NOD2-Mediated Innate Immune Signaling Regulates the Eicosanoids in Atherosclerosis

Hui-Qing Liu, Xiao-Ying Zhang, Kristina Edfeldt, Manon Ouode Nijhuis, Helena Idborg, Magnus Bäck, Joy Roy, Ulf Hedin, Per-Johan Jakobsson, Jon D. Laman, Dominique P. de Kleijn, Gerard Pasterkamp, Göran K. Hansson, Zhong-Qun Yan

Objective—The activity of eicosanoid pathways is critical to the inflammatory and immune responses that are associated with the progression of atherosclerosis. Yet, the signals that regulate these pathways are poorly understood. Here, we address whether the innate immune signals of nucleotide-binding oligomerization domain–containing protein (NOD) 2 affect eicosanoids metabolism in atherosclerosis.

Approach and Results—Analysis of human carotid plaques revealed that NOD2 was abundantly expressed at both mRNA and protein levels by endothelial cells and macrophages. Stimulation of NOD2 in ex vivo–cultured carotid plaques by muramyl dipeptide, an extrinsic ligand of NOD2, led to release of prostaglandin E₂, upregulation of cyclooxygenase-2 and microsomal prostaglandin E synthase-1, and to downregulation of cyclooxygenase-1. NOD2 was coexpressed with cyclooxygenase-2 in lesional macrophages. NOD2-induced cyclooxygenase-2 expression in macrophages was dependent on p38 mitogen-activated protein kinase activation and was mediated by interleukin-1β and tumor necrosis factor-α. Selective lipidomic analysis of the eicosanoids released by the carotid plaques characterized the metabolites of 12-, 5-, and 15-lipoxygenase as the predominant eicosanoids that were produced by the atherosclerotic lesion in the absence of additional stimuli. Unlike the prostaglandin E₂ pathway, metabolic activity of the lipoxygenase pathways was not altered on the short-term activation of NOD2 in carotid plaques.

Conclusions—These results suggest that atherosclerosis may involve enhanced NOD2-mediated innate immunity. Activation of NOD2 preferentially upregulates the prostaglandin E₂ pathway. Nevertheless, lipoxygenase pathways, such as 12-lipoxygenase, predominate the basal synthesis and metabolism of eicosanoids in atherosclerotic plaques. These findings provide new insights into the regulation of eicosanoids in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:2193-2201.)

Key Words: atherosclerosis ■ eicosanoids ■ inflammation ■ immunity, innate ■ pattern recognition receptors ■ peptidoglycan

Innate immune activation by pathogen pattern recognition receptors has been implicated in the pathogenesis of atherosclerosis, on the basis of evidence that toll-like receptors (TLRs), such as TLR4 and TLR2, are linked to active inflammatory signaling pathways in human atherosclerotic lesions. In addition to TLRs, cytoplasmic nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs) have recently been reported as central regulators of innate and inflammatory responses in human inflammatory diseases, including atherosclerosis.

NLR comprise a diverse group of ≥25 receptors. NOD2, the NLR member to be initially characterized, contains 2 caspase recruitment domains (CARD) in its N-terminal region for signaling, a nucleotide-binding and oligomerization domain in its central region, and leucine-rich repeats in its C terminus for sensing muramyl dipeptide (MDP), a component of most types of peptidoglycan in Gram-positive and Gram-negative bacteria.

On stimulation with MDP, NOD2 oligomerizes through its central NOD domain and recruits the serine/threonine kinase receptor-interacting protein 2 (also called RICK2) through CARD–CARD interactions, ultimately activating nuclear factor κB and mitogen-activated protein kinase (MAPK) pathways. NOD2 has also been implicated in the activation of interferon response factor 3–dependent interferon-β.
production on viral RNA recognition through a receptor-interacting protein 2–independent mechanism.\textsuperscript{8}

The involvement of NOD2 in inflammatory diseases was initially suggested by the observation that mutations in \textit{CARD15}, encoding NOD2, were linked to the susceptibility to Crohn disease.\textsuperscript{9,10} \textit{CARD15} variants are also associated with an increased risk of developing angiographically documented coronary atherosclerosis and clinical destabilization of coronary plaques.\textsuperscript{11} A recent study on NOD2-deficient mice demonstrated that NOD2 inhibits the migration and proliferation of vascular smooth muscle cells on tissue injury.\textsuperscript{12} Notably, bacterial peptidoglycan, the natural ligand of NOD1 and NOD2, is present in atheromatous lesions, particularly those that showed marked inflammation and a vulnerable plaque phenotype.\textsuperscript{13} However, little is known about the involvement of NOD2 in human atherosclerosis and its relevance to vascular inflammation.

