The potential use of N-octanoyl-dopamine (NOD) in organ transplantation
Wedel, Johannes

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
N-octanoyl-dopamine inhibits the expression of a subset of κB regulated genes: Potential role of p65 Ser276 phosphorylation

Maximilia C. Hottenrott*
Johannes Wedel*
Sophie Gärtner
Eleni Stamellou
Tineke Kraaij
Linda Mandel
Ralph Lösel
Christian Sticht
Simone Höger
Lamia Ait-Hsiko
Angelika Schedel
Mathias Hafner
Benito A. Yard
Charalambos Tsagogiorgas

* equally contributed

Public Library of Science One 2013;8(9):e73122.
Abstract
Catechol containing compounds have anti-inflammatory properties, yet for catecholamines these properties are modest. Since we have previously demonstrated that the synthetic dopamine derivative N-octanoyl-dopamine (NOD) has superior anti-inflammatory properties compared to dopamine, we tested NOD in more detail and sought to elucidate the molecular entities and underlying mechanism by which NOD down-regulates inflammation. Genome wide gene expression profiling on human umbilical vein endothelial cells (HUVEC) was performed after stimulation with TNF-α or stimulation with TNF-α and NOD. Confirmation of these differences, NF-κB activation and the molecular entities that were required for the anti-inflammatory were assessed in subsequent experiments.
Down-regulation of inflammatory genes by NOD occurred predominantly for κB regulated genes, however not all κB regulated genes were affected. These findings were explained by inhibition of RelA phosphorylation at Ser276. Leukocyte adherence to TNF-α stimulated HUVEC was inhibited by NOD and was reflected by a diminished expression of adhesion molecules on HUVEC. NOD induced HO-1 expression, but this was not required for inhibition of NF-κB. The anti-inflammatory effect of NOD seems to involve the redox active catechol structure, although the redox active para-dihydroxy benzene containing compounds also displayed anti-inflammatory effects, provided that they were sufficiently hydrophobic.
The present study highlighted important mechanisms and molecular entities by which dihydroxy benzene compounds exert their potential anti-inflammatory action. Since NOD does not have hemodynamic properties, NOD seems to be a promising candidate drug for the treatment of inflammatory diseases.
Introduction
Regulated transmigration of leukocytes across the endothelial lining of the vasculature is critical to both innate and acquired immunity. Inappropriate or excessive transendothelial migration is however undesired and can initiate many pathological processes. Temporal spatial regulation of the inflammatory response is therefore of utmost importance to prevent excessive inflammation in organs, and yet, to function adequately in combating infections. The inflammatory response is tightly regulated by mediators that activate the endothelium to express cell-associated adhesion molecules. Leukocyte transmigration starts by P- and E-selectin-mediated transient binding to and rolling along the endothelium. Upon cytokine or chemokine activation, leukocytes firmly adhere to the endothelium [1] and subsequently leave the bloodstream using either of two fundamentally different pathways, \textit{i.e.} the para-cellular route requiring the opening of cell contacts [2] or the trans-cellular route through the body of endothelial cells [3-5].

The transcription factor NF-κB is a family of closely related protein dimers that regulate inducible gene expression of pro-inflammatory mediators [6]. This family consists of five related proteins, \textit{i.e.} p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB1) and p52 (NF-κB2), which bind as dimer to common κB sequence motifs in promoters or enhancers of target genes. Subsequently, transcription is regulated through the recruitment of transcriptional co-activators and transcriptional co-repressors [7]. Inhibition of NF-κB effectively down-regulates inflammation as has been shown in a number of experimental studies [8].

Plant-derived polyphenols are increasingly receiving attention as potential drugs for the treatment of a variety of pathological conditions [9-11]. Their beneficial effect seems to be directly related to structural entities within these compounds as reflected by differences in efficacy amongst individual polyphenols [12,13]. The ability of para- and ortho-hydroquinone moieties within polyphenols to activate the Keap-1/Nrf-2/ARE pathway underscores the relevance of these entities for displaying cyto-protective properties [14]. While it has been unambiguously demonstrated that a number of polyphenols possess strong anti-inflammatory action, the underlying mechanism have been equivocally discussed in recent years. Although tested in different cells or cell lines obtained from different species, Nrf-2-mediated induction of HO-1 [15,16], inhibition of NF-κB [16,17] and inhibition of PLA2 [13] all seems to be pivotal or contributing to the anti-inflammatory action.
In addition to polyphenols, there is also a huge body of evidence indicating that catecholamines have the propensity to modulate immune function in a pleiotypic manner affecting a variety of immune cells including monocytes, lymphocytes and natural killer (NK) cells [18,19]. Modulation of the cytokine network by catecholamines occurs at (patho)-physiological concentrations and is mediated via engagement of adrenergic receptors [19]. Like polyphenols, catecholamines have the propensity to induce HO-1 [20,21] and to inhibit the expression of inflammatory mediators in cultured endothelial and renal epithelial cells in a receptor independent fashion [22,23]. Yet in vitro, their effective concentration to exert these anti-inflammatory properties by far surpasses clinical relevant concentrations, making as to whether catecholamines exert these anti-inflammatory properties in vivo questionable. Nonetheless, it should be emphasized that dopamine-treatment in brain dead rats [24] or in rats subjected to renal ischemia [25] is associated with a reduction of inflammation, albeit that the mechanisms by which this occur may largely differ from the in vitro findings.

