y1905/1928F, Y1928/2064F and Y1905/2064F) in Notch1-IC, and then examined their sensitivity to NO. The mutation of Y1928/2604F did not abolish the nitration level of Notch1-IC, whereas the mutations of either Y1905/1928F or Y1905/2064F significantly reduced it (Fig. 7B), supporting that Y1905 is a possible target site for NO in Notch1-IC. Next, we examined the effect of NO on the transcriptional activity of Notch1-IC (Y1905F) in NIH3T3 and RAW264.7 cells. SNAP or LPS pre-treatment did not prevent Notch1-IC (Y1905F) activities in NIH3T3 or RAW264.7 cells, respectively (Fig. 7C). Moreover, we ascertained that Notch1-IC (Y1905F) is resistant to the NO-induced suppression of physical binding with RBP-Jk, which implies that the NO-induced nitration of Notch1-IC is crucial for the downregulation of Notch1 signaling (Fig. 7D).

Discussion

To our knowledge, our data provide the first evidence for crosstalk between Notch1-IC–RBP-Jk and the NO signaling pathway. The activation of Notch via ligand binding induces the sequential processing of Notch1, and the intracellular domain of Notch undergoes nuclear translocation (Gordadze et al., 2001; Kao et al., 1998). Therefore, Notch1-IC induces the expression of Hes1, Hes5 and other members of the bHLH protein family (de Celis et al., 1996; Iso et al., 2002; Iso et al., 2001; Jennings et al., 1994; Tapanes-Castillo and Baylies, 2004). Here, we determined that Notch signaling is regulated by NO via the nitration of Notch, with modification occurring at the tyrosine residue.

Previous reports have indicated the possible involvement of Notch signaling in inflammation. NF-kB signaling performs a principle function in inflammation by its regulation of a variety of gene expressions, particularly iNOS induction (Bethea et al., 1998). However, NF-kB signaling is known to be negatively or positively regulated by Notch via direct binding with the p50 subunit of the NF-kB complex (Shin et al., 2006; Wang et al., 2001). Under inflammatory conditions, levels of endogenous NO increase significantly and this is thought to be responsible for the differential expression of a host of genes. A recent report showed that NO generation is diminished in activated macrophages, and there is upregulation of Notch1 and Jagged mRNA and protein levels in a p38 MAPK-dependent manner (Monsalve et al., 2006). In human liver, Notch1, but not other Notch receptors, was upregulated, dependent upon iNOS, and iNOS expression also facilitated Notch signaling by inducing the nuclear translocation of its intracellular domain and the expression of a transcriptional target, the Hairy and enhancer of split (Hes)1 (Ishimura et al., 2005). A role of