Eicosanoids are potent bioactive lipid molecules, the synthesis of which is initiated by the release of arachidonic acid (AA) from membrane glycerophospholipids through cytosolic phospholipase \textit{A}_\text{2}. Subsequently, the AA is converted into prostaglandins, leukotrienes, lipoxins, and hydroxyeicosatetraenoic acids (HETEs)—collectively termed eicosanoids—by cyclooxygenases, lipoxygenases, and terminal enzymes.

Eicosanoids have a complex profile of activities with regard to pro- and antiatherogenic processes.\textsuperscript{14} Eicosanoids affect the immune system by modulating cytokine signaling, cell

\begin{figure}
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\includegraphics[width=\textwidth]{figure1}
\caption{Enhanced expression of nucleotide-binding oligomerization domain–containing protein (NOD) 2 in atherosclerotic lesions. \textbf{A}, Analysis of NOD2 mRNA in internal mammary arteries (healthy; \textit{n}=10) and human carotid atherosclerotic plaques (atherosclerotic; \textit{n}=38) by quantitative real-time polymerase chain reaction. Box plots show relative abundance of NOD2 transcript normalized to cyclophilin A. \textit{P}<0.05 vs control. \textbf{B}, Western blotting analysis of NOD2 in internal mammary arteries (healthy; \textit{n}=6) and atherosclerotic plaques of carotid arteries (atherosclerotic; \textit{n}=120). Box plots represent protein levels in arbitrary units. \textit{P}<0.05 vs the healthy. \textbf{C}, Immunohistochemical analysis of NOD2 in internal mammary arteries (healthy, \textit{left}) and atherosclerotic plaques (atherosclerotic, \textit{middle, right}) stained with anti-NOD2 (\textit{left, middle}) or an isotype control (IgG; \textit{right}). \textbf{D}, Representative images of double immunolabeling for NOD2 and CD68 (macrophage marker), CD163 (macrophage marker), or von Willebrand Factor (vWF, endothelial marker) in atherosclerotic lesions. DAPI indicates 4',6-diamidino-2-phenylindole.}
\end{figure}
differentiation, survival, migration, antigen presentation, and cell death. By acting on various aspects of immunity and inflammation, eicosanoids mediate acute inflammation and govern chronic inflammation in atherosclerosis.15,16

Prostaglandin E2 (PGE2), an inflammatory lipid mediator in the eicosanoid family, has been studied extensively.15 PGE2 production depends on the activity of 2 cyclooxygenases—cyclooxygenase-1 and cyclooxygenase-2—and prostaglandin E synthases (PGESs), such as cytosolic PGES and type 1 and type 2 membrane-bound PGES (mPGES-1 and mPGES-2).15,17 Cyclooxygenase-1 and cytosolic PGES are constitutively expressed in most cell types, including vascular endothelial cells and smooth muscle cells. In contrast, basal expression of cyclooxygenase-2 and mPGES-1 is low, but both are rapidly induced at the site of inflammation.

In vivo activation of NLRs, such as NLR family, apoptosis inhibitory protein 5/NLR family, CARD-domain-containing 4 inflammasome has been shown to induce strong generation of prostaglandins and leukotrienes,18 suggesting that NLRs are the crucial regulator of eicosanoids. Here, we examined the regulation of eicosanoid pathways by a previously unrecognized NLR member, NOD2, in ex vivo–cultured human carotid plaques.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**NOD2 Is Abundant in Atherosclerosis**

To characterize the involvement of NOD2 in atherosclerosis, we first measured NOD2 transcript levels in 38 human carotid plaques. NOD2 transcript levels were upregulated 4-fold in atherosclerotic plaques versus healthy mammary arteries (Figure 1A), consistent with the significant increase in NOD2 protein in atherosclerosis, as validated in an analysis of 120 carotid plaque samples from a prospective cohort, AtheroExpress (Figure 1B). By immunostaining analysis, NOD2 localized preferentially to inflamed areas in the atherosclerotic lesion and was expressed by endothelial cells that lined the lumen of the diseased vessel. NOD2 was virtually undetectable in healthy arteries (Figure 1C). Double immunofluorescent staining for NOD2 and CD163, CD68, or von Willebrand Factor demonstrated that lesional NOD2 was expressed predominantly by macrophages and endothelial cells (Figure 1D).

These expression data suggest that atherosclerosis is associated with increased NOD2-mediated innate immunity, secondary to its upregulation in endothelial cells and macrophage infiltration.