We have recently synthesized a more hydrophobic dopamine derivative, i.e. N-octanoyl-dopamine (NOD), which compared to dopamine displayed improved cellular uptake and does not elevate mean arterial blood pressure [26]. In vitro, NOD is approximately 40 times more effective than dopamine in protecting endothelial cells against hypothermic cell injury [26]. Moreover, not only the anti-inflammatory action of NOD is superior to that of dopamine, it is also more effective in reducing ischemia-induced acute kidney injury in rats [27]. In the present study we investigated the anti-inflammatory properties of NOD in more detail. By making use of genome wide gene expression profiling, functional studies and structural variants of dihydroxy benzene derivatives we sought to elucidate the underlying molecular mechanism and molecular entities by which NOD down-regulates TNF-α-mediated inflammatory responses.
Material and Methods

Ethics statement
Human umbilical vein endothelial cells (HUVEC) were received in collaboration with the Institute of Transfusion Medicine and Immunology, Medical Faculty Mannheim, Heidelberg University. Permission for isolation and propagation of endothelial cell from umbilical cords for research purposes was granted by the local ethic committee of the clinical faculty Mannheim, University of Heidelberg Germany, with informed consent in writing.

Cell culture
HUVEC were grown in basal endothelial medium supplemented with 10% FBS and essential growth factors (Promo Cell, Heidelberg, Germany). Only cells in passage 4-6 were used in all experiments.

Gene expression profiling
Sample preparation and processing was performed according to the Affymetrix GeneChip Expression Analysis Manual (http://www.Affymetrix.com). Total RNA from HUVECs was isolated using Trizol reagent (Life Technologies, Rockville, USA). DNase-treatment was carried out using RNase free DNase I (Ambion, Woodward, Austin, USA). RNA-concentration and quality were assessed by RNA 6000 nano assays on a Bioanalyser 2100 system (Agilent, Waldbronn, Germany). 5 µg total RNA was converted into cDNA using T7-(dT)24 primers and the SuperScript Choice system for cDNA synthesis (Life Technologies, Rockville, USA). Biotin-labelled cRNA was prepared by in silico transcription using the BioArray high yield RNA transcript labelling kit (Enzo Diagnostics, Farmingdale, USA). The resulting cRNA was purified, fragmented and hybridized to U133A gene chips (Affymetrix, Santa Clara, USA). After hybridization the chips were stained with streptavidin–phycoerythrin (MoBiTec, Goettingen, Germany) and analysed on a GeneArray scanner (Hewlett Packard Corporation, Palo Alto, USA). The Raw fluorescence intensity values were normalized applying quantile normalization.
**Flow cytometric analysis**

FACS analysis was performed as described previously [28] using FITC-conjugated monoclonal antibodies directed against ICAM-1 (BBIG-I1), VCAM-1 (BBIG-V3) or E-selectin (BBIG-E5) (all from R&D Systems, Wiesbaden-Nordenstadt, Germany). FACS analysis was performed on a FACScalibur (Becton Dickinson, Heidelberg, Germany) equipped with the CELLQuest software. The data were analyzed by Windows Multiple Document Interface (WinMDI) software (Version 2.8).

**Adhesion assays**

HUVEC were seeded either in collagen coated 24 well plates or in flow chambers (ibidi, Munich, Germany) at a concentration of $10^6$ cells per ml. For cell adhesion under static conditions, the plates were washed and incubated for 30 min with 1 ml of $10^6$ carboxyfluorescein succinimidyl ester (CSFE) (Invitrogen, Darmstadt, Germany) labelled peripheral blood mononuclear cells (PBMC). PBMC were isolated using Ficoll gradient centrifugation. CSFE labelling was performed according to the manufacturer’s instructions. The plates were extensively washed with PBS and remaining cells lysed with distilled water. The fluorescence in cell lysates was measured on a Tecan Infinite 200 with the appropriate filters (Tecan Group, Männedorf, Switzerland). For cell adhesion under flow conditions (0.6 dyn/cm$^2$), ibidi chambers were subsequently perfused for 10 min with normal cell culture medium, than perfused for 10 min with cell culture medium containing $10^6$ PBMC/ml and finally perfused for 5 min with normal cell culture medium to remove non-adherent cells. All conditions were performed in triplicate. Each individual chamber PBMC was counted in five random non-coincident microscopic fields (phase contrast). Counting was performed by two investigators without prior knowledge of the experimental conditions.

**Electrophoretic mobility shift assay (EMSA)**

HUVEC were stimulated for different time periods with 10 ng/ml TNF-α alone or in combination with 50 µM NOD. In some experiments the cells were pretreated for 2 hours with 5 µg/ml cyclohexamide (CyHx) before stimulation. In these experiments the cells were stimulated for 8 hours in the continued presence or absence of CyHx. Nuclear extracts were prepared as previously described [29]. Protein-concentrations were determined by Bradford assay. EMSA was performed essentially as previously published [30,31]. Briefly,
N-octanoyl-dopamine inhibits expression of a subset of κB regulated genes