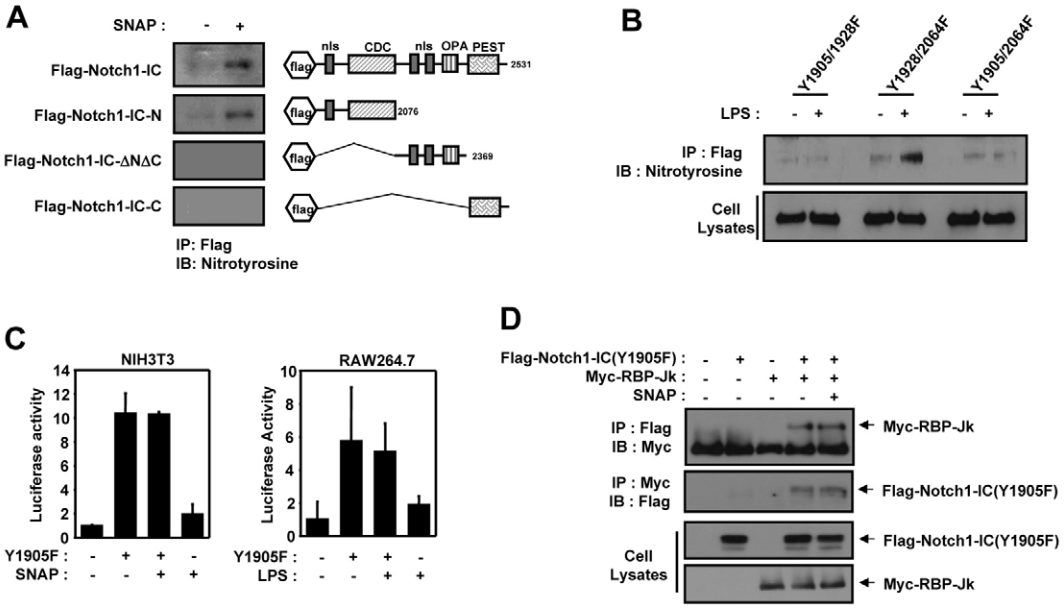


Fig. 7. Mapping of nitrated tyrosine residues in Notch1-IC. (A) HEK293 cells were transfected with *Flag–Notch1-IC*, *Flag–Notch1-IC-N*, *Flag–Notch-IC-ΔNΔC* or *Flag–Notch1-IC-C*, after which the cells were exposed to 200 μM SNAP for 8 hours. The cells were lysed and immunoprecipitated against anti-Flag antibody and immunoblotted against anti-nitrotyrosine antibodies. (B) RAW264.7 cells were transfected for 24 hours with the indicated expression vectors for *Flag–Notch1-IC (Y1905/1928F)*, *Flag–Notch1-IC (Y1928/2064F)* or *Flag–Notch1-IC (Y1905/2064F)* and then exposed to 5 μg/ml LPS for 24 hours. The cells were lysed and immunoprecipitated against anti-Flag antibody. The immunocomplexes were analyzed via SDS-PAGE and immunoblotting against anti-nitrotyrosine or anti-Flag monoclonal antibody. (C) NIH3T3 and RAW264.7 cells were transfected with the indicated expression vector for 4×CSL-Luc, β-galactosidase and Notch1-IC (Y1905F), and then exposed to 200 μM SNAP for 8 hours or 5 μg/ml LPS for 24 hours, respectively. The cells were then lysed and assayed for luciferase activity. The activity of the luciferase reporter in each of the samples was normalized according to the β-galactosidase activity measured in the same sample. These results represent the means ± average deviation of triplicates from one of three independent experiments. (D) HEK293 cells were transfected with *Flag–Notch1-IC (Y1905F)* and *Myc–RBP-Jk*, after which the cells were exposed to 200 μM SNAP for 8 hours. The cells were lysed and immunoprecipitated against anti-Flag or anti-Myc antibody and immunoblotting was conducted against anti-Myc or anti-Flag antibody.

inflammation in modulating the extent of angiogenesis has been shown for many organs. A recent study has provided an approach and baseline data to address the expression of genes, by examining functionally altered endothelial cells at sites of angiogenesis in tumors, inflammation or other pathological conditions (Favre et al., 2003). This report indicates that Notch-related genes are involved in vascular development and angiogenesis (Favre et al., 2003). However, the functional correlation between Notch and inflammatory signaling remains poorly understood. Our results demonstrate that Notch transcriptional activity was inhibited by the presence of endogenous NO in intact cells, thereby suggesting that NO, which was triggered by inflammation signals, might also involve the suppression of the transcriptional activity of Notch.

Notch1-IC and RBP-Jk exist predominantly in the nucleus. The subcellular locations of these two proteins can be observed in the nucleus in both the presence and absence of NO, suggesting that NO does not influence the subcellular distribution of Notch1-IC and RBP-Jk to any significant degree. Furthermore, we determined that the Notch1-IC and RBP-Jk transcription complexes could be dissociated from the *Hes1* promoter region in intact cells. This result suggests that NO plays a negative role in the regulation of the formation with the *Hes1* promoter in Notch1-IC and RBP-Jk transcriptional complexes. Our subsequent experiments demonstrated that Notch1-IC and/or RBP-Jk appear to be impaired by endogenous NO with regards to binding activity, both in vivo and in vitro. Furthermore, RBP-Jk is a DNA-binding transcription factor that regulates the transcription of Notch target genes by interacting with co-regulators. Transcriptional activation requires the displacement of co-repressors from RBP-Jk by Notch1-IC, and the recruitment of the co-activator protein Mastermind to a groove at the interface between Notch-IC and RBP-Jk (Jeffries et al., 2002; Kovall, 2007; Wilson and Kovall, 2006). We determined that there is negative regulation of Notch1-IC–RBP-Jk–Mastermind complex formation by NO. Obviously, we are unable to dismiss the possibility of their direct interaction in processes such as nitration.