**Transcriptional Upregulation of NOD2 in Atherosclerosis**

Recent studies suggest that NOD2 transcription is regulated by proinflammatory cytokines and TLR signaling.19,20 To

![Figure 2. Selective functional lipidomic analysis of eicosanoids produced by atherosclerotic plaques. Ex vivo cultures of human carotid atherosclerotic plaque (0.1 g/well) were treated with muramyl dipeptide (MDP; 10 μg/mL) or left untreated (basal) for 24 hours. Eicosanoids in the supernatant of ex vivo cultures were measured by lipidomic analysis (see Materials and Methods). A, Spontaneous production of major eicosanoids by carotid plaques. B, MDP-induced alterations in eicosanoid production. C through I, Comparative analysis of eicosanoid production by carotid plaques under basal conditions (basal) and after stimulation with MDP. Data are presented as mean±SD; n=4. HETE indicates hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; PG, prostaglandin; PGE2, prostaglandin E receptor 2; and Tx, thromboxane.](http://ahajournals.org/doi/abs/10.1161/CIRCRESAHA.116.304589)
determine whether the increase in NOD2 expression in atherosclerosis results from transcriptional upregulation in response to immune stimuli, NOD2 and NOD1 expressions were measured in atherosclerotic plaques after treatment with lipopolysaccharide and MDP, selective agonistic ligands of TLR4 and NOD2, respectively. NOD2 was upregulated on lipopolysaccharide or MDP stimulation, whereas expression of NOD1 was unaltered (Figure I in the online-only Data Supplement). These results suggest that the increased expression of NOD2 in atherosclerotic lesions can be attributed in part to in situ transcriptional upregulation in the inflammatory milieu.

Analysis of NOD2 Responsive Eicosanoids in Human Atherosclerosis

Atherosclerotic tissues synthesize various eicosanoids, each of which can function as an innate immune signal with specific effects. To determine the eicosanoid pathways that react specifically to NOD2 activation in atherosclerosis, we performed liquid chromatography coupled with tandem mass spectrometry to simultaneously analyze representative products of the major cyclooxygenase and lipoxygenase pathways in human atherosclerotic tissue.

We observed distinct eicosanoid profiles between basal conditions and NOD2 activation. Under basal conditions, carotid plaques synthesized predominantly 6-keto-PGF\(_1\alpha\) (a metabolite of PGI\(_2\))—along with relatively little PGE\(_2\), PGF\(_2\alpha\), and the thromboxane A\(_2\) metabolite thromboxane B\(_2\). These data indicate that the intrinsic biosynthesis of PGI\(_2\) was preserved in atherosclerotic tissue (Figure 2A). Notably, activation of NOD2 in carotid plaques affected a ≈10-fold increase in supernatant PGE\(_2\) levels from baseline, whereas the levels of PGF\(_2\alpha\), 6-keto-PGF\(_1\alpha\), and thromboxane B\(_2\) rose to a lesser extent compared with their baselines (Figure 2C–2F). A similar pattern of prostaglandin responses was observed in 3 other atherosclerotic tissue cultures. These results establish that NOD2 activation in atherosclerotic tissue preferentially upregulates PGE\(_2\).

We also examined lipoxygenase pathway activity by analyzing the 5-lipoxygenase metabolite 5-HETE, 12-lipoxygenase metabolite 12-HETE, and 15-lipoxygenase-1 metabolite 13-hydroxyoctadecadienoic acid. 12-HETE was the most abundant eicosanoid, at levels ≈40-fold greater than the PGI\(_2\) metabolite 6-keto-PGF\(_1\alpha\) in carotid plaques without NOD2 stimulation (Figure 2A). 5-HETE and 13-hydroxyoctadecadienoic acid levels were 6- and 7-fold higher than those of 6-keto-PGF\(_1\alpha\), respectively. Notably, the levels of these lipoxygenase products were unaltered from baseline after NOD2 activation. These results suggest that lipoxygenase pathways, and in particular the 12-lipoxygenase pathway, dominate eicosanoid biosynthesis and metabolism in the basal condition.