NF-κB (5’-AGTTGAGGGGACTTTCCCAGGC-3’) double-stranded consensus oligonucleotide (Promega Corp., Madison, USA) was end-labeled with γ-32P-ATP using T4-polynucleotide kinase, ethanol precipitated and finally dissolved in 20 µl distilled water. 1 µl 32P-labeled probe (~30,000 cpm) and 15 µg nuclear extracts were added to a binding reaction mixture containing 10 mmol/l HEPES (pH 7.5), 0.5 mmol/l EDTA, 70 mmol/l KCl, 2 mmo/l DTT, 2% glycerol, 0.025% NP-40, 4% Ficoll, 0.1 mol/l PMSF, 1 mg/ml bovine serum albumin and 0.1 mg/ml poly di/dc and incubated for 30 min at room temperature. DNA–protein complexes were separated by electrophoresis through a 5% non-denaturing acrylamide:bis-acrylamide gel in 0.5x Tris–borate/EDTA (TBE) for 3 hours at 220 V. Gels were analyzed by autoradiography using an Amersham Hyperfilm ECL (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). In each experiment, specificity of binding was demonstrated by pre-incubation of cold consensus (100x excess of unlabeled oligonucleotide) or mutated NF-κB oligonucleotide to the nuclear extracts. In addition, supershifts were performed by adding p50, p52, p65, RelB and c-Rel antibodies (all Santa Cruz Biotechnology, Heidelberg, Germany) to the samples.

**Western blot analysis**

HUVEC were lysed in lysis buffer (10 mM Tris, 2% SDS, 0.5% beta-mercaptoethanol) (all from Sigma-Aldrich, St. Louis, USA). Protein concentrations were measured using Coomassie reagent (Pierce, Rockford, USA). Samples (20 µg protein extract) were heated to 95°C for 5 min, loaded and separated on 10-20% SDS-polyacrylamide gels followed by semi-dry blotted onto PVDF membranes (Roche, Mannheim, Germany). Staining of blots was performed by standard operating procedures using polyclonal anti-VCAM-1, anti-HO-1, anti-Nrf-2 antibodies (all Santa Cruz Biotechnology, Heidelberg, Germany). To confirm equal protein loading, membranes were re-probed with monoclonal anti-GAPDH antibody (Abcam, Cambridge, UK).
Cell transfection with siRNA

HUVEC were seeded in 6 well plates at a density of 0.5-2×10^5 one day before transfection with HO-1 siRNA, Nrf-2 siRNA or control siRNA (Santa Cruz Biotechnology, Heidelberg, Germany). Transfection was performed according to the manufacturer’s instructions. Briefly, cells were incubated for 6 hours in transfection medium supplemented with siRNA and transfection reagent. Hereafter, endothelial cell culture medium containing 20% FBS was added without removing the transfection solution and the cells were allowed to grow for additional 24 hours. For each experiment the efficacy of siRNA was demonstrated by disappearance of the specific band in Western blot analysis.

Synthesis of dihydroxybenzoic acid derivatives

2 g 2,5-dihydroxybenzoic acid was suspended in 5 ml acetic anhydride under magnetic stirring. When two drops of sulphuric acid were added, the suspension turned clear and stirring was continued for one hour. Diluted hydrochloric acid (5 ml) was added and 30 min later the reaction mixture was poured into 200 ml ice water. The precipitated product was collected by vacuum filtration and dried under vacuum to yield 2,5-bisacetoxybenzoic acid, pure as judged by thin layer chromatography (TLC). Bisacetoxybenzoic acid was reacted with stoichiometric amounts of ethyl chloroformate to obtain the mixed anhydride which was used without purification. The anhydride was dissolved in dimethyl formamide and the respective amine added in equal stoichiometric quantity. After reacting overnight, the mixture was diluted with ethyl acetate and the organic phase was extracted subsequently with neutral phosphate buffer, brine, diluted sulphuric acid and again brine. Drying over MgSO_4 and removal of the solvent under vacuum yielded the crude product, which was recrystallized from aqueous ethanol.

Statistical analysis

Differential gene expression was analysed based on loglinear mixed model ANOVA, using a commercial software package SAS JMP7 Genomics, version 3.1, from SAS (SAS Institute, Cary, USA). A false positive rate of α=0.05 with Holm correction was taken as the level of significance. Pathways belonging to various cell functions such as cell cycle or apoptosis were obtained from public external databases (KEGG, http://www.genome.jp/kegg/). A Fisher’s exact test was performed to detect the significantly regulated pathways.
N-octanoyl-dopamine inhibits expression of a subset of κB regulated genes

Statistical analyses of cell adhesion assays under static and flow conditions were performed using SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany). Data were compared with the Kruskal-Wallis signed-rank test and Dunn’s post hoc test when required. Statistical significance was defined as \( p < 0.05 \). Descriptive statistics are expressed as mean ± SD.

For Western blots optical density of bands of all blots were assessed using ImageJ 1.46 and Student’s t-test with previous testing of normal distribution by SigmaPlot 11.0 (Systat Software GmbH, Erkrath, Germany) was performed. If distribution test failed, the Kruskal-Wallis-test was performed.
Results

