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RESEARCH PAPER

Nucleotide oligomerization domain 1 is a dominant pathway for NOS2 induction in vascular smooth muscle cells: comparison with Toll-like receptor 4 responses in macrophages

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Background and purpose: Gram-negative bacteria contain ligands for Toll-like receptor (TLR) 4 and nucleotide oligomerization domain (NOD) 1 receptors. Lipopolysaccharide (LPS) activates TLR4, while peptidoglycan products activate NOD1. Activation of NOD1 by the specific agonist FK565 results in a profound vascular dysfunction and experimental shock *in vivo*.
Experimental approach: Here, we have analysed a number of pharmacological inhibitors to characterize the role of key signalling pathways in the induction of NOS2 following TLR4 or NOD1 activation.
Key results: Vascular smooth muscle (VSM) cells expressed NOD1 mRNA and protein, and, after challenge with *Escherichia coli* or FK565, NOS2 protein and activity were induced. Macrophages had negligible levels of NOD1 and were unaffected by FK565, but responded to *E. coli* and LPS by releasing increased NO and expression of NOS2 protein. Classic pharmacological inhibitors for NF-κB (SC-514) and mitogen-activated protein kinase (SB203580, PD98059) signalling pathways inhibited responses in both cell types regardless of agonist. While TLR4-mediated responses in macrophages were specifically inhibited by the pan-caspase inhibitor z-VAD-fmk and the PKC inhibitor Gö6976, NOD1-mediated responses in VSM cells were inhibited by the Rip2 inhibitor PP2.
Conclusions and implications: Our findings suggest a selective role for NOD1 in VSM cells, and highlight NOD1 as a potential novel therapeutic target for the treatment of vascular inflammation.
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Abbreviations: NOD1, nucleotide oligomerization domain 1; PAMPs, pathogen-associated molecular patterns; TLR, Toll-like receptor; VSM, vascular smooth muscle

Introduction

It is now clear that pattern recognition receptors (PRRs) are intrinsically involved in cardiovascular diseases including atherosclerosis (Michelsen *et al.*, 2004). Moreover, Toll-like receptor (TLR) 4, the receptor for Gram-negative lipopolysaccharide (LPS), or TLR2, the receptor for Gram-positive lipoteichoic acid, is expressed on vascular cells and in vessels where they sense pathogens directly (without the requirement of macrophages), leading to induction of inflammatory genes including NOS 2 (Cartwright *et al.*, 2007a,b) and COX-2 (Jimenez *et al.*, 2005). Our recent work has shown that activation of the PRR nucleotide oligomerization domain (NOD) 1 on vascular smooth muscle (VSM) cells leads to profound induction of NOS2 *in vitro* and vascular collapse *in vivo* (Cartwright *et al.*, 2007a). NOD1 and NOD2 are receptors for products of the synthesis and degradation of peptidoglycan (PGN) from Gram-negative bacteria (Chamaillard *et al.*, 2003; Masumoto *et al.*, 2006; Park *et al.*, 2007). Thus, TLR4 and NOD1 are both receptors for different pathogen-associated molecular patterns (PAMPs) from Gram-negative bacteria. Importantly, others have shown that, in leucocyte populations, activation

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of NOD1 receptors alone can result in weak activation, and that co-stimulation with TLR agonists can result in increased responses (Chamaillard *et al*., 2003; Fritz *et al*., 2005; van Heel *et al*., 2005; Tada *et al*., 2005; Masumoto *et al*., 2006). However, work from our group and others have shown that activation of NOD receptors alone results in induction of inflammatory genes in some types of cell, including VSM cells (Cartwright *et al*., 2007b; Park *et al*., 2007). However, the mechanism by which activation of NOD1 in VSM cells results in inflammation was not previously studied. Thus, in the current study, we have compared responses in VSM and macrophages to specific ligands for TLR4 and NOD1, and measured NOS2 activity as a relevant readout of cell activation. Our findings show, for the first time, that NOD1 receptors are selectively expressed in VSM cells. We also go on to establish similarities and differences in how NOD1 versus TLR4 activation (in VSM cells vs. macrophages, respectively) signal resulting in the induction of NOS2. These observations provide important new information about how vascular cells sense pathogens and danger signals, which may well have direct relevance to our understanding of cardiovascular disease in more complex settings.

Methods

Cell culture

Clonal populations of VSM cells with a contractile ('spindle') phenotype derived from the mixed polyclonal rat aortic VSM cell line WKY12-22 were kindly provided by Professor Timothy Warner (William Harvey Research Institute, London, UK). J774.2, a murine macrophage cell line, was obtained from the European collection of cell cultures. Murine peritoneal macrophages were collected from *C57BL/6* mice or Wistar–Kyoto rats by peritoneal lavage (Terenzi *et al*., 1995). Cells were cultured as described previously (Jimenez *et al*., 2005) using Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotic/mycotic mix. Cells were treated for 24 (VSM and J774.2 cells) or 48 (rat peritoneal macrophages) hours with either FK565 (10 nM, NOD1 agonist), LPS (1 $\mu g \cdot mL^{-1}$, TLR4 agonist) or whole heat-killed *Escherichia coli* (3×10^7 $CFU \cdot mL^{-1}$). In some experiments, cells were pretreated for 60–90 min with pharmacological inhibitors of different signalling cascades before the addition of bacterial ligands.

Measurement of NO production

NO production by cells was measured by the accumulation of its oxidation product, nitrite, using the Griess reaction, as we have described previously (Jimenez *et al*., 2005; Paul-Clark *et al*., 2006). Nitrite levels obtained in the presence of different inhibitors are expressed as a percentage of the response to the agonist alone.

Measurement of 6-keto prostaglandin (PG) $F_{1\alpha}$

COX activity was measured by the accumulation of 6-keto $PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin (PGI_2), by specific radioimmunoassay (Bishop-Bailey *et al*., 1997; Jimenez *et al*., 2005).

Assessment of cell respiration by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Cell respiration, a marker of viability, was assessed after all treatments by measuring the reduction of MTT (Sigma Chemical Company, Poole, Dorset, UK) to formazan. Unless shown in Supporting Information Tables S1 and S2, none of treatments affected cell viability.

Measurement of NOS2, COX-2 and NOD1 protein expression

Western blot analysis using specific NOS2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NOD1 (1:1000, Cell Signaling Technology, New England Biolabs Ltd., Hitchin, Hertfordshire, UK) and COX-2 (1:1000; Cayman Chemical, Ann Arbor, MI, USA) antibodies was performed as previously described (Jimenez *et al*., 2005; Paul-Clark *et al*., 2006; Cartwright *et al*., 2007a). The expression of NOD1 was also analysed on murine macrophages collected from *C57BL/6* mice by peritoneal lavage (Bosca *et al*., 2005).