Regulation of PGE\(_2\) Pathway by the Activation of NOD2 in Atherosclerosis

To determine the mechanism of NOD2-induced PGE\(_2\), we measured the expression of cyclooxygenase-2, mPGES-1, and mouse cyclooxygenase (COX)-2/prostaglandin (PG)E2 pathway and cytokines in atherosclerotic plaques. Ex vivo cultures of human carotid atherosclerotic plaque were stimulated with lipopolysaccharide (LPS; 1 μg/mL), C12DAP (1 μg/mL), or muramyl dipeptide (MDP; 10 μg/mL) in triplicate for 24 hours. A, PGE\(_2\) release was measured by enzyme-linked immunosassay; mRNA levels of COX-2 (B), microsomal prostaglandin E synthase (mPGES)-1 (C), COX-1 (D), EP\(_2\) (E), and EP\(_4\) (F) in plaque tissue were measured by quantitative real-time polymerase chain reaction (qRT-PCR). Interleukin (IL)-6 (G), IL-8 (H and I), and IL-10 (H and L) were measured in the supernatant by ELISA and in tissue by qRT-PCR on MDP treatment. Data are mean±SEM of 10 plaques. *P<0.05 vs control. PGF indicates prostaglandin E receptor 2.
cyclooxygenase-1 in atherosclerotic plaques after activation of TLR4 with lipopolysaccharide, NOD1 with lauroyl-γ-glutamyl-meso-diaminopimelic acid (C12DAP), and NOD2 with MDP. Similar to the effects of TLR4, NOD2 activation enhanced PGE\(_2\) production, which was accompanied by upregulation of cyclooxygenase-2, mPGES-1 and PGE\(_2\). By immunostaining analysis, we detected that cyclooxygenase-2 and mPGES-1 colocalized with NOD2 in lesional macrophages (Figure 3A–3F). In contrast, cyclooxygenase-1 mRNA levels fell after lipopolysaccharide, C12DAP, and MDP treatment (Figure 3A–3F). In addition, stimulation of plaque NOD2 also induced cytokine expression and release (Figure 3G–3L), indicative of an important role for NOD2 in the regulation of atherosclerosis-associated inflammation.

By immunostaining analysis, we detected that cyclooxygenase-2 colocalized with NOD2 in lesional macrophages (Figure IIA–IIC in the online-only Data Supplement). Moreover, on analysis of the data in BiKE, a human carotid plaque biobank, we also noted a positive correlation between NOD2 and cyclooxygenase-2 and mPGES-1 (Figure IID and IIE in the online-only Data Supplement). Together, these data suggest that NOD2 and the PGE\(_2\) pathway may interact in macrophages in atherosclerosis.

**PGE\(_2\) Is the Downstream Signaling Mediator of NOD2 Activation in Human Macrophages**

To examine the relationship between NOD2 and the PGE\(_2\) pathway in macrophages, we treated human monocyte-derived macrophages with MDP. MDP upregulated cyclooxygenase-2 and mPGES-1 (Figure 4A and 4B) and affected a rapid increase in PGE\(_2\) production (Figure 4C). By Western blot, we confirmed that MDP induced cyclooxygenase-2 and mPGES-1 dose dependently and downregulated cyclooxygenase-1 (Figure 4D). Stimulation of macrophage TLR4 or NOD2 also upregulated EP4 and EP2 (Figure 4E–4G). These results indicate that NOD2 elicits PGE\(_2\) by inducing the expression of enzymes that mediate its biosynthesis, and further escalates the effect of PGE\(_2\) by upregulating its receptors.

**Activation of p38 MAPK and Interleukin-1β Mediate NOD2-Induced PGE\(_2\) Production**

To determine the signaling mechanisms of NOD2-driven upregulation of the PGE\(_2\) pathway, we examined the responses of MAPK and nuclear factor-κB in macrophages to NOD2 stimulation. Stimulation of macrophages by MDP was shown to provoke robust and prolonged phosphorylation of p38 and activation of nuclear factor-κB, as evidenced by the increase in phosphorylated p65 and the decline in IκB\(\alpha\) (Figure 5A). Furthermore, MDP also weakly activated extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase (Figure 5A). However, pharmacological inhibition of individual MAPKs suggested that p38 activation was indispensable for NOD2-mediated induction of PGE\(_2\) pathways (Figure 5B).

Previous studies have suggested that interleukin-1β and tumor necrosis factor (TNF)-α are the potent inducer of PGE\(_2\).\(^{21,22}\) To verify their function in NOD2-induced PGE\(_2\), we blocked interleukin-1β and TNF-α with an interleukin-1β...
receptor antagonist (interleukin-1RA) and TNF-α–neutralizing antibody (anti–TNF-α), respectively. Consistent with previous findings, our data show that interleukin-1β and TNF-α are critically involved in the induction of cyclooxygenase-2 and PGE₂ by NOD2 (Figure 5C and 5D).

**Discussion**

Although significant progress has recently been made in understanding of NOD2-mediated innate immune mechanisms in the pathogenesis of inflammatory diseases, this study provides additional insights on NOD2 in atherosclerosis. Our findings show that human atherosclerosis is characterized by high NOD2 expression in endothelial cells and macrophages. Because NOD2 is readily induced in macrophages and endothelial cells by proinflammatory cytokines and innate immune signaling, the rise in NOD2 in atherosclerotic lesions is attributable to transcriptional upregulation in the local inflammatory milieu and massive infiltration of macrophages. Compared with the little NOD2 in health arteries, its abundance in lesions signifies that the activity of human atherosclerosis is associated with enhanced NOD2-mediated innate immunity.

