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.

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; So 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 ubiquitination 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; Breit 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 sulphydryl 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×CSSLuc 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×CSSLuc 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-

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. 2FH). 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.

Fig. 1. LPS inhibits Notch1 transcriptional activity. (A) RAW264.7 cells were transfected for 24 hours with the expression vector for 4×CSSLuc 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×CSSLuc (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/m²) (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.
involved in signaling downstream of Notch; however, LPS attenuated their expression (Fig. 2J). Furthermore, NOS-inhibitor treatment restored the LPS-induced suppression of Hes1 and Hes5 expression (Fig. 2J). 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). 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).

**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 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 mean ± 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 200 μM SNAP for 8 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.
Negative regulation of Notch signaling by LPS 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/10⁶ 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/10⁶ 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 NO 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’-monophosphatase (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 not participate in the regulation of Notch signaling by LPS.
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 Notch1 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 nitrtaion of Notch1-IC and nitrtaion-induced conformational change**

We then attempted to determine whether NO directly affects the Notch1–RBP-Jk complex via nitrtaion. 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-
Negative regulation of Notch signaling by LPS

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
The expression of Notch1-IC or RBP-Jκ was analyzed in macrophage cells from 1 mM L-NNA- and 5 μM SNAP-treated rats in vivo (Fig. 6E).

To study the mechanism of nitration of Notch1-IC, we first determined 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 nitration 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-ΔNAC 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 nitration of Notch1-IC-ΔNAC or 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. Mutation of Y1928/2064F 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-Jκ, 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-Jκ 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-κB 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-κB signaling is known to be negatively or positively regulated by Notch via direct binding with the p50 subunit of the NF-κB 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...
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 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.

Fig. 7. Mapping of nitrated tyrosine residues in Notch1-IC. (A) HEK293 cells were transfected with Flag–Notch1-IC, Flag–Notch1-IC-N, Flag–Notch1-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 immunoblottling was conducted against anti-Myc or anti-Flag antibody.
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% CO2. 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 5% fetal bovine serum. All immune cells were obtained from BALB/c mice. TG-elicted 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, CCGGGGAA, with the luciferase gene), Hes1-Luc (~467 to +46 of the Hes1 promoter with the luciferase gene) and Hes5-Luc (~750 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 luciferase assay (Calbiochem). The nitrated proteins were visualized using horseradish peroxidase-conjugated antibody against goat IgG (Amersham Biosciences), with the SuperSignal Chemiluminescent detection system (PIERC).

Detection of nitration

Sprague-Dawley rats or BALB/c mice were injected with 5 μg/ml LPS and/or 1 mM NOS inhibitor into the tails 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% Nonidet P-40, 0.5% deoxycholate, 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 containing 0.1% Tween 20) and then incubated with anti-nitrotyrosine or anti-nitrocysteine antibody (Abcam). The nitrated proteins were visualized using horseradish peroxidase-conjugated antibody against goat IgG (Amersham Biosciences), with the SuperSignal Chemiluminescent detection system (PIERC).

Fluorescence measurements for structural change

Native Notch-1C 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 sought 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 Notch-1C (~3-6 μg of protein/ml) was measured routinely in 50 mM Tris-HCl at a pH of 7.4.

Immunochemical staining

Assays were conducted as previously described with RAW264.7 cells plated at 1×106 cells per 10 cm dish. A total of 0.5 μg of appropriate DNA per well was then transfected using Geneporter2 (Genethernity). 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-Notch-1C (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-phenylindole dihydrochloride (DAPI). The stained cells were evaluated for localization via fluorescence microscopy (Leica DM LB2).

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