Anti-inflammatory potential of NOD

To investigate the anti-inflammatory potential of NOD, we screened by genome wide gene expression profiling in HUVEC for genes that were down-regulated by NOD. To this end, three different primary cultures of HUVEC were stimulated with TNF-α alone or in combination with 100 µM NOD. Two major differences were observed when an arbitrary cut-off for a fold change of at least 2 was chosen. Firstly, the expression of a number of genes encoding chemokines or adhesion molecules was strongly down-regulated, and secondly, down-regulation in genes which are believed to be involved in the ubiquitin-proteasome system (UPS) was noted. Enlisted in table 1 are chemokines and adhesion molecules that were more than two-fold down-regulated by NOD, when comparing TNF-α vs. TNF-α + 100 µM NOD. Changes in chemokine expression were found for the CCL and CXCL family members, but also for fractalkine (CX3CL1). Similarly, the expression of three major adhesion molecules, i.e. VCAM1, ICAM1 and SELE (E-selectin), was significantly reduced in the presence of NOD (Table 1). Changes in gene expression for genes belonging to the UPS included ubiquitin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change$^a$</th>
<th>p-value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chemokines</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>1.35</td>
<td>9.70E-07</td>
</tr>
<tr>
<td>CCL5</td>
<td>3.4</td>
<td>3.10E-11</td>
</tr>
<tr>
<td>CCL20</td>
<td>3.09</td>
<td>5.30E-08</td>
</tr>
<tr>
<td>CXCL1</td>
<td>3.07</td>
<td>2.00E-13</td>
</tr>
<tr>
<td>CXCL2</td>
<td>2.66</td>
<td>5.20E-09</td>
</tr>
<tr>
<td>CXCL3</td>
<td>3.26</td>
<td>7.80E-12</td>
</tr>
<tr>
<td>CXCL5</td>
<td>4.26</td>
<td>2.20E-33</td>
</tr>
<tr>
<td>CXCL6</td>
<td>4.05</td>
<td>1.30E-08</td>
</tr>
<tr>
<td>CXCL10</td>
<td>5.42</td>
<td>5.50E-21</td>
</tr>
<tr>
<td>CXCL11</td>
<td>5.47</td>
<td>1.80E-29</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>4.28</td>
<td>4.50E-18</td>
</tr>
<tr>
<td></td>
<td>Adhesion molecules</td>
<td></td>
</tr>
<tr>
<td>VCAM1</td>
<td>6.11</td>
<td>7.90E-21</td>
</tr>
<tr>
<td>ICAM1</td>
<td>2.47</td>
<td>1.60E-18</td>
</tr>
<tr>
<td>SELE</td>
<td>4.94</td>
<td>5.70E-24</td>
</tr>
</tbody>
</table>

Table 1: Down-regulation of chemokines and adhesion molecules by NOD.

$^a$fold change values are expressed as log$_2$, TNF-α vs. TNF-α + 100 µM NOD. $^b$p-values for the comparison TNF-α vs. TNF-α + 100 µM NOD are given as log$_{10}$.
N-octanoyl-dopamine inhibits expression of a subset of κB regulated genes

ligases (UBE2L6 and HERC6), ubiquitin like modifiers (ISG15 and UBD) and several proteasome subunits (PSME1, PSMB10, PSMB9 and PSMB8) (Table 2). Although in affymetrix analysis some of the signalling molecules belonging to the NF-κB pathway were slightly reduced by NOD (TNF-α vs. TNF-α + NOD fold change as log₂: RELB: 0.73; NFKB1: 0.66; NFKBIA: 0.80 and IKBKE: 0.86), qPCR analysis revealed only a significant change for RELA, RELB and NFKBIE in independent experiments (data not shown). The expression of 95 genes was more than two-fold up-regulated by TNF-α + NOD compared to TNF-α alone. With the exception of HO-1 (HMOX1: fold change (log₂) 4.37; p-value: 1.9E-22), these differences were not further analysed. The complete dataset, including normalised and raw data, are available at the GEO repository http://www.ncbi.nlm.nih.gov/geo/ with accession number (GSE34059). The influence of NOD on VCAM1, ICAM1, SELE and HMOX1 was confirmed by qPCR in independent experiments (data not shown).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin ligases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBE2L6</td>
<td>2.04</td>
<td>5.80E-11</td>
</tr>
<tr>
<td>HERC6</td>
<td>2.48</td>
<td>2.10E-12</td>
</tr>
<tr>
<td>Ubiquitin like</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBD</td>
<td>5.6</td>
<td>2.10E-22</td>
</tr>
<tr>
<td>ISG15</td>
<td>2.68</td>
<td>3.70E-15</td>
</tr>
<tr>
<td>Proteasome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSME1</td>
<td>1.55</td>
<td>6.80E-12</td>
</tr>
<tr>
<td>PSMB10</td>
<td>1.75</td>
<td>7.00E-12</td>
</tr>
<tr>
<td>PSMB8</td>
<td>1.96</td>
<td>3.10E-11</td>
</tr>
<tr>
<td>PSMB9</td>
<td>3.46</td>
<td>2.20E-10</td>
</tr>
</tbody>
</table>

Table 2: Down-regulation of UPS associated genes by NOD.

*afold change values are expressed as log₂, TNF-α vs. TNF-α + 100 µM NOD. bp-values for the comparison TNF-α vs. TNF-α + 100 µM NOD are given as log₁₀.*
**NOD impairs PBMC adhesion to endothelial cells**

Western blotting revealed that NOD dose-dependently inhibits TNF-α-mediated VCAM-1 expression on protein level and confirmed that NOD induces the expression of HO-1 (Figure 1). An almost complete inhibition of VCAM-1 was observed at a concentration of 12 µM of NOD, while induction of HO-1 was already noticed at 1 µM NOD (Figure 1A). Similar as demonstrated for VCAM-1 expression, FACS analysis revealed that TNF-α-mediated up-regulation of E-selectin and ICAM-1 was blunted when the cells were stimulated with TNF-α in the presence of NOD (Figure 1B). Induction of HO-1 expression was completely independent of TNF-α as HO-1 was also induced when cells were stimulated with NOD alone (Figure 1C).