Despite an apparently simple diatomic structure, NO has a wide variety of functions in both physiology and pathology, and within every major organ system. Two particular modifications have recently received much attention: S-nitrosylation of cysteine residues to produce S-nitrosothiol and the nitration of tyrosine residues to produce nitrotyrosine. NO-mediated post-translational modification is a well-known essential feature of diverse cellular signaling, including inflammation, neurodegeneration and aging (Benhar et al., 2006; Gaston et al., 2006; Lipton et al., 2007; Nakamura et al., 2007; Nakamura and Lipton, 2007; Satoh and Lipton, 2007). A recent study determined that Notch protein can be modified by endogenous NO in aging skeletal muscle (Kanski et al., 2005); the results of this study also implied that nitration might play an important role in the regulation of Notch signaling. Therefore, we can expect that the functional relationship between Notch and NO signaling is based, to some extent, on nitration. Our findings might shed some light on the functional roles played by Notch in the context of inflammation. We determined that the nitration of Notch1 at the 1905 tyrosine residue exerts regulatory effects on Notch signaling. This is the first finding suggesting that

Notch protein modification by nitration might contribute to the functional regulation of Notch signaling.

In conclusion, the findings of this study amply demonstrate that endogenous NO, which can be generated under inflammation conditions, is capable of suppressing Notch signaling via protein nitration. Our results provide further support for the existence of signal crosstalk in other contexts, including inflammation and wound healing, in which both signaling pathways are determined to play important roles.

Materials and Methods

Cell culture

RAW264.7, NIH3T3, HEK293 and HEK293 cells stably expressing NOS (Kim et al., 1997) were all separately cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with an atmosphere containing 5% CO_2. Rat or mouse alveolar macrophages were isolated from the lungs of Sprague-Dawley rats or BALB/c mice as described elsewhere (Park et al., 2000). The isolated alveolar macrophages were grown in DMEM without fetal bovine serum for 1 hour, and were then cultivated in the same medium containing 3% fetal bovine serum. All immune cells were obtained from BALB/c mice. TG-elicited macrophages were obtained as reported previously (Tannenbaum et al., 1988).

Cell transfection

For transfection, cells were grown in adequate dishes to ~50-60% confluence, and were transiently transfected with expression vectors using calcium phosphate for the NIH3T3 and HEK293 cells (Park et al., 2001). The RAW264.7 cells were transfected with expression vectors by using Fugene 6 reagent (Roche), and the alveolar macrophage cells were transfected with expression vectors by using Metafectin reagent (Biontex), following the manufacturer's recommendations. The amount of transfected DNA was kept constant by the addition of appropriated amounts of the parental empty vectors. β-galactosidase or GFP were used as internal controls for transfection efficiency.

Luciferase reporter assay

The luciferase assay was conducted as described previously (Park et al., 2001). The luciferase reporter plasmids were under the control of *4×CSL-Luc* (a four-time repeating section of the RBP-Jk target sequence, CGTGGGAA, with the luciferase gene), *Hes1-Luc* (–467 to +46 of the *Hes1* promoter with the luciferase gene) and *Hes5-Luc* (–800 to +32 of the *Hes5* promoter with the luciferase gene). In brief, Myc–Notch1-IC and the Notch transcription reporter system (*4×CSL-Luc*, *Hes1-Luc* or *Hes5-Luc*) were transfected along with *lacZ* in either NIH 3T3 cells or 293-NOS cell lines (12-well plates). After 48 hours of transfection, the cells were treated with SNAP or L-Arg and L-NNA. Then, the cells were lysed using chemiluminescent lysis buffer and were analyzed with a Luminometer (Berthold) for the luciferase assays. The luciferase reporter activity in each sample was normalized according to the β-galactosidase activity, which had been measured in the same sample.

Determination of nitrate and nitrite

The measurement of nitrate+nitrite concentration was performed using a commercial nitrate/nitrite assay kit (Cayman) with samples run in triplicate, as described previously (Park et al., 2000). This assay involves the conversion of nitrate to nitrite using nitrate reductase. A Greiss reagent is then added to convert the nitrite into a purple compound, and measurement of the absorbance of this compound indirectly determines the nitrate+nitrite concentration.