Measurement of NOD1 mRNA expression

Reverse transcription–PCR (RT–PCR) analysis was performed as previously described (Cartwright *et al*., 2007a). Total RNA was extracted using RNeasy Minikit (Qiagen, Crawley, UK), and converted into complementary DNA by RT following the protocol of the manufacturer (Omniscript Reverse Transcription kit, Qiagen). Primers for the PCR were selected from the regions of the NOD1 mRNA sequence with the highest degree of homology in rat (NM_001109236.1, GenBank) and mouse (NM_172729.2, GenBank). Both sequences were compared with GenBank using the Blast software (National Center for Biotechnology Information website). The following primers, synthesized on request by Invitrogen (Paisley, UK), were used for the amplification of NOD1; sense: 5′- GTC CTC AAC GAG CAT GGC GAG ACT -3′ and antisense: 5′-TGC AGC TCA TCC AGG CCG TCA A-3′ resulting in a product of 302 bp; and for β-actin, sense: 5′-GAT CAT GTT TGA GAC CTT CAA CAC C-3′ and antisense: 5′-GTT TCA TGG ATG CC ACA GGA TTC-3′, which yielded a product of 467 bp. In preliminary experiments, we tested a number of cycles ranging from 24 to 35, with cDNA diluted over a range of three magnitudes, to establish the linearity of the reaction. PCR of NOD1 was performed using a MyCycler Thermal cycler (Bio-Rad, Hercules, CA, USA) by using *Taq* PCR Master Mix Kit (Qiagen) for 30 cycles (94°C for 45 s, 64.7°C for 45 s and 72°C for 45 s) followed by a final extension step at 72°C for 10 min. The same protocol was used for β-actin except for the annealing temperature, which was 60.4°C. The products of the PCR were separated in 2% agarose gels stained with ethidium bromide, and bands were quantified using image analysis software (Quantity One, Bio-Rad). Results were normalized to the bands of β-actin from the same samples.

Drugs and bacterial ligands

Heat-killed *E. coli* suspensions were prepared as previously described (Cartwright *et al*., 2007a) from a clinical blood

culture isolate of *E. coli* 0111.B4. The NOD1 agonist FK565 (heptanoyl-gamma-D-glutamyl-L-meso-diaminopimelyl-D-alanine), a generous gift from Professor David Van Heel (QMUL, London, UK), was obtained from Fujisawa Pharmaceutical (Osaka, Japan), the NOD2 agonist MDP (muramyl *N*-acetylmuramyl-L-alanyl-D-isoglutamine, also known as muramyl dipeptide) was purchased from Invivogen (San Diego, CA, USA), the cell-permeable JNK peptide inhibitor D-JNKi ((D)-hJIP[175-157]-DPro-DPro-(D)-HIV-TAT[57-48]) was purchased from Enzo Life Sciences (Exeter, UK) and the TLR4 agonist LPS (from *E. coli* 055:B5) was purchased from Sigma. All other drugs were obtained from Calbiochem (San Diego, CA, USA). Drugs were dissolved initially in dimethyl sulphoxide (DMSO) (except for LPS and FK565, which were dissolved in PBS) to prepare a 0.1 or 0.01 mol·L^{-1} stock solution; further dilutions were made in DMEM. The maximum concentration of DMSO achieved in any experiment, 0.3%, did not show significant effects on cell viability.

Statistical analysis

Results were normalized to 'control' in most figures, expressed as mean ± SEM, and were analysed by Student's one sample *t*-test. Values of $P < 0.05$ were considered to be significant.

Results

Effect of NOD1 or TLR4 activation on NOS2 activity in VSM cells and macrophages

We have recently shown that FK565 activates the VSM component of intact blood vessels via a NOD1-specific pathway resulting in NOS2 induction and vascular dysfunction (Cartwright *et al*., 2007b). The effect of FK565 was found to be independent of TLR4 or TLR adapter proteins (Cartwright *et al*., 2007b). Here, we have confirmed that FK565 induces NOS2 activity (Figure 1A and Supporting Information Figure S1) and protein expression (Figure 1C) in cultured VSM cells. Furthermore, NOS2 induction was accompanied by an increase in the activity and protein expression of COX2 (Supporting Information Figure S1), another enzyme involved in the production of key vasoactive inflammatory mediators (such as prostacyclin). NOS2 activity and protein were also induced in VSM cells stimulated with whole Gram-negative *E. coli* and, to a lesser extent, LPS (Figure 1A,C). Similarly, J774.2 murine macrophages showed increased levels of NOS2 activity (Figure 1B) and protein (Figure 1D) when stimulated with *E. coli* or LPS. However, in direct contrast to results seen in VSM cells, FK565 had no effect on NOS2 activity in macrophages (Figure 1B,D). VSM cells and J774.2 macrophages were unaffected by the NOD2 agonist MDP (nitrite μM; VSM cells: basal, 0.0 ± 0.0; plus 10 μM MDP, 0.8 ± 0.4; macrophages: basal, 1.0 ± 0.9, plus 10 μM MDP, 0.0 ± 0.0; $n = 9$; 24 h). In order to ensure these observations were not due to any species differences, we performed separate studies using primary cultures of rat macrophages. Similar to experiments using murine J774 macrophages, rat macrophages released increased levels of NO when stimulated with LPS, but not FK565 (nitrite μM; basal, 0 ± 0; plus 1 μg·mL^{-1} LPS, 28 ± 10; plus 10 nM FK565, 0 ± 0; $n = 6$; 48 h). In line with these observations, VSM cells expressed clear detectable levels of NOD1 protein, while both J774.2 and freshly elicited mouse macrophages expressed low or undetectable levels of NOD1 protein (Figure 1E,F). These findings were confirmed by RT–PCR (Figure 1G,H). Levels of NOD1 gene expression (NOD1/β-actin) were not altered by stimulation of cells with FK565 for 24 h (VSM cells: control 0.100 ± 0.030, plus FK565 0.147 ± 0.033; J774.2 macrophages: 0.006 ± 0.002, plus FK565 0.003 ± 0.006). In previous studies, enhancement of TLR4-induced secretion of pro-inflammatory cytokines (including IL-1β, IL-6 or IL-8) by NOD1 stimulation has been shown in macrophages and dendritic cells (Chamaillard *et al*., 2003; Fritz *et al*., 2005; Masumoto *et al*., 2006). In our study, we found no interaction between FK565 and LPS for the induction of NOS2 in macrophages (co-stimulation with 10 nM FK565 plus 1 μg·mL^{-1} LPS induced 105 ± 5.2% of nitrite produced by 1 μg·mL^{-1} LPS alone). In VSM cells, there was a small, but statistically significant, increase in the ability of FK565 to induce NOS2 activity when cells were co-treated with threshold concentrations of LPS (co-stimulation with 0.1 μg·mL^{-1} LPS plus 10 nM FK565 induced 132 ± 5.6% of nitrite produced by FK565 alone, $P < 0.05$).

Role of NF-κB, MAP kinases, PKC and RIP2 in responses induced by NOD1 compared to those induced by TLR4 in VSM cells and macrophages

In different cell types, induction of NOS2 by a variety of agents is sensitive to inhibition by corticosteroids. In this study, we found that NOS2 activity and protein expression induced by either FK565 in VSM cells or by LPS in macrophages were inhibited by dexamethasone (Figure 2A–D; Supporting Information Table S3). The I-κB kinase (IKK)-2-inhibitor SC-514 (4-amino-[2′,3′-bithiophene]-5-carbox amide) (Kishore *et al*., 2003) inhibited NOS2 activity and protein expression in VSM cells stimulated with FK565 (Figure 2E,G; Supporting Information Table S3) or macrophages (Figure 2F,H; Supporting Information Table S3). Similarly, SC-514 inhibited NOS2 activity induced by whole *E. coli* in VSM cells and macrophages (Supporting Information Table S4). The p38 and extracellular signal-regulated (ERK) mitogen-activated protein kinase (MAPK) inhibitors SB 203580 (4-[5-(4-fluorophenyl)-2-[4-(methylsulphonyl) phenyl]-1H-imidazol-4-yl]pyridine) and PD 98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (Davies *et al*., 2000), respectively, inhibited NOS2 activity and protein expression in VSM cells stimulated with FK565 or macrophages stimulated with LPS (Figure 3; Supporting Information Table S3). NOS2 activity induced in macrophages by LPS was inhibited by the c-Jun N-terminal kinase (JNK) inhibitor SP 600125 (anthra[1-9-cd]pyrazol-6(2H)-one) (Bennett *et al*., 2001) (Figure 3; Supporting Information Table S4), but not by the cell-permeable JNK peptide inhibitor D-JNKi ((D)-hJIP[175-157]-DPro-DPro-(D)-HIV-TAT[57-48]) (Borsello and Bonny, 2004). By contrast, SP 600125 showed a weak trend to inhibit NOS2 activity induced by FK565 in VSM cells, while D-JNKi induced a statistically significant inhibition of activity in these cells (Figure 3; Supporting Information Tables S3 and S4). These results are in contrast to those found in cells stimu-