To obtain direct proof of the function of NOD2 in human atherosclerosis, we developed an in vivo tissue culture system that mimics aspects of the inflammatory milieu in atherosclerosis. Using this model, we examined the function of NOD2 in regulating eicosanoid pathways on innate immune activation in atherosclerosis.
Our data also suggest that metabolites of lipoxygenase pathways, including 5-lipoxygenase, 15-lipoxygenase-1, and 12-lipoxygenase, represent the dominant eicosanoids in atherosclerosis. In particular, the levels of 12-HETE were the highest of the eicosanoids that we analyzed (Figure 2). In humans, 12-HETE is formed by oxidation of AA by 12-lipoxygenase. On the basis of potential association between 12-lipoxygenase in atherosclerosis and atherothrombotic events and on its function in eliciting inflammation, targeting the respective 12-lipoxygenase pathway is a potential therapeutic approach for atherosclerosis.

Because of limited quantities of the samples, our study was unable to assess activities of the 15-lipoxygenase pathway in human atherosclerosis. By comparative analysis of the 12- and 15-lipoxygenase pathways, a recent study reported that the expression and the activity of 15-lipoxygenase-2 are higher than other 12- and 15-lipoxygenase in human atherosclerosis. These observations suggest that in addition to 12-lipoxygenase, 15-lipoxygenase-2 is another prevalent eicosanoid pathway in atherosclerosis. However, further study is required to define the function of innate immunity in regulation of the 15-lipoxygenase-2 pathway in atherosclerosis.

Macrophages are the primary source of eicosanoids, which are fundamental to the inflammation and immune activity in atherosclerosis. Previous studies showed that activation of macrophage TLR4 leads to the increased production of PGE2 through upregulation of cyclooxygenase-2 indicative of PGE2 as a central signaling component that contributes to TLR4-mediated innate immunity. However, the potential pattern recognition receptor that governs PGE2 synthesis in human atherosclerosis has not been defined. We demonstrated that NOD2 may represent another important innate immune recognition response that regulates the PGE2 pathway in atherosclerosis. Short-term stimulation of NOD2 in macrophages evokes the production of PGE2 through upregulation of cyclooxygenase-2 and the PGE2-specific synthase mPGES-1. In addition, we showed that NOD2 is also linked to the production of cytokines that are critical to vascular inflammation. These findings establish the involvement of NOD2-mediated innate immunity in atherosclerosis.

We found that activated p38 is a central transcriptional signaling mechanism that relays NOD2-induced innate eicosanoid signaling. p38 is the most pathophysiologically significant MAPK with regard to the inflammatory responses that are associated with atherosclerosis. Activated p38 orchestrates the expression of many proinflammatory mediators, including cytokines, chemokines, and adhesion molecules, that promote atherosclerosis. p38 also governs the expression of macrophage scavenger receptors, thus controlling the uptake of oxidized low-density lipoprotein. Finally, p38 is a central signal that directs cell growth and migration.

Thus, we postulate that through activation of p38, NOD2 participates in various pathogenic processes of atherosclerosis. Consistent with previous observations, NOD2 also signals to nuclear factor-κB, interleukin-1β, and TNF-α in human macrophages. Notably, NOD2-induced interleukin-1β and TNF-α are indispensable in the upregulation of the cyclooxygenase
2-PGE$_2$ axis, possibly through autocrine regulation of MAPK. These observations increase our understanding of the NOD2-mediated signaling network in atherosclerosis.

The signal-activated generation of bioactive eicosanoids is initiated by liberating AAs from phospholipids by activation of phospholipase A$_2$ (PLA$_2$). Whether PLA$_2$ regulates NOD2-triggered PGE2 production remains to be defined. Recent studies have shown that stimulation of TLR2, TLR3, or TLR4 leads to MAPK-dependent, coordinated activation of cytosolic PLA$_2$ and secretory PLA$_2$. On the basis of resemblance of NOD2 to TLR4 in downstream signaling pathways, it is plausible that activation of PLA$_2$ by NOD2 signaling is an important mechanism in the regulation of eicosanoid pathways.

The implications of NOD2-mediated PGE2 signaling in atherosclerosis are likely to be intricate because PGE$_2$ has proinflammatory and anti-inflammatory effects depending on the context of cell type and PGE$_2$ receptor subtype. NOD2-induced PGE$_2$ production is concurrent with the enhanced expression of EP$_3$ and EP$_4$ in human atherosclerotic plaques, and EP$_4$ is preferentially upregulated in macrophages by NOD2, potentially favoring EP$_4$-related activities. These findings increase our understanding of how NOD2 shapes innate immune responses in atherosclerosis.