![Western blot and FACS analysis](image)

**Figure 1: Influence of NOD on the expression of adhesion molecules and HO-1.**

A: HUVEC were stimulated for 24 hours with 10 ng/ml TNF-α in the presence of different concentrations of NOD. The expression of VCAM-1 and HO-1 was assessed by Western blotting. GAPDH was used as loading control.

B: HUVEC were stimulated as described in A. The expression of ICAM-1 and E-selectin was assessed by FACS analysis.

C: To demonstrate that the induction of HO-1 by NOD was independent of TNF-α, HUVEC were stimulated for 24 hours with 10 ng/ml of TNF-α alone, 100 µM of NOD alone or in combination of both. HUVEC cultured in medium served as control. The expression of VCAM-1 and HO-1 was assessed by Western blotting.

The results shown in A, B and C are representative experiments. A total of 6 independent experiments with different HUVEC cultures were performed. All Western blots have been scanned and statistics was performed on the ratio of the optical density of protein of interest/GAPDH. Significant inhibition of VCAM-1 expression occurred at NOD-concentrations above 12.5 µM and HO-1 induction already at 1 µM.
Under static conditions, adhesion of peripheral blood mononuclear cells (PBMC) to HUVEC was significantly impaired when HUVEC were stimulated with the combination of TNF-α + NOD as compared to TNF-α alone (Figure 2A). Also under flow conditions adhesion of PBMC to HUVEC was strongly impaired when HUVEC were stimulated with TNF-α + NOD (Figures 2B+C).

Figure 2: Influence of NOD on the adherence of PBMC to endothelial cells.
A: Adherence of PBMC was assessed under static conditions. To this end, HUVEC were seeded in 24-well plates and stimulated for 24 hours with 10 ng/ml TNF-α alone, 100 µM NOD alone or with the combination of both. HUVEC cultured in medium served as control. CSFE-labelled PBMC were added to the plates for 30 min in a concentration of 10⁶ cells/well. Hereafter the plates were thoroughly washed and the fluorescence signal was measured in the cell lysates. All conditions were tested in triplicates and at least 4 independent experiments were performed. The results are expressed as mean fluorescence ± SD.
B: Adherence of PBMC under flow conditions. HUVEC were seeded in ibidi flow chambers and stimulated as described in A. The chambers were flushed as described in the Methods section and adherent PBMC were counted by two investigators without prior knowledge of the experimental conditions. All conditions were performed in triplicate and for each individual chamber five random microscopic fields (phase contrast) were counted. A total of 4 different experiments were performed the results are expressed as mean cell count ± SD.
C: A representative microscopic field is shown.
NOD inhibits activation of NF-κB

TNF-α-mediated expression of chemokines and adhesion molecules critically depends on activation of the NF-κB transcription factor. We therefore tested if an impaired activation of NF-κB could underlie the decreased mRNA expression of chemokines and adhesion molecules when NOD was present during TNF-α stimulation. NF-κB was activated by TNF-α in a time dependent manner with maximal activation occurring at 24 hours of stimulation. Although in the presence of NOD activation of NF-κB also occurred, it was at all studied time-points clearly diminished (Figure 3A). Since Affymetrix analysis revealed that the expression of genes belonging to the UPS were significantly changed by NOD, we anticipated that NOD could potentially interfere with the degradation of IκBα. 10 minutes after TNF-α stimulation IκBα was not anymore detected in Western blot analyses but re-appeared after 30 min. The presence of NOD during stimulation did not influence degradation of IκBα (Figure 3B). To exclude that the time of TNF-α stimulation would be too short for reaching sufficient intra-cellular concentrations of NOD, HUVEC were pretreated for 24 hours with NOD and subsequently stimulated over an 1 hour-period with TNF-α in the continued presence of NOD. Degradation of IκBα still occurred in this experimental setting (data not shown) suggesting that NOD does not interfere with the initial events of NF-κB activation.

Post-translational modifications of NF-κB proteins are of eminent importance for transcriptional regulation of a number of NF-κB regulated genes. In particular, phosphorylation of NF-κB p65 on serine 276 (Ser276) seems to be instrumental for the recruitment of co-activators and subsequently gene transcription during inflammation [32,33]. We therefore tested if Ser276 phosphorylation of NF-κB p65 was affected by NOD and to what extent the expression of total NF-κB p65 in nuclear and cytoplasmic extracts was influenced. In cells that were not stimulated, the expression of NF-κB p65 was more prevalent in the cytoplasmic extract as compared to the nuclear extract. Upon stimulation with TNF-α, there was a shift towards a higher expression of NF-κB p65 in the nuclear extract, compatible with its nuclear translocation. Interestingly, in cells that were stimulated with the combination of TNF-α + NOD the expression of NF-κB p65 in both the cytoplasmic and nuclear extract was lower compared to cells that were not stimulated or stimulated with TNF-α alone (Figure 3C, panel in the middle). Phosphorylation on Ser276 only occurred in the nucleus when the cells were stimulated with TNF-α. This was completely prevented when NOD was present during stimulation (Figure 3C, upper panel).
N-octanoyl-dopamine inhibits expression of a subset of κB regulated genes

Figure 3: Influence of NOD on TNF-α-mediated NF-κB activation.