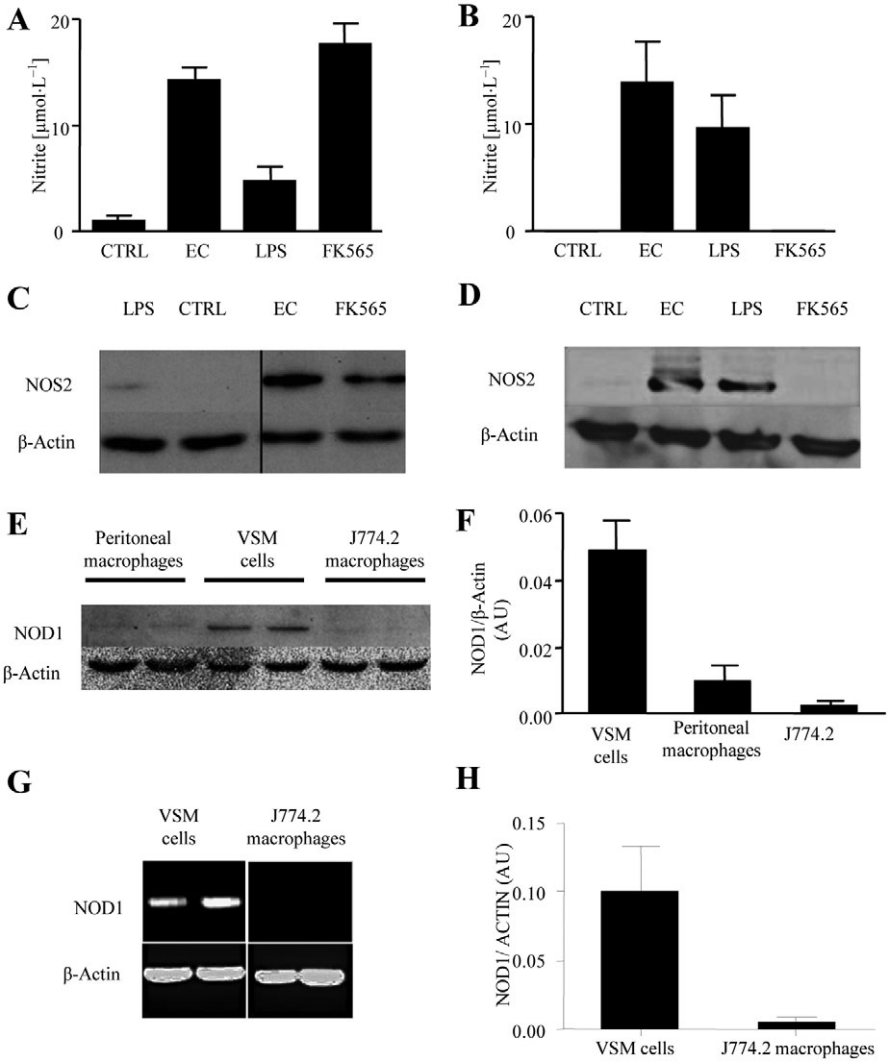

Figure 1 Effects of Gram-negative PAMPs on NO release by cultured VSM cells and macrophages, and expression of NOD1 in VSM cells and macrophages. (A) At 24 h, *Escherichia coli* (EC; 3×10^7 CFU·mL^{-1}), LPS (1 μg·mL^{-1}) or FK565 (10 nM) induced significant increases in release of NO from VSM cells. NO was measured by the oxidation product nitrite by Griess assay, and compared to cells incubated in media alone (CTRL). (B) *Escherichia coli* and LPS induced significant release of NO in J774.2 macrophages. In contrast, FK565 did not significantly increase the release of NO. Data are presented as the mean ± SEM from $n = 6$ determinations for J774.2 cells and $n = 18$ for VSM cells. (C,D) Representative Western blots of NOS2 (≈130 kDa) and β-actin (≈47 kDa) from VSM cells (C) and macrophages (D) treated with *E. coli*, LPS or FK565 for 24 h. (E) Representative Western blot of NOD1 protein (≈95 kDa) in VSM, J774.2 or freshly isolated peritoneal macrophages from *C57 BL/6* mice. Detectable NOD1 expression was only seen in VSM cells. (F) Densitometric data analysis of NOD1 protein normalized to β-actin levels and expressed as arbitrary units (AU). Data are presented as the mean ± SEM from $n = 3$. (G) Ethidium bromide-stained agarose gels containing RT–PCR products of NOD1 (302 bp) and β-actin (467 bp) in VSM cells and J774.2 macrophages. Detectable NOD1 expression was only seen in VSM cells. (H) Densitometric data analysis of NOD1 mRNA normalized to β-actin levels and expressed as AU. Data are presented as the mean ± SEM from $n = 3$.

lated with whole *E. coli* (Supporting Information Table S4), where SP 600125 inhibited NOS2 activity in both VSM cells and macrophages. Gö6983 (3-[1-[3-(dimethylamino) propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione), which at the concentration tested is a selective inhibitor of conventional (α, βI, βII, γ), some novel (δ) and atypical (ι/λ, ζ) isoforms of PKC (Way *et al*., 2000), had no effect on NOS2 activity induced in VSM cells by FK565 or macrophages induced by LPS (Figure 4). Gö6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile), which, in addition to conventional isoforms, inhibits PKCμ (Way *et al*., 2000), also had no effect on NOS2 activity induced by FK565 in VSM cells. By contrast, Gö6976 inhibited NOS2 activity induced by LPS in macrophages (Figure 4). Similar results were found when cells were stimulated with whole *E. coli*. The Src family kinase inhibitor PP2 (3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine), at concentrations reported to inhibit RIP2 [EC_{50} = 100 nM for Src and EC_{50} = 10 nM for RIP2 (Windheim *et al*., 2007)] inhibited NOS2 activity induced in VSM cells stimulated with either FK565 or whole *E. coli*, but had little or no effect on activity induced in macrophages stimulated with LPS or whole *E. coli* (Figure 5, Supporting Information Table S4).