Leukotrienes are another group of eicosanoids mediators with significant functions in atherosclerosis. Leukotrienes are produced in various leukocytes—preferentially in macrophages and mast cells—through the concerted activity of 5-lipoxygenase and 5-lipoxygenase–activating protein in diseased vessels. In addition to transforming growth factor β and 1,25(H)D$_3$, lipopolysaccharide, and the TLR7/8 agonist Resiquimod (R-848) upregulate leukotriene B$_4$, suggesting that increased leukotriene levels result from innate immune activation. However, the significance of NOD2 in the regulation of 5-lipoxygenase pathway and biosynthesis of leukotrienes is unknown.

In summary, these results imply that atherosclerosis is accompanied by enhanced NOD2-mediated innate immune activity. NOD2 is implicated for the induction of PGE$_2$ through upregulation of cyclooxygenase-2 and mPGES-1 in lesional macrophages on activation. However, lipoxygenase pathways dominate the basal process of eicosanoid synthesis and metabolism in atherosclerosis without additional innate immune activation. This study affords new perspectives on the functional relevance of NOD2 to heightened inflammation in atherosclerosis.

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Disclosures
None.

References
Atherosclerosis has long been acknowledged to progress with chronic vascular inflammation. In this study, we demonstrate that atherosclerotic plaques express an intracellular innate immune signaling receptor, called nucleotide-binding oligomerization domain containing 2 (NOD2). Using an ex vivo culture system of human atherosclerotic plaque, we find that stimulation of NOD2 in carotid plaques upregulates cytokines that have crucial functions in inflammation. In addition, NOD2 has a significant function in the regulation of eicosanoid pathways.


**Significance**

Atherosclerosis has long been acknowledged to progress with chronic vascular inflammation. In this study, we demonstrate that atherosclerotic plaques express an intracellular innate immune signaling receptor, called nucleotide-binding oligomerization domain containing 2 (NOD2). Using an ex vivo culture system of human atherosclerotic plaque, we find that stimulation of NOD2 in carotid plaques upregulates cytokines that have crucial functions in inflammation. In addition, NOD2 has a significant function in the regulation of eicosanoid pathways, characterized by enhanced prostaglandin E2 production through the upregulation of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in lesional macrophages. Finally, we show that NOD2 signals to multiple signaling pathways that regulate inflammation, such as mitogen-activated protein kinase and nuclear factor-κB. These findings suggest the involvement of innate immune NOD2 in atherosclerosis-associated inflammation.
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Supplemental Figure I. Transcriptional upregulation of NOD2 in atherosclerotic plaques.
Levels of NOD1 mRNA (A) and NOD2 mRNA (B) were assessed in ex vivo cultured human carotid plaques 20 h after treatment with LPS (1 μg/mL) or MDP (10 μg/mL). Results are presented as mean ± SEM of 9 plaques. *p<0.05 vs the basal levels.
Materials and Methods

Human atherosclerotic tissue
For analysis of NOD2 expression, carotid plaques from the Atherosclerotic Plaque Expression (AtheroExpress) study were used. AtheroExpress is an ongoing longitudinal multicenter cohort study, initiated in 2002 and currently being executed in two Dutch hospitals: the University Medical Center Utrecht and Sint Antonius Hospital Nieuwegein. Recruitment of patients undergoing carotid endarterectomy started in April 2002. All cohort members are followed for the occurrence of adverse cardiovascular events for a minimum of 3 years. The objective of AtheroExpress is to evaluate differential atherosclerotic plaque expression of protein in relation to future cardiovascular events and patient characteristics. 1 38 additional human atherosclerotic lesions were collected from patients undergoing carotid endarterectomy at Karolinska University Hospital, Sweden. And 10 human internal mammary arteries obtained from patients undergoing coronary artery bypass surgery were used as non-atherosclerotic control arteries. Written informed consent was obtained from all participants according to the declaration of Helsinki. The investigations were approved by the Ethical Committee of Northern Stockholm and by the Medical Ethical Committees of the participating hospitals in the Netherlands.

Ex vivo atherosclerotic plaque tissue cultures
Ex vivo atherosclerotic plaque tissue cultures were set up as previously described.2 In brief, fresh plaque tissues were processed to remove calcified tissue, cut into small pieces (about 1.5 mm³), and washed with cold PBS. The tissue was distributed equally in a 48-well plate (~ 0.1 g tissue/well). 4 h after incubation in RPMI with 10% FCS, the tissues were incubated with indicated reagents. Thereafter, supernatants and tissue were snap-frozen and kept at -80 °C.

Macrophage cultures
Human peripheral blood from healthy volunteers was obtained from the Blood Center, Karolinska University Hospital, Sweden. Peripheral blood mononuclear cells (PBMC) were prepared using Lymphoprep™ gradient medium (density 1.077 g/ml; Axis-Shield, Oslo, Norway) according to the manufacturer’s instructions. Monocytes purified from PBMC were cultured for 7 days in the medium that contained 60% AIM V® medium and 30% Iscove’s Modified Eagle’s Medium (IMDM) and was supplemented with 10% inactivated human AB+ serum. The media was changed every 3 days. Subsequently, 5 × 10⁵ macrophages/ml were plated in RPMI 1640 with 1% FCS and treated with different reagents for the indicated time and concentrations at 37°C, 5% CO₂.