ChIP assay

The cells were washed with PBS and crosslinked with 1% formaldehyde at room temperature for 10 minutes. After crosslinking, the cells were treated with 0.125 M glycine, which halted the crosslinking reaction. The cells were then washed twice with ice-cold PBS and treated with 1:5 diluted trypsin at a concentration of 1 ml/plate for 10 minutes at 37°C. The cells were then sequentially collected, incubated for 15 minutes at 30°C in 100 mM Tris-HCl (pH 9.4) and centrifuged for 5 minutes at 2000 ***g***. The cells were washed with 1 ml of ice-cold PBS, buffer I [0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5)] and buffer II [200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES (pH 6.5)]. Then, the cells were resuspended in 300 μl (containing cell pellet volumes) of lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl, (pH 8.1)] and protease inhibitor cocktail (PMSF, aprotinin, leupeptin), and each of the samples was sonicated three times for 10 seconds at a strength setting of 3 (Ultrasonic Processor, GE 600). After sonication, the lysates were centrifuged for 10 minutes. The supernatants were then collected and immunoprecipitated with specific antibodies. The immunoprecipitation was conducted overnight at 4°C, using specific antibodies. Next, 2 μg of salmon sperm DNA and protein A-agarose (Peptron) were added to the samples, which were then left to stand for 1 hour at 4°C. After immunoprecipitation, the precipitates were washed for 10 minutes each in sequential mixtures of TSE I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl] and buffer III [0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)]. The precipitates were then washed three times with TE buffer and extracted three times with elution buffer (1% SDS, 0.1 M $NaHCO_3$). The elutants were heated at 65°C overnight in order to reverse the formaldehyde crosslinking. The eluted DNAs were purified and 50 μl of autoclaved ddH_2O was added. For the PCR, 1 μl of each of the sample DNAs was amplified for 35 cycles and then visualized with agarose gel.

Co-immunoprecipitation

Cells were washed with ice-cold PBS and lysed with RIPA lysis buffer. The lysates were then subjected to immunoprecipitation with specific antibodies. The immunoprecipitation was conducted overnight at 4°C. After the overnight incubation, protein A-agarose was applied to the samples for 3 hours at 4°C. Then, the precipitates were washed three times in ice-cold PBS and 5× protein sample buffer was added. The precipitates were separated by SDS-PAGE and visualized via immunoblotting.

In vitro binding assay

The whole-cell lysates of the HEK 293 cells, which had been transiently transfected with the indicated expression vectors, were incubated with pre-purified GST or GST–Notch1-IC immobilized onto GSH-agarose beads, for 10 hours at 4°C. The GST-fusion proteins were expressed in *Escherichia coli*, using pGEX-4T (Pharmacia), and purified with glutathione-agarose (Sigma), as was previously described (Park et al., 2001). The precipitates were extensively washed three times with PBS and then analyzed via immunoblotting.

Detection of nitration

Sprague-Dawley rats or BALB/c mice were injected with 5 μg/ml LPS and/or 1 mM L-NNA into the tail vein. After 24 hours, alveolar macrophage cells were isolated from the lungs. The cells were then lysed in RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonident P–40, 0.5% dexoycholate, 10% SDS]. The lysates were quantified using Bradford reagent and then the proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After SDS-PAGE, the gels were transferred to PVDF membranes (Millipore). The membranes were blocked with 5% non-fat milk in PBS-T buffer (PBS buffer containing 0.1% Tween 20) and then incubated with anti-nitrotyrosine or anti-nitrocysteine antibody (Calbiochem). The nitrated proteins were visualized using horseradish peroxidase-conjugated antibody against goat IgG (Amersham Biosciences), with the SuperSignal Chemiluminescent detection system (PIERCE).

Titration of thiol groups using DTNB

The purified Notch1-IC proteins were pre-incubated with 200 mM SNAP in 100 mM Tris-HCl (pH 7.0) at 25°C and then treated with 0.20 mM DTNB (in 50 mM HEPES, pH 7.0) to give a final volume of 1.0 ml. After incubation, the absorbance at 412 nm was determined against a control buffer and the reagents (Ellman, 1959).

Fluorescence measurements for structural change

Native Notch1-IC proteins were prepared as previously described (Park and Park, 1998). The steady-state fluorescence measurements were conducted on a Shimadzu RF-5301 PC spectrofluorophotometer, with the sample compartment maintained at 22°C. We used a 150 W xenon source. The slit-width was fixed at 5 nm for both excitation and emission. Unless otherwise stated, the samples were excited at a wavelength of 278 nm, and emission was monitored at between 300 and 400 nm. Each of the recorded spectra was taken as an average of three separate scans and we corrected for the background fluorescence of the relevant control. The intrinsic fluorescence of the native Notch1-IC (~3-6 μg of protein/ml) was measured routinely in 50 mM Tris-HCl at a pH of 7.4.