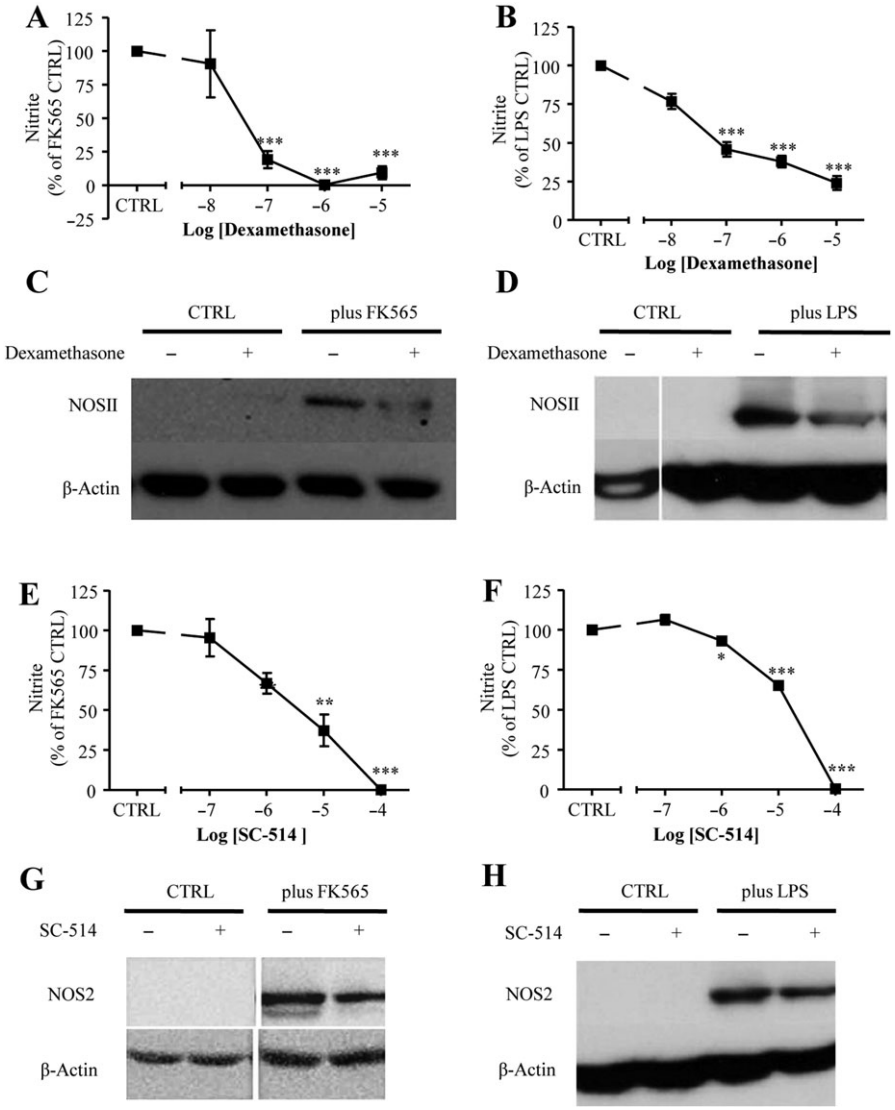

Figure 2 Role of corticosteroids and NF-κB on TLR4 and NOD1-induced NO production and NOS2 expression in VSM and J774 cells. VSM cells (A,E) and J774.2 macrophages (B,F) were treated with increasing concentrations of dexamethasone (0.01–10 μM; A,B) or the IKK 2 inhibitor SC-514 (0.1–30 μM; E,F). NOS2 was induced in VSM cells with FK565 (10 nM) and in macrophages with LPS (1 $\mu g \cdot mL^{-1}$). Data were collected from n = 9–12 for J774.2 cells and n = 6 for VSM cells and expressed as a percentage of the response induced by FK565 or LPS, respectively (CTRL). Western blotting (C,D,G,H) for NOS2 protein showed that dexamethasone (1 μM; C,D) and SC-514 (10 μM; G,H) inhibited protein expression in both VSM cells (C,E) and macrophages (D,H) at 24 h (n = 3–4). Data were analysed using one-sample t-test for normalized data, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Role of the inflammasome in NOD1- versus TLR4-induced responses in VSM cells and macrophages

Activation of several NOD-like receptors (NLRs) from the NALP subfamily has also been linked to the formation of an inflammasome, leading to the activation of caspase, which processes pro-IL-1β into mature IL-1β to initiate an inflammatory response. Neither the pan-caspase inhibitor (Garcia-Calvo *et al.*, 1998) Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; Figure 6A) nor binding antibodies for IL-1β or IL-18 (Figure 6C) affected NOS2 activity induced in VSM cells stimulated with FK565. By contrast, Z-VAD-fmk inhibited NOS2 activity and protein expression in J774.2 macrophages stimulated with whole *E. coli* or LPS (Figure 6B,D; Supporting Information Tables S3 and S4). However, this was associated with a direct effect of the drug on cell viability (Supporting Information Tables S1 and S2). The selective inhibition of caspase 1 and caspase 8 isoforms by Z-WEHD-fmk (benzyloxycarbonyl-Trp-Glu(OMe)-His-Asp(OMe)-fluoromethylketone) and Z-IETD-fmk (benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethylketone) (Maelfait *et al.*, 2008), respectively, mimicked the effects of Z-VAD-fmk on NOS2 induction while preserving cell viability (Figure 6F).

Discussion

It is now well accepted that stromal cells of the vasculature, including VSM cells, sense pathogens and danger signals directly, and that this is important for the development of

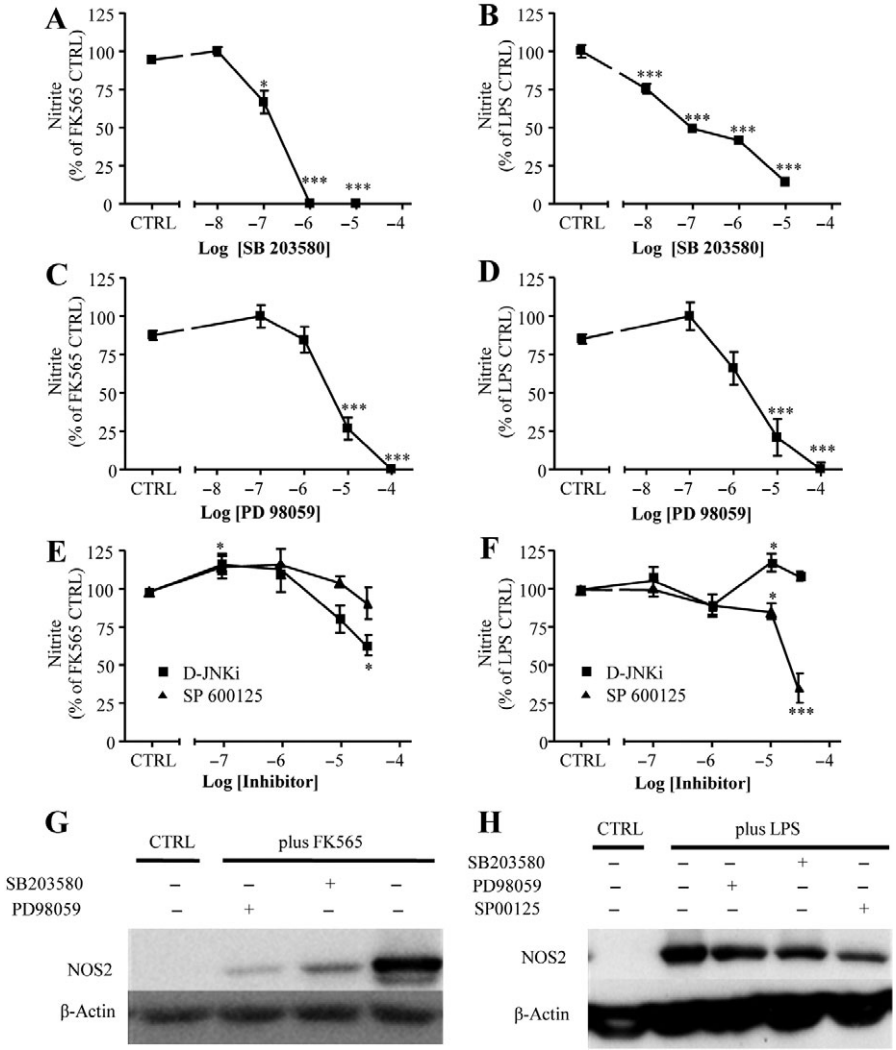

Figure 3 Role of MAPKs in TLR4 and NOD1 signalling pathways. Regulation of nitrite production in VSM cells (A, C, E, G) and macrophages (B, D, F, H) by the p38 MAPK inhibitor SB 203580 (0.01–10 μM), the ERK 1/2 inhibitor PD 98059 (0.01–100 μM) and the JNK inhibitors SP 600125 (0.1–30 μM) and D-JNKi (0.1–30 μM). Data are presented as the mean ± SEM of $n = 6–9$ for J774.2 cells, and $n = 9–15$ for VSM cells, and are expressed as a percentage of the response to the FK565 or LPS alone (CTRL). Representative Western blotting images for NOS2 protein (G,H) showing the effects of SB 203580 (1 μM), PD 98059 (10 μM) and SP 600125 (30 μM) on NOS2 expression in VSM cells (G) and macrophages (H). Data were analysed using one-sample *t*-test for normalized data, $^*P < 0.05$ and $^{***}P < 0.001$, $n = 3–4$.