In some experiments, THP-1 cells were cultured in RPM1 1640 containing 2 mM glutamine; 10 mM HEPES; 1mM sodium pyruvate; 4.5g/L glucose; 1.5g/L sodium bicarbonate and 10% FCS.

RNA isolation and real time PCR
Total RNA was isolated from carotid plaque tissue or macrophages with RNeasy Mini kits (QIAGEN, Germany) with an on-column DNase digestion step. RNA quantity and quality were assessed by NanoDrop (ND-1000) Spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively. First-strand of cDNA was synthesized using random hexamers and SuperScript II (Invitrogen Life Technologies, Paisley, UK). Real-time PCR on cDNA was performed in an ABI 7900 Sequence Detector (Applied Biosystems, Carlsbad, CA) with Assay on
demand primer and probe for NOD2 (assay ID Hs01550762_g1) and Sybr Green primers as listed in the table below. Cyclophilin A was used as housekeeping gene.

### Table S1. Primer sets for SYBR-Green based real time PCR

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<th>Target gene</th>
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<th>Sequence of reverse primer (5’-3’)</th>
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<td>Cyclophilin A</td>
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### LC-MS/MS of eicosanoids

In brief, supernatant of carotid plaques was centrifuged for 1 min (14 000 rpm) to remove cellular debris. Deuterated standards of PGE$_2$, PGD$_2$, PGF$_{2\alpha}$, TxB$_2$ and 6-keto-PGF$_{1\alpha}$ were added and solid phase extraction of eicosanoids was performed on 30 mg Oasis HLB columns (Waters Corporation, Milford, MA, USA). After applying the sample, the columns were washed with 1 ml 5% (v/v) methanol in MilliQ water acidified with 0.05% (v/v) formic acid. Retained material was then eluted with 1 ml methanol and the eluent was dried under vacuum and reconstituted in 50 µl 7% acetonitrile in MilliQ water. Eicosanoid analysis was performed by LC-MS/MS on a Waters 2795 HPLC coupled to an Acquity TQ Detector triple quadrupole mass spectrometer (Waters Corporation). The mobile phase composed of MilliQ water as solvent A and acetonitrile acidified with 0.05% formic acid as solvent B. Separation of the analyses was achieved on a Synergy Hydro-RP column (100 x 2 mm, 2.5 µm particle size and 100 Å pore size) by a 45 min linear stepwise gradient running from 10 to 90% B. Eicosanoids were identified in samples by matching their MRM signal and LC retention time (Table S2) with those of a pure standard.

### Table S2. MS/MS settings for eicosanoid profiling
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</table>
| 6-keto-PGF
1α-d₄ | 16.9                 | 373.1               | 249.2             | 53      | 21            |
| 6-keto-PGF
1α    | 17.0                 | 369.1               | 245.2             | 53      | 21            |
| TxB₂-d₄      | 21.6                 | 373.1               | 173.1             | 30      | 13            |
| TxB₂         | 21.6                 | 369.1               | 169.1             | 30      | 13            |
| PGF₂α-d₄      | 22.6                 | 357.1               | 313.2             | 30      | 17            |
| PGF₂α        | 22.6                 | 353.1               | 309.1             | 30      | 17            |
| PGE₂-d₄       | 23.2                 | 355.1               | 319.2             | 29      | 10            |
| PGE₂         | 23.3                 | 351.1               | 315.1             | 29      | 10            |
| PGD₂-d₄       | 24.2                 | 355.1               | 319.1             | 17      | 10            |
| PGD₂         | 24.3                 | 351.1               | 315.1             | 17      | 10            |
| 13-HODE₁      | 40.0                 | 295.2               | 277.1             | 45      | 25            |
| 15-HETE₁      | 40.3                 | 319.5               | 219.5             | 30      | 20            |
| 12-HETE₁      | 40.9                 | 319.5               | 179.5             | 30      | 20            |
| 5-HETE₁       | 41.2                 | 319.5               | 115.4             | 30      | 20            |