A: HUVEC were stimulated for different time periods with 10 ng/ml TNF-α. During stimulation NOD (50 µM) was absent (-) or present (+). Nuclear extracts were prepared and assessed for NF-κB activation by means of EMSA.

B: In separate experiments the influence of NOD on the degradation of IκBα was assessed. HUVEC were stimulated for different time periods in the presence (+) or absence (-) of 50 µM NOD. HUVEC grown in medium served as control. IκBα degradation was assessed by Western blotting.

C: HUVEC were stimulated for 24 hours with 10 ng/ml TNF-α in the presence (+) or absence (-) of 50 µM NOD. Cells that were left untreated (medium) served as control. Nuclear- and cytoplasmic extracts were prepared and assessed for the expression of phosphorylated p65 at Ser276 (p65-Ser276) and total p65 (p65) by Western blotting. GAPDH was used as loading control. Note that in the condition of TNF-α + NOD phosphorylation of p65 at Ser276 is not detectable in the nuclear extracts and that expression of total p65 in both nuclear and cytoplasmic extracts is decreased. The results of a representative experiment are shown. A total of 4 independent experiments with different HUVEC cultures were performed.
**NOD-mediated inhibition of NF-κB does not require HO-1**

Since it has been demonstrated that HO-1 is able to inhibit NF-κB p65 phosphorylation at Ser276 [34] and because HO-1 was strongly induced by NOD, we sought to assess the contribution of HO-1 on NOD-mediated inhibition of NF-κB. To this end, we employed an siRNA approach to either knock-down NF-E2 related factor-2 (Nrf-2) expression, a transcription factor that drives the expression of HO-1, or by knock-down of HO-1 expression directly (Figure 4). Neither Nrf-2 nor HO-1 siRNA completely blocked HO-1 expression, yet, even though HO-1 expression was significantly diminished by these siRNAs the inhibitory effect of NOD on VCAM-1 expression was not affected (Figure 4).

To formerly exclude a role for HO-1 in inhibition of NF-κB by NOD, we used a second approach by blocking de novo protein synthesis. HUVEC were either pre-incubated with cyclohexamide (CyHx) for 2 hours or left untreated and subsequently stimulated for 8 hours with TNF-α alone or in combination with NOD in the continued presence of CyHx. The 8 hours time period of stimulation was chosen on the basis of CyHx associated cell toxicity that usually occurred after 12 hours of CyHx-treatment. In this experimental setting protein synthesis was effectively blocked by CyHx since induction of VCAM-1 by TNF-α alone or induction of HO-1 by NOD was not observed in the presence of CyHx (Figure 5A). Similar as shown in figure 3A, activation of NF-κB was evident after

![Figure 4: Partial silencing of Nrf-2 and HO-1 expression does not abrogate NOD-mediated inhibition of VCAM-1.](image)

HUVEC were transfected with control siRNA, HO-1 siRNA or Nrf-2 siRNA. One day after transfection the cells were stimulated for 24 hours with TNF-α alone (10 ng/ml), NOD alone (50 µM) or in combination of both. Cells that were not stimulated were included in each experiment. The expression of VCAM-1, Nrf-2 and HO-1 was assessed by Western blotting. GAPDH was used as loading control. The results of a representative experiment are shown. A total of 3 independent experiments with different HUVEC cultures were performed.
8 hours of stimulation with TNF-α alone, while it was strongly diminished in combination with NOD (Figure 5B). In CyHx-pretreated HUVEC NF-κB activation was less pronounced after TNF-α stimulation, yet inhibition was still observed in the combination of TNF-α + NOD (Figure 5B).

![Image of Western blot and EMSA](image)

**Figure 5: HO-1 induction by NOD is not required for inhibition of NF-κB.**

**A:** HUVEC were pretreated for 2 hours with 5 mg/ml of cyclohexamide (+CyHx) or left untreated (-CyHx). Hereafter, cells were stimulated for 8 hours with 10 ng/ml TNF-α in the presence (+) or absence (-) of 50 µM NOD. In case the cells were pretreated with CyHx, this was present during the whole period of stimulation. In case cells were not treated with CyHx, this was absent during stimulation. Western blotting of the cytoplasmic fractions revealed that de novo protein synthesis was effectively inhibited by CyHx. Note that VCAM-1 is not induced by TNF-α in the presence of CyHx. Also in the combination of TNF-α + NOD the induction of HO-1 did not occur. GAPDH was used as loading control.

**B:** Nuclear extracts were prepared and assessed for NF-κB activation by means of EMSA. Specificity of the bands was assessed by adding an excess of unlabelled NF-κB consensus (cold consensus [CC]) or mutated (cold mutated [CM]) oligonucleotides to the samples. To demonstrate the presence of p50 and p65 in the shifted bands super-shifts (SS) were performed by adding anti-p50 or anti-p65 monoclonal antibodies to the samples. In A and B the results of a representative experiment are shown. A total of 4 independent experiments with different HUVEC cultures were performed.
**Structural requirement for inhibition of VCAM-1 by NOD**