septic shock and atherosclerosis. In the current study, we identified a clear difference in how VSM and macrophages sense NOD1 agonists. NOD1 receptors are PRR for PAMPs of Gram-negative bacteria. VSM cells express NOD1 receptors and respond to agonists by expressing increased NOS2. By contrast, macrophages do not express NOD1 receptors and are unresponsive to the selective agonist FK565. Our findings also suggest that the signalling pathways for NOD1 and TLR4 are separate, but converge at the level of NF-κB and MAPK activation for NOS2 induction.

In the current study, we found that the synthetic NOD1 agonist FK565 induced profound increases in NO release and increased expression of NOS2 protein in VSM cells. Similarly VSM cells showed increased levels of COX-2 activity and expression when stimulated with FK565. These observations confirm our previous results showing that activation of NOD1 in murine blood vessels induces NOS2 activity and vascular dysfunction (Cartwright *et al*., 2007a). Our previous studies confirmed that these actions of FK565 are totally specific to NOD1 receptors *in vitro* and are accompanied by extensive vascular collapse and shock *in vivo* (Cartwright *et al*., 2007a). In that model of shock, the decline in blood pressure is directly associated with the induction of NOS2 in blood vessels (Szabo *et al*., 1993). We also found that the PAMP for TLR4, LPS induced NOS2 activity in our cultures of VSM cells, although the magnitude of response was reduced compared to the response we obtained when cells were stimulated with FK565. This observation is also in line with, and explains, our previous study where the 'shock' induced by FK565 *in vivo* was far greater than that induced by LPS (Cartwright *et al*., 2007a). It is therefore significant to note that in VSM, NOD1 appears to dominate TLR4 responses when it comes to induction of NOS2. Importantly, the reverse seems to be true for macrophages. As expected, we found that LPS induced a robust induc-

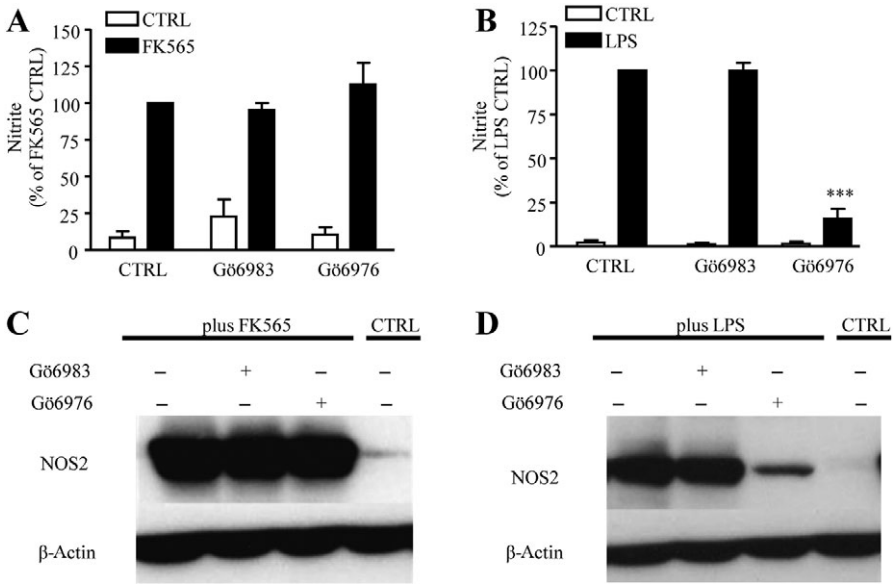

Figure 4 Role of protein kinase C isoforms (PKC) in TLR4 and NOD1 signalling pathways. VSM cells (A) and J774.2 macrophages (B) were treated with the PKC inhibitors Gö6983 (0.1 μM) and Gö6976 (0.1 μM). NOS2 activity was induced by FK565 (10 nM) in VSM cells and by LPS (1 $\mu g \cdot mL^{-1}$) in macrophages. Data are the mean ± SEM of $n = 6$ for J774.2 cells and $n = 15$ for VSM cells and are expressed as a percentage of the response to the FK565 or LPS alone (CTRL). Western blotting images for NOS2 protein (C,D) showing the effects of Gö6976 and Gö6983 (both at 0.1 μM) on NOS2 expression in VSM cells (C) and macrophages (D) ($n = 3$). Data were analysed using one-sample *t*-test for normalized data, $^*P < 0.05$ and $^{***}P < 0.001$.

tion of NOS2 activity in the murine macrophage cell line, J774, and in primary cultures of rat macrophages. By contrast, we found that the NOD1 agonist FK565 was completely inactive as an inducer of NOS2 activity or protein. In line with these observations, we found that VSM cells expressed detectable levels of NOD1 mRNA and protein, while macrophages did not. NOD2 signalling is often studied in parallel with NOD1. We have previously shown that the NOD2 agonists, MDP, have little effect on NOS2 activity in VSM cells. In the current study, we have confirmed these observations in experiments where VSM cells and macrophages were treated in parallel. Specifically, we found that MDP did not induce NOS2 activity in either cell type. These observations strengthen our assertion that NOD1 is the predominant pathway for NOS2 induction in these cells. This result supports published data showing that NOD2 agonists are poor inducers of cytokine production (Park *et al*., 2007) and vascular dysfunction (Cartwright *et al*., 2007b). In other studies, activators of NOD receptors and activators of TLRs were found to have a synergistic effect (Chamaillard *et al*., 2003; Fritz *et al*., 2005; van Heel *et al*., 2005; Tada *et al*., 2005; Masumoto *et al*., 2006). However, in our study, where NOS2 was the gene of interest, we found no interaction between LPS and FK565 in macrophages, and only a very modest interaction in VSM cells. Nevertheless, because macrophages represent a phenotypically highly diverse cell population, the role of NOD1 signalling pathways in macrophages from different sources may well vary and requires further study before extensive conclusions can be drawn.

The pathway by which TLR4 signals in the VSM component of blood vessels is well worked out (Yang *et al*., 2005; Cartwright *et al*., 2007a; Son *et al*., 2008). However, this is the first study to investigate NOD1-mediated NOS2 induction in a vascular model. Firstly, we demonstrated that similar to NOS2 induced via the TLR4 pathway, the glucocorticoids dexamethasone and prednisolone inhibited NOS2 induced by FK565 in VSM cells. NF-κB is a major inflammatory factor triggered by both TLRs and NLRs (Shaw *et al*., 2008). Accordingly, the effects induced by TLR4 and NOD1 agonists were blocked by the IKK-2 inhibitor SC-514 in both types of cell. More recently, it was shown that Gram-negative bacterial LPS is also able to activate MAPK signalling pathways in macrophages (Shaw *et al*., 2008). Our data confirm that the p38 MAPK and ERK1/2 pathways are needed for NOS2 induction in VSM cells and J774.2 macrophages. The role of JNK is, however, uncertain in this cell system and requires further study. In addition, the apparent lack of specificity of SP 600125 in murine macrophages highlights that caution must be applied when drawing conclusions using these compounds.