**Western blotting**

Protein of carotid plaque tissues or cells was washed with PBS and harvested following indicated treatments. Total proteins from carotid plaques or cells were extracted using T-PER tissue protein extraction reagent with protease inhibitor cocktail and EDTA (Thermo Scientific, Rockford, IL). Protein concentration was measured with the BCA assay (Bio Red, Hercules, CA). Protein samples were mixed with 2× loading buffer and boiled for 5 min. An equal amount of protein per lane was separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel electrophoresis before being electroblotted onto PVDF (Polyvinylidene fluoride) membranes. The membranes were blocked in 5% non-fat milk in TBS-T (tris buffer PBS/0.1% Tween 20) and incubated with the following primary antibodies: mouse anti-human NOD2 (Cayman Chemical Co.), rabbit anti-COX-1 (Santa Cruz, CA.), mouse anti-human COX-2 (Dako, Glostrup, Denmark), mPGES-1 (provide by Dr. Per-Johan Jakobsson, Karolinska Institute), rabbit anti-human EP₂ and rabbit anti-human EP₄ (Cayman Chemical Co.), rabbit anti-human phospho-NF-
κB p65 (Ser468, Cell Signaling Technology, Beverly, MA, USA) and anti-IκBα (Cell Signaling Technology) in 3% non-fat milk overnight at 4°C, washed with TBS-T before incubation with the horseradish peroxidase-conjugated secondary antibody. To document the loading controls, the membrane was reprobed with a monoclonal antibody against α-smooth muscle actin (Sigma-Aldrich). After successive washes, the protein bands were detected with an enhanced chemiluminescence (ECL) kit (GE Healthcare, Piscataway, NJ). Optical densities of specific bands were measured with the use of the GelDoc system (Biorad, Hercules, CA) and expressed in arbitrary units.

**Immunostaining**

Acetone-fixed 10 μm cryosections were preincubated for 30 min with 5% normal fetal calf serum or 2% BSA and 3% non-fat dry milk. Subsequently, sections were incubated with rabbit anti-NOD2 antibody (Cayman Chemical Co.) at 4°C overnight, followed with biotinylated goat-anti rabbit or horse anti-mouse secondary antibody followed by avidin-biotin peroxidase complex and developed with diaminobenzidine (all from Vector Laboratories, Burlingame, CA). The specificity of the NOD2 antibody was confirmed by incubation with isotype-matched control IgG.

For double-staining, sections were first incubated with rabbit anti-NOD2 antibody (Cayman Chemical Co.) at 4°C overnight, followed with Alexa fluor 488 labeled goat anti-rabbit IgG (Life technologies, Stockholm, Sweden) for 30 min. Subsequently, the sections were incubated overnight with anti-human CD163 antibody (BioLegend, San Diego, CA), anti-CD68 antibody (BioLegend), mouse anti-human von Willebrand Factor antibody (DAKO) or anti-human COX-2 antibody (DAKO).

**Enzyme Immunoassays**

PGE$_2$, LTB4 and LTE4 in supernatants of carotid plaques and macrophage cultures were determined by enzyme immunoassays (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer’s instructions.

**Data analysis**

Prism software (GraphPad) was used for statistical analysis. Results were analyzed by Student t test or One-way ANOVA followed by the appropriate post hoc comparison. $p < 0.05$ was regarded statistically significant.

**References**


apolipoprotein b100 (apobds-1) that triggers innate proatherogenic responses. *Circulation.* 2011;124:2433-2443, 2431-2437
Figure S3. Regulation of the 5-LOX pathway in atherosclerosis. Expression of 5-LOX (A) and FLAP (B) was determined by real-time-PCR in human monocyte derived macrophages treated with TLR4 agonist LPS (1 μg/ml) or NOD2 agonist MDP (10 μg/ml). Data are expressed as mean ± SEM of 3 independent experiments. (C and D) Ex vivo cultures of atherosclerotic plaques (n=8) were treated with MDP for 24 h. Leukotriene B4 (LTB4) and leukotriene E4 (LTE4) in the culture supernatants were analyzed by enzyme-linked immunoassay. Data are expressed as mean ± SEM of 3 independent experiments.
Figure S2. Co-expression of NOD2 and COX2 in lesional macrophages. Double immunofluorescent staining for COX2 (red), CD163 (green), CD68 (green) or NOD2 (green) was performed to determine cellular localization of NOD2 and COX2 in human carotid atherosclerotic lesions. Nuclei were labeled with DAPI (blue). Original resolution: 63x. (A) Image of COX2 expression in CD163 positive macrophages as indicated by arrowheads, and an image of COX2 and CD163 double positive cell at high magnification in the right panel. (B) COX2 expression in CD68 positive macrophages, a representative cell is illustrated in the right panel at high magnification. (C) Co-localization of NOD2 and COX2 in atherosclerotic lesions. Original magnification: 200x. In addition, analysis of mRNA levels for NOD2, COX2 and mPGES-1 in a carotid plaque biobank, BiKE study, supports a positive correlation between NOD2 and mPGES-1 (D), NOD2 and COX2 (E) in carotid plaques.