Immunofluorescence staining

Assays were conducted as previously described with RAW264.7 cells plated at 1×10^5 cells per well onto cover slips (Fisher). A total of 0.5 μg of appropriate DNA per well was then transfected using Geneporter2 (Genetherapysystems). The transfected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), and then permeabilized with 0.1% triton X-100 in PBS. Goat anti-Notch1-IC (Santa Cruz) and rabbit anti-RBP-Jk (Santa Cruz) antibodies were used as primary antibodies at a dilution of 1:100. Fluorescein-conjugated anti-goat or Rhodamine Red-conjugated anti-rabbit secondary antibody (1:100) was added, then stained with 4′,6-diamidino-2-phenyl-indole dihydrochloride (DAPI). The stained cells were evaluated for localization via fluorescence microscopy (Leica DM LB2).

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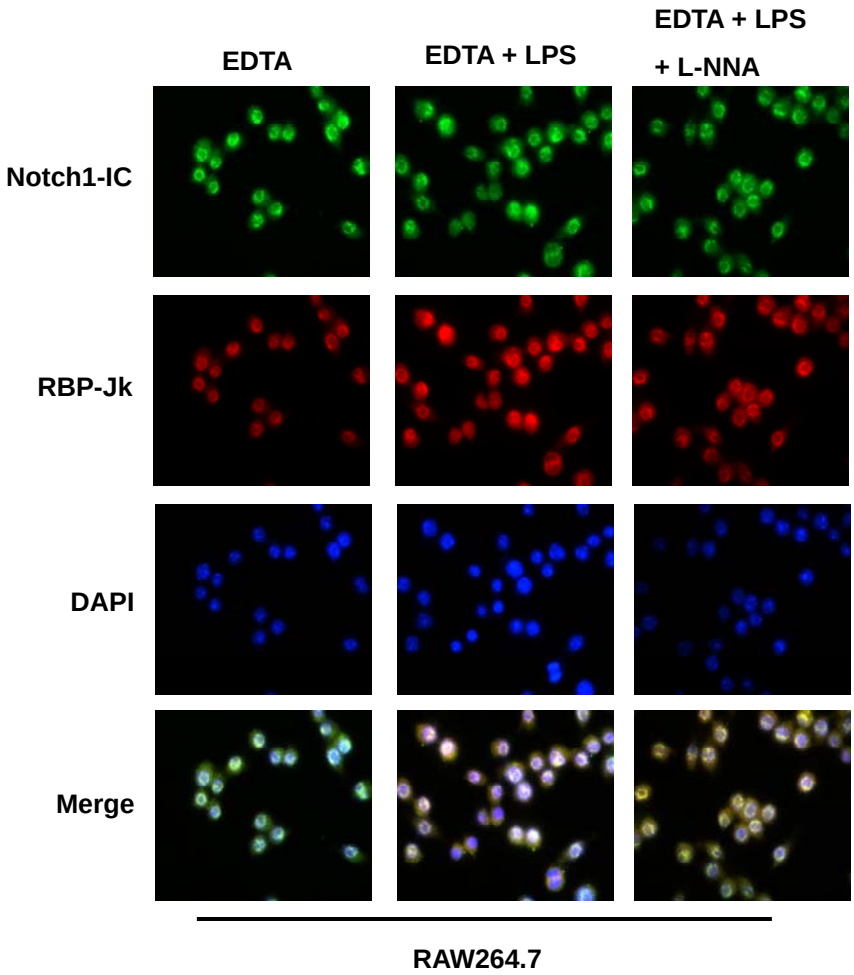

Supplemental Figure S1. NO has no effect on Notch1-IC and RBP-Jk subcellular localization.

RAW264.7 cells growing on slides were pretreated with 2 mM L-NNA for 30 min and then exposed to 5 μg/ml LPS for 24 hr. After pretreatment, cells were exposed to 0.5 mM EDTA for 15 min at 37°C, allowed to recover in complete media for 2 hr and then fixed and co-stained with antibodies to Notch1-IC (Green) and RBP-Jk (Red).