In other cell types, the details of how activation of NF-κB and MAPK is linked to TLR4 and NOD1 are not clearly established, but different reports have suggested that PKC and some tyrosine kinases could be involved in these signalling pathways. Several broad inhibitors of PKC isoforms have been demonstrated to be effective in inhibiting secretion of pro-inflammatory cytokines following TLR4 activation in macrophages, neutrophils and neurones (Asehnoune *et al*., 2005; Song *et al*., 2007). Gö6983 and Gö6976 were used in this study to assess the role of PKC in the induction of NOS2 by TLR4 and NOD1 agonists. Gö6983, which at the concentration tested shows selectivity for the conventional (α, βI, βII, γ), some novel (δ) and atypical (ι/λ, ζ) PKC isoforms (Way *et al*., 2000), had no effect on NOS2 activity induced in VSM cells by FK565 or that in macrophages induced by LPS. By contrast, Gö6976, which in addition to the conventional isoforms, also inhibits PKCμ (Way *et al*., 2000) inhibited NOS2 activity

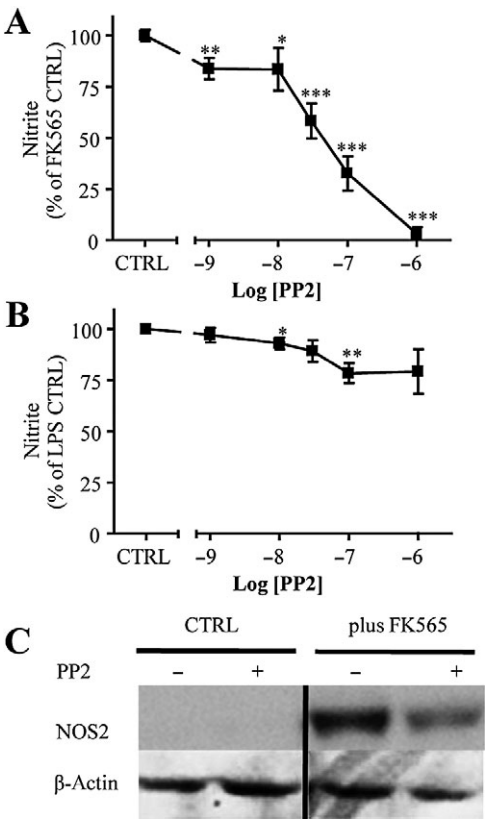

Figure 5 Effects of Src and serine/threonine kinase inhibitor PP2 on the induction of NOS2 by TLR4 and NOD1 agonists. Effects of the Src and serine/threonine kinase inhibitor PP2 (1 nM–1 μM) on the ability of FK565 or LPS to induce nitrite production in VSM cells (A) and macrophages (B). The data are the mean ± SEM of $n = 9$ for J774.2 cells and $n = 15$ for VSM cells, and are expressed as a percentage of the response to the FK565 or LPS alone (CTRL). (C) Representative image showing that 0.1 μM PP2 inhibited NOS2 induction in VSM cells by NOD1 activation ($n = 3$). Data were analysed using one-sample *t*-test for normalized data, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

induced by LPS in macrophages, but had no effect on the activity induced in VSM cells by FK565. As the only isoform known to be sensitive to Gö6976 and insensitive to Gö6983 is PKCμ (also known as protein kinase D or PKD), our data suggest that this isoform might be involved in the responses induced by LPS in macrophages. PKD isoforms are present in a wide variety of cells where they control cellular processes such as cell proliferation, apoptosis or immune responses (Van Lint *et al*., 2002; Song *et al*., 2007). PKDs have been shown to control gene transcription by activating NF-κB and MAPK pathways, and by inhibiting HDACs (Matthews *et al*., 2006). PKD can be activated by a variety of stimuli via PKC-dependent and -independent pathways (Johannessen *et al*., 2007). Previous reports have shown that the involvement of specific PKC isoforms seems to be time dependent. Because we analysed the effects of these inhibitors after 24 h, the possibility that other PKC isoforms are involved in earlier events cannot be ruled.

As pointed out in the introduction, the signalling events associated with NOD1 are not completely understood. It is clear that RIP2 and NOD1 interact, in order for downstream signalling to occur (Kobayashi *et al*., 2002), but whether this interaction provides a mere physical link between NLR and downstream effectors molecules or whether the kinase activity of RIP2 plays a direct role in transduction mechanisms remains to be determined (Windheim *et al*., 2007). In the present study, PP2, a potent inhibitor of RIP2 (Windheim *et al*., 2007), inhibited the production of NO induced by FK565 in VSM cells. Because activation of NF-κB by TLR agonists has also been shown to signal through this kinase, RIP2 was proposed as a point of synergy (Kobayashi *et al*., 2002). However, in our model, inhibition of RIP2 had little or no effect on macrophages stimulated with either LPS or *E. coli*. These results suggest that RIP2 kinase activity is required for NOS2 induction following NOD1 activation. SB 203580, which is commonly used as a specific inhibitor of p38 MAPK, has also been found to inhibit RIP2 kinase activity *in vitro* with even greater potency (Argast *et al*., 2005; Windheim *et al*., 2007). Therefore, the inhibition observed in the production of NO by VSM cells following treatment with SB 203580 might be revealing the consequences of inhibiting both p38 MAPK and RIP2.

Activation of TLRs can result in the activation of caspase 1 and caspase 8 (Maelfait *et al*., 2008; Shaw *et al*., 2008) by the inflammasome complex, which induces the maturation of the pro-inflammatory cytokines IL-1β and IL-18. Functional co-operation has also been observed between inflammasome NLRs and NOD1 receptors, generating controversy over whether inflammasome NLRs are able to sense products of the degradation of PGN (Shaw *et al*., 2008). When we analysed the role of the inflammasome in NOD1 responses using FK565 or whole Gram-negative bacteria in VSM cells, we found that neither the pan-caspase inhibitor Z-VAD-fmk nor the binding antibodies for IL-1β or IL-18 affected the production of NO. By contrast, Z-VAD-fmk inhibited TLR4-mediated NOS2 activity and protein expression, and reduced cell viability in macrophages stimulated with LPS or *E. coli*. The effects of Z-VAD-fmk on NOS2 induction, but not on macrophage viability, were mimicked when caspase 1 and caspase 8 were inhibited selectively using Z-WEHD-fmk and Z-IETD-fmk respectively. It should be noted that, in contrast to some preceding reports, in the current study where NOS2 activity was our readout, no account was taken of any influence of exogenous or endogenous ATP. Finally, our data suggest that activation of caspases and PKD are both necessary and specific events for the induction of NOS2 following TLR4 activation in macrophages. It is noteworthy that caspase-dependent activation of PKD has been demonstrated both *in vitro* and *in vivo* (Endo *et al*., 2000; Van Lint *et al*., 2002).