To assess the structural entities within NOD that are responsible for its anti-inflammatory effect, we synthesized structurally related compounds that differ in their redox activity or in their hydrophobicity (Figure 6). To this end, dopamine or tyramine were covalently bound to octanoic acid at the amine side chain, yielding NOD or N-octanoyl-tyramine (NOT). These compounds differ in redox activity, while the hydrophobicity of both is not significantly different as calculated by the engine at www.molinspiration.com (3.7 vs. 4.0). In addition we synthesized compounds by sequential modification of the redox active 2,5-dihydroxy-benzoic acid (genestic acid). The dihydroxy moieties were first acetylated resulting in 2,5-bisacetoxy-benzoic acid (BB). This prevents oxidation of the compound, unless redox activity is restored through the action of intracellular esterase activity, and facilitates cellular uptake by reducing polarity. In a second step the 2,5-bisacetoxy-benzoic acid was reacted at the free carboxy group with either n-butylamine or n-octylamine, resulting in 2,5-bisacetoxybenzoyl-N-butylamide (BBNB) and 2,5-bisacetoxybenzoyl-N-octanoylamide (BBNO) respectively. These compounds differ in hydrophobicity compared to the parent compound BB, while the redox activity is similar provided that the dihydroxy moiety is restored by intracellular esterase activity.

All compounds were tested for their ability to inhibit TNF-α-mediated VCAM-1 expression (Figure 7). In contrast to NOD NOT was not able to inhibit VCAM-1 expression indicating that a redox active moiety might be essential for VCAM-1 inhibition. In addition, VCAM-1 inhibition only occurred if the free 2,5-bisacetoxy-benzoic acid was linked to butylamine or octylamine indicating that inhibition requires sufficient hydrophobicity of the compound. All compounds that were able to inhibit VCAM-1 expression also induced the expression of HO-1.
N-octanoyl-dopamine inhibits expression of a subset of κB regulated genes

Figure 6: Structure of the compounds used in this study.
N-octanoyl-dopamine (NOD); N-octanoyl-tyramine (NOT); 2,5-bisacetoxy-benzoic acid (BB); 2,5-bisacetoxy-benzoyl-N-butylamide (BBNB); 2,5-bisacetoxy-benzoyl-N-octanoylamide (BBNO).

Figure 7: A redox moiety and sufficient hydrophobicity are required for NOD-mediated inhibition of VCAM-1 expression and for induction of HO-1.
HUVEC were stimulated with 10 ng/ml TNF-α alone (-) or stimulated with TNF-α in the presence of the different compounds shown in figure 6. Cells that were left untreated (medium) served as control. The expression of VCAM-1 and HO-1 was assessed by Western blotting. GAPDH was used as loading control. The results of a representative experiment are shown. A total of 3 independent experiments with different HUVEC cultures were performed.
Discussion

In the present study we assessed the anti-inflammatory potential of NOD and sought to elucidate the underlying molecular mechanism by which this was mediated. The main findings of this study are the following. Firstly, NOD down-regulates a wide range of κB regulated pro-inflammatory mediators, e.g. chemokines and adhesion molecules, yet not all κB regulated genes were affected by NOD. Down-regulation of inflammatory mediators had functional consequences for the adherence of PBMC to endothelial cells and was associated with inhibition of NF-κB. Secondly, inhibition of NF-κB occurred independently of IκBα degradation and was reflected by an overall decrease in p65 expression and a decreased phosphorylation of p65 at Ser276. Thirdly, de novo protein synthesis was not required for inhibition of NF-κB, hence excluding that up-regulation of HO-1 was involved in the anti-inflammatory properties of NOD. In line with this, it was found that in HO-1 siRNA transfected cells NOD-mediated inhibition of VCAM-1 expression was not impaired. Finally, we provide evidence that redox activity and hydrophobicity are important molecular entities that are required for the anti-inflammatory properties of NOD.

Owing to its ability of simultaneously activating multiple signalling pathways, TNF-α regulates a plethora of biological responses in cells, e.g. cell death, proliferation, differentiation and inflammation [35,36]. This is essentially mediated through the formation of two signalling complexes, mediating NF-κB activation and initiating apoptosis respectively [37,38]. Cross-talk between both platforms occurs via NF-κB dependent transcription of anti-apoptotic genes. Inhibition of NF-κB therefore instantaneously results in cell death [38]. Interestingly, our data seem to be in contradiction with this notion, as apoptosis was not noticed when HUVEC were simultaneously stimulated with TNF-α and NOD, despite the fact that NF-κB was clearly inhibited under this condition. Because degradation of IκBα, was not influenced by NOD and not all κB regulated gene transcription was equally affected, our data suggest that NOD does not completely prevent activation of NF-κB. Limited activation of NF-κB that occurs in the presence of TNF-α and NOD might still be sufficient to drive the transcription of the anti-apoptotic proteins Bcl-xl, c-FLIP and XIAP, which is in line with the observation that Affymetrix analysis did not reveal statistical differences in their expression.

Transcription factor-selective and signal-specific cofactor recruitment is
important to ensure restricted gene transcription despite redundancy in signalling pathways [39-41]. For many NF-κB regulated genes, accessibility of κB sites in promoter regions requires DNA uncoiling, a process which is regulated by histone acetyltransferase (HAT) activity [42,43]. Evidence has culminated in recent years demonstrating that HAT activity is provided to p50/RelA NF-κB dimers by the recruitment of CBP/p300. This is essential for transcription of a number of inflammatory genes [44,45], particularly for genes in which the κB sites are not directly accessible [43]. Although our study did not address if CBP/p300 recruitment is impaired by NOD, we did observe that phosphorylation of RelA (p65) at Ser276 was inhibited by NOD. In keeping with the essential role of p65 Ser276 phosphorylation for recruitment of CBP/p300 [32,33], it is likely that the selective inhibitory effect of NOD on the transcription of pro-inflammatory genes might be related to the lack of DNA uncoiling required for transcription of these genes, leaving κB regulated genes with direct accessibility unaffected.

Apart from the lack of p65 Ser276 phosphorylation, we also observed an overall decrease in cellular p65 expression in TNF-α plus NOD stimulated cells. The mechanism that underlies this observation is still elusive. In addition to IκBα-mediated nuclear export of NF-κB, proteasomal degradation of DNA-bound p65 regulates NF-κB dependent gene expression [46,47]. Hence, an increased proteasomal degradation of p65 might account for an overall diminished p65 expression in TNF-α plus NOD stimulated cells. Yet, it should be emphasized that NOD down-regulates several genes involved in ubiquitination and proteasomal degradation, including proteasomal subunits, ubiquitin ligases and ubiquitin like modifiers (Table 2). Amongst these genes, the ubiquitin like modifier UBD appeared to be the one that was the most affected by NOD. Recent studies have demonstrated that UBD expression is important for appropriate NF-κB activation [48,49]. In renal tubular epithelial cells derived from UBD−/− mice TNF-α-induced NF-κB activation is abrogated as the result of an altered proteasomal subunit expression [48,49]. Although our data demonstrate a decreased expression for both UBD and proteasomal subunits (LMP2 [PSMB9], LMP7 [PSMB8], LMP10 [PSMB10]), our results do not allow the conclusion that an altered proteasomal subunit expression, as observed in cells that were stimulated in the presence of NOD, is causally related to inhibition of NF-κB. Because transcription of UBD and proteasomal subunits is also regulated by NF-κB [50,51], it is more likely that the expression of genes belonging to the ubiquitin proteasomal system is modulated by NOD through an inadequate NF-κB activation.
NF-κB activation has a dual and opposite dependence on oxidative events, because its translocation is favoured by an oxidative milieu in the cytosol while binding to DNA requires a reductive environment in the nucleus [52-54]. Therefore the finding that the anti-inflammatory properties of NOD rely on its redox activity was not surprising and compatible with published studies on polyphenols [55,56]. Our data are also in line with a previously published study in which it was shown that catechols in caffeic acid phenethyl ester (CAPE) selectively inhibit NF-κB target genes [17].

Activation of the Keap-1/Nrf-2 pathway determines the ability of multicellular organisms to adapt to conditions of stress caused by oxidants and electrophiles via induction of proteins with versatile cytoprotective functions such as HO-1. Para- and ortho-dihydroxybenzene derivatives (catechols and hydroquinones) were among the first identified small-molecule inducers of this pathway. Oxidation of these hydroquinones to their corresponding electrophilic quinones is a requisite step for the activation of the Keap-1/Nrf-2 pathway. In line with this, it was found that HO-1 was induced by the catechol NOD and that this was partly dependent on Nrf-2. Also the BBNB and BBNO derivatives, which by virtue of intracellular esterase activity are converted to para-hydroquinones, strongly increased HO-1 expression. In contrast, the free BB did not induce HO-1 expression, which might be explained by its lower hydrophobicity and thereby its inefficient cellular uptake. Therefore, apart from its redox activity a sufficient degree of hydrophobicity seems to be important for the anti-inflammatory effect of NOD. This is also supported by our previous finding that inhibition of VCAM-1 expression by dopamine is only partial and occurs at much higher concentrations (300 µM) [22] compared to the present finding on the more hydrophobic NOD.

We are aware of the studies performed by Soares et al. [57] and Seldon et al. [34] showing a pivotal role for HO-1 in down-regulation of VCAM-1 expression. According to their data HO-1 down-regulates the inflammatory phenotype of endothelial cells by reducing intracellular nonprotein-bound iron [34]. Accordingly, reduction of the labile iron pool results in hypophosphorylation of RelA Ser276. Although we can exclude that the inhibitory effect of NOD on NF-κB is mediated via up-regulation of HO-1, a reduction of the labile iron pool as cause for RelA Ser276 hypophosphorylation might still be valid. Microorganisms circumvent low iron availability by secreting siderophores that complex ferric iron with high affinity [58]. The chelating functionalities of siderophores include catecholates, hydroxamates, and α-hydroxycarboxylates [59]. Hence, both NOD (catecholate)
and the from genetic acid (α-hydroxycarboxylates) derived BB compounds have iron chelating functionalities. In contrast to these compounds NOT has no iron chelating properties and is not able to inhibit VCAM-1 expression. It remains to be assessed if the importance of the redox moiety of NOD for its inhibitory effect is related to impairment of the redox milieu within intracellular compartments, its iron chelating functionality or both.

In conclusion our data demonstrate that NOD has a potent inhibitory effect on TNF-α-mediated inflammatory processes. This occurs, most likely through its action on post-translational modification of NF-κB. Inhibition seems to be selective, affecting only the expression of a subset of κB regulated genes. Although redox modulation of chromatin remodeling may account for selectivity in this regard, the role of iron in RelA Ser276 phosphorylation, its role in chromatin remodelling and if the effect of NOD is only restricted to the TNF-α signalling cascade need to be addressed in further studies.

Acknowledgements

We thank Paula Sternik for her technical support.
References


N-octanoyl-dopamine inhibits expression of a subset of κB regulated genes