In addition to immune cells, structural cells of the cardiovascular system including VSM cells sense pathogens and danger signals directly, clearly contributing to the development of septic shock and atherosclerosis. In sepsis or vascular inflammation, the endothelium tends to become activated, resulting in recruitment of immune cells to the site of injury. However, if the inflammation persists, the endothelium can become dysfunctional with reduced endothelium-dependent dilator responses (Clapp *et al*., 2004). Under these conditions, the underlying VSM becomes activated by PAMPs or cytokines, and expresses inducible enzymes including NOS2 (Cartwright *et al*., 2007b; Mitchell *et al*., 2007), leading to hypo-responsiveness to constrictor agents. This impaired vascular contractility is independent of the endothelium (Julou-

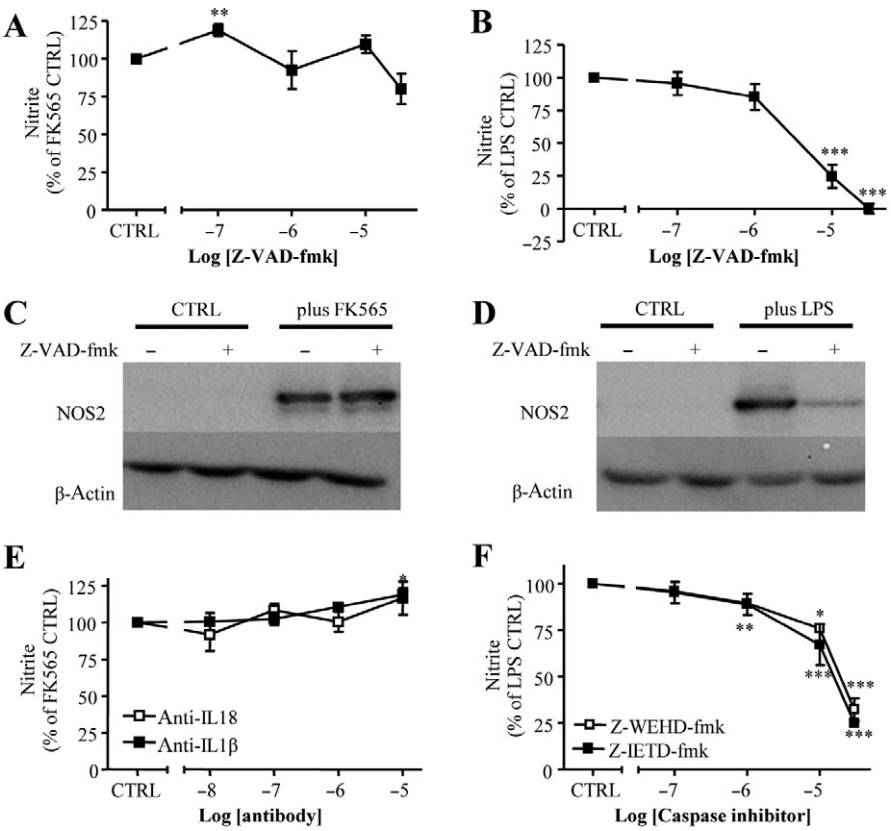

Figure 6 Role of the inflammasome in the induction of NOS2 by TLR4 and NOD1 agonists. (A,B) Effects of the pan-caspase inhibitor Z-VAD-fmk (0.1–30 μM) on the ability of FK565 or LPS to induce nitrite production in VSM cells (A) and macrophages (B). (C,D) Representative Western blotting images showing the effects of Z-VAD-fmk (10 μM) on NOS2 induction in VSM cells (C) and J774.2 macrophages (D) ($n = 3$–6). (E) Increasing concentrations of anti-IL1β- and anti-IL18-neutralizing antibodies (10^{-8} to 10^{-5} g·mL^{-1}) did not inhibit NOS2 activity in VSM cells stimulated with FK565. (F) The selective caspase-1 inhibitor Z-WEDH-fmk and the caspase-8 inhibitor Z-IETD-fmk (both at 0.1–30 μM) similarly inhibited NOS2 induction by the TLR4 agonist LPS in macrophages. The data are the mean ± SEM of $n = 6$–9 for VSM cells and $n = 6$–12 for J774.2 cells, and are expressed as a percentage of the response to the FK565 or LPS alone (CTRL). Data were analysed using one-sample *t*-test for normalized data, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Schaeffer *et al*., 1990; Szabo *et al*., 1993) and associated specifically with the induction of NOSII in the VSM component of blood vessels (Bishop-Bailey *et al*., 1997)

In vivo, the host is exposed to multiple ligands derived from Gram-negative bacteria, including both NOD1 and TLR4 agonists. Here, we have identified some similarities and differences in how VSM cells and macrophages sense Gram-negative PAMPs. Our findings show a novel and selective role for NOD1 receptors in VSM cells. Our data also show that the signalling pathways resulting in NOS2 induction are separate for NOD1 and TLR4, but converge at the level of NF-κB and MAPK. An effective host response to infection and injury relies on the ability to regulate the spatial and temporal patterns of the signalling pathways activated during the inflammatory response. The data presented here suggest that NOD1 is a central PRR pathway in VSM cells, and highlight NOD1 as a potential novel therapeutic target for the treatment of vascular inflammation.

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Conflicts of interest

The authors declare no conflicts of interest.

References

Argast GM, Fausto N, Campbell JS (2005). Inhibition of RIP2/RIck/CARDIAK activity by pyridinyl imidazole inhibitors of p38 MAPK. *Mol Cell Biochem* **268**: 129–140.

Asehnoune K, Strassheim D, Mitra S, Yeol Kim J, Abraham E (2005). Involvement of PKCalpha/beta in TLR4 and TLR2 dependent activation of NF-kappaB. *Cell Signal* **17**: 385–394.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W *et al*. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci U S A* **98**: 13681–13686.

Bishop-Bailey D, Larkin SW, Warner TD, Chen G, Mitchell JA (1997). Characterization of the induction of nitric oxide synthase and cyclo-oxygenase in rat aorta in organ culture. *Br J Pharmacol* **121**: 125–133.

Borsello T, Bonny C (2004). Use of cell-permeable peptides to prevent neuronal degeneration. *Trends Mol Med* **10**: 239–244.

Bosca L, Zeini M, Traves PG, Hortelano S (2005). Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *Toxicology* **208**: 249–258.

Cartwright N, McMaster SK, Sorrentino R, Paul-Clark M, Sriskandan S, Ryffel B *et al*. (2007a). Elucidation of Toll-like receptor and adapter protein signaling in vascular dysfunction induced by Gram-positive *Staphylococcus aureus* or Gram-negative *Escherichia coli*. *Shock* **27**: 40–47.

Cartwright N, Murch O, McMaster SK, Paul-Clark MJ, van Heel DA, Ryffel B *et al*. (2007b). Selective NOD1 agonists cause shock and organ injury/dysfunction *in vivo*. *Am J Respir Crit Care Med* **175**: 595–603.

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L *et al*. (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* **4**: 702–707.

Clapp BR, Hingorani AD, Kharbanda RK, Mohamed-Ali V, Stephens JW, Vallance P *et al*. (2004). Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. *Cardiovasc Res* **64**: 172–178.

Davies SP, Reddy H, Caivano M, Cohen P (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351** (Pt 1): 95–105.

Endo K, Oki E, Biedermann V, Kojima H, Yoshida K, Johannes FJ *et al*. (2000). Proteolytic cleavage and activation of protein kinase C [micro] by caspase-3 in the apoptotic response of cells to 1-beta-D-arabinofuranosylcytosine and other genotoxic agents. *J Biol Chem* **275**: 18476–18481.

Fritz JH, Girardin SE, Fitting C, Werts C, Mengin-Lecreulx D, Caroff M *et al*. (2005). Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol* **35**: 2459–2470.

Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA (1998). Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem* **273**: 32608–32613.

van Heel DA, Ghosh S, Butler M, Hunt K, Foxwell BM, Mengin-Lecreulx D *et al*. (2005). Synergistic enhancement of Toll-like receptor responses by NOD1 activation. *Eur J Immunol* **35**: 2471–2476.

Jimenez R, Belcher E, Sriskandan S, Lucas R, McMaster S, Vojnovic I *et al*. (2005). Role of Toll-like receptors 2 and 4 in the induction of cyclooxygenase-2 in vascular smooth muscle. *Proc Natl Acad Sci U S A* **102**: 4637–4642.

Johannessen M, Delghandi MP, Rykx A, Dragset M, Vandenheede JR, Van Lint J *et al*. (2007). Protein kinase D induces transcription through direct phosphorylation of the cAMP-response element-binding protein. *J Biol Chem* **282**: 14777–14787.

Julou-Schaeffer G, Gray GA, Fleming I, Schott C, Parratt JR, Stoclet JC (1990). Loss of vascular responsiveness induced by endotoxin involves L-arginine pathway. *Am J Physiol* **259** (4 Pt 2): H1038–H1043.

Kishore N, Sommers C, Mathialagan S, Guzova J, Yao M, Hauser S *et al*. (2003). A selective IKK-2 inhibitor blocks NF-kappa B-dependent gene expression in interleukin-1 beta-stimulated synovial fibroblasts. *J Biol Chem* **278**: 32861–32871.

Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA *et al*. (2002). RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* **416**: 194–199.

Maelfait J, Vercammen E, Janssens S, Schotte P, Haegman M, Magez S *et al*. (2008). Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8. *J Exp Med* **205**: 1967–1973.

Masumoto J, Yang K, Varambally S, Hasegawa M, Tomlins SA, Qiu S *et al*. (2006). Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment *in vivo*. *J Exp Med* **203**: 203–213.

Matthews SA, Liu P, Spitaler M, Olson EN, McKinsey TA, Cantrell DA *et al*. (2006). Essential role for protein kinase D family kinases in the regulation of class II histone deacetylases in B lymphocytes. *Mol Cell Biol* **26**: 1569–1577.

Michelsen KS, Doherty TM, Shah PK, Arditi M (2004). TLR signaling: an emerging bridge from innate immunity to atherogenesis. *J Immunol* **173**: 5901–5907.

Mitchell JA, Ryffel B, Quesniaux VF, Cartwright N, Paul-Clark M (2007). Role of pattern-recognition receptors in cardiovascular health and disease. *Biochem Soc Trans* **35** (Pt 6): 1449–1452.

Park JH, Kim YG, Shaw M, Kanneganti TD, Fujimoto Y, Fukase K *et al*. (2007). Nod1/RICK and TLR signaling regulate chemokine and antimicrobial innate immune responses in mesothelial cells. *J Immunol* **179**: 514–521.

Paul-Clark MJ, McMaster SK, Belcher E, Sorrentino R, Anandarajah J, Fleet M *et al*. (2006). Differential effects of Gram-positive versus Gram-negative bacteria on NOSII and TNFalpha in macrophages: role of TLRs in synergy between the two. *Br J Pharmacol* **148**: 1067–1075.

Shaw MH, Reimer T, Kim YG, Nunez G (2008). NOD-like receptors (NLRs): bona fide intracellular microbial sensors. *Curr Opin Immunol* **20**: 377–382.

Son YH, Jeong YT, Lee KA, Choi KH, Kim SM, Rhim BY *et al*. (2008). Roles of MAPK and NF-kappaB in interleukin-6 induction by lipopolysaccharide in vascular smooth muscle cells. *J Cardiovasc Pharmacol* **51**: 71–77.

Song MJ, Wang YQ, Wu GC (2007). Lipopolysaccharide-induced protein kinase D activation mediated by interleukin-1beta and protein kinase C. *Brain Res* **1145**: 19–27.

Szabo C, Mitchell JA, Thiemermann C, Vane JR (1993). Nitric oxide-mediated hyporeactivity to noradrenaline precedes the induction of nitric oxide synthase in endotoxin shock. *Br J Pharmacol* **108**: 786–792.

Tada H, Aiba S, Shibata K, Ohteki T, Takada H (2005). Synergistic effect of Nod1 and Nod2 agonists with Toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect Immun* **73**: 7967–7976.

Terenzi F, Diaz-Guerra MJ, Casado M, Hortelano S, Leoni S, Bosca L (1995). Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages. *J Biol Chem* **270**: 6017–6021.

Van Lint J, Rykx A, Maeda Y, Vantus T, Sturany S, Malhotra V *et al*. (2002). Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol* **12**: 193–200.

Way KJ, Chou E, King GL (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol Sci* **21**: 181–187.

Windheim M, Lang C, Peggie M, Plater LA, Cohen P (2007). Molecular mechanisms involved in the regulation of cytokine production by muramyl dipeptide. *Biochem J* **404**: 179–190.

Yang X, Coriolan D, Murthy V, Schultz K, Golenbock DT, Beasley D (2005). Proinflammatory phenotype of vascular smooth muscle cells: role of efficient Toll-like receptor 4 signaling. *Am J Physiol Heart Circ Physiol* **289**: H1069–H1076.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of the NOD1 agonist FK565 on COX and NOS activity in cultured VSM cells. At 24 h, FK565 (0.1–100 nM) induced a concentration-dependent increase in both COX (A, measured by the accumulation of 6-ketoPGF$_{1\alpha}$) and NOS2 (B, measured by the accumulation of nitrite) activity in

VSM cells. Data are expressed as mean ± SEM. $n = 6$. (Inset) Representative Western blots of COX2 (≈72 kDa) and β-actin (≈47 kDa) from VSM cells.

Table S1 Effects of drug treatments on VSM cell viability assayed by MTT assay. Results shown are selected concentrations (μM) from experiments where drugs were found to affect viability in either VSM cells or J774 macrophages. Where concentrations of drugs are not shown on S1 (or S2), no effect on cell viability was seen. Data are expressed as mean absorbance values ± SEM, and were analysed using one-way ANOVA followed by Dunnett's post-test; * denotes $P < 0.05$ for each inhibitor compared to respective control. $n = 6–12$.

Table S2 Effects of drug treatments on J774 cell viability assayed by MTT assay. Results shown are selected concentrations (μM) from experiments where drugs were found to affect viability in either VSM cells or J774 macrophages. Where concentrations of drugs are not shown on S1 (or S2), no effect on cell viability was seen. Data are expressed as mean absorbance values ± SEM, and analysed using one-way ANOVA followed by Dunnett's post-test; * denotes $P < 0.05$ for each inhibitor compared to respective control. $n = 6–18$.

Table S3 Densitometry analysis for NOS2 protein in cells treated with various inhibitors. Results were normalized by β-actin and expressed as a percentage of the response to FK565 (10 nM) or LPS (1 $\mu g \cdot mL^{-1}$). Data were analysed using one sample *t*-test; * denotes $P < 0.05$ for each inhibitor compared to respective control. $n = 3–5$. Inhibitors were used at the concentration shown in parentheses (μM).

Table S4 Effect of signalling pathway inhibitors on the ability of *Escherichia coli* (3×10^7 CFU·mL^{-1}), FK565 (10 nM) and LPS (1 $\mu g \cdot mL^{-1}$) to induce nitrite production in VSM cells and macrophages. Results were expressed as a percentage of the corresponding response to *E. coli*, FK565 or LPS alone. Results were analysed using one sample *t*-test, and * denotes $P < 0.05$ for treatments versus respective control (100%). $n = 6–15$. Inhibitors were used at the concentration shown in parentheses (μM).

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