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 is a potent inhibitor of platelet function
Abstract

Dopamine (DA) is a co-agonist for platelet activation; yet, donor DA-treatment is associated with improved transplantation outcome in renal and heart recipients. Recently, N-octanoyl-dopamine (NOD) was developed which displays superior effects compared to DA in terms of graft protecting properties. Whereas DA is a known platelet co-agonist the effect of NOD on platelet function is unknown. This is a hypothesis generating study with the aim to assess the effects and molecular mechanisms of NOD and NOD-like compounds on platelet function.

The influence of DA, NOD and NOD-like compounds on platelet responses to classical agonists (ADP, U46619) was investigated in 6 healthy donors by applying whole blood aggregometry (Multiplate) and flow cytometry for Pac-1, CD62P and CD63 expression. Changes in platelet cAMP-concentrations were assessed by ELISA.

While DA showed synergy in platelet activation by ADP and U46619, NOD caused significant inhibition of platelet function both in whole blood aggregometry and flow cytometry. The inhibitory effect of NOD was not mediated via cAMP levels. The nonredox-active NOD-analog N-octanoyl-tyramine (NOT) had no effects on platelet function. Acetylated-NOD (A-NOD) that is conferred to NOD by intracellular esterases showed similar inhibitory effects as NOD.

In contrast to DA, NOD is a potent inhibitor of platelet function most likely through intracellular redox-active processes. This adds to the overall protective effect of NOD on pre-transplantation injury and makes NOD an attractive candidate compound for donor or organ conditioning prior to transplantation.
Introduction

Since the seminal finding of Takada et al. [1] that brain death leads to immune activation in peripheral organs, donor brain death and its implications for transplantation outcome has received much scientific attention. The awareness that immune activation already occurs in the donor and the perception that this represents a therapeutic target for maintaining organ quality in brain dead donors, has paved the way for donor orientated clinical transplantation research. Implementation of hormonal resuscitation therapy (HRT) in donor management [2], the use of methylprednisolone, either alone [3] or as part of HRT, or protective lung ventilatory strategies [4] are only a few examples that convincingly demonstrate that the concept of donor treatment not only increases the number of eligible and harvested organs but also has a major impact on transplantation outcome.

In line with this concept, we have shown in both retro- [5,6] and prospective [7] studies that low-dose donor dopamine (DA)-treatment has a salutary effect on transplantation outcome in recipients who received a renal or heart allograft [8]. Although the underlying mechanism seems to be related to graft protecting properties of DA [9], particularly in the setting of organ preservation, we also have shown that DA limits inflammation in end-organs in the course of brain death [10]. Platelet activation and adherence to the endothelium are prominent features of the inflammatory cascade and may occur in donors with severe traumatic brain injury [11,12]. In addition, platelet activation may occur in recipients as a consequence of cold ischemia/reperfusion-injury [13]. Since platelets express different adrenergic and dopaminergic receptors [14-16], the release of catecholamines at the onset of brain death or catecholamine infusion to stabilize blood pressure in brain dead donors might affect platelet function. Indeed, we and others have shown that DA is an ADP-dependent platelet agonist [17-19] that acts via D2-like but not D1-like receptors [17].

Inasmuch as the salutary effect of donor DA-treatment seems to be mediated independently of receptor engagement, we have synthesized a DA derivative, i.e. N-octanoyl-dopamine (NOD), which is devoid of hemodynamic action and yet is more efficacious than DA in terms of protecting cells against cold preservation injury [20]. In the present study we compared the effects of DA, NOD and NOD-like compounds on platelet function.
Materials and Methods

Reagents and compounds

Pac-1 antibody (FITC-conjugated monoclonal antibody), CD62P (PE-conjugated mouse-anti-human monoclonal antibody), CD63 (R-PE-conjugated mouse-anti-human monoclonal antibody) were obtained from BD Bioscience (Heidelberg, Germany). Adenosine 5′-diphosphate (ADP) was purchased from Dynabyte GmbH, Munich, Germany, dopamine hydrochloride from Sigma-Aldrich (Taufkirchen, Germany) and the thromboxane A₂ analog U46619 from Tocris Bioscience (Ellisville, USA).

Synthesis of dopamine derivates

NOD was synthesized from commercially available precursors as previously described [20]. Briefly, octanoic acid was dissolved in tetrahydrofurane and N-ethylispropylamine before conversion to their mixed anhydride by the reaction with ethyl chloroformate. For coupling dopamine hydrochloride was dissolved in N,N-dimethylformamide in the presence of diisopropylamine. Sodium hydrogen carbonate and sodium sulfate were added and after evaporation of the solvent the acylated product was obtained. N-octanoyl-tyramine (NOT) was synthesized according to NOD using the analogue mixed anhydride coupled to tyramine coupling material. 3,4-bisacetoxy-N-octanoyl-dopamine (A-NOD) was synthesized in one step by mixing NOD with acetic anhydride and sodium acetate. All compounds were purified by twofold recrystallisation to homogeneity which was proved by thin layer chromatography (TLC). Samples investigated by ¹H-NMR spectroscopy yielded spectra in accordance with the expected structure.

Blood samples and platelet preparation

Whole blood was collected from 6 healthy nonsmokers, who had not taken any drugs for the last 10 days to exclude interference with platelet function. Platelet-rich-plasma (PRP) was obtained by centrifugation of citrated blood at 200 xg for 15 min. For platelet aggregometry blood was anticoagulated with hirudin.
Flow cytometric analysis of fibrinogen receptor activation and platelet degranulation

Binding of Pac-1 antibody as a marker for active confirmation of the fibrinogen receptor (GPIIb/IIIa) as well as P-selectin (CD62P) and LAMP3 (CD63) surface expression as markers for the release of α-granules and dense granules, respectively, were measured in PRP by flow cytometry (FACSCalibur, BD Bioscience, Heidelberg, Germany). 10 µl PRP were added to 100 µl HBS buffer (150 mM NaCl, 5 mM KCl, 1 mM MgSO₄ and 10 mM HEPES pH 7.4) and incubated in a dark environment at room temperature for 20 min containing 10 µl of the desired agonist and saturating antibody-concentrations. To investigate the effects of dopamine and NOD-like substances, platelets were treated either with dopamine (100 µM), the NOD-likes (NOD, NOT, A-NOD; all at 100 µM) alone or in combination with classical platelet agonists 5 µM ADP and 5 µM U46619. Cell wash solution (BD Bioscience, Heidelberg, Germany) was added and samples were employed to flow cytometric analysis. Based on forward and side scatters, a gate was set around the platelet population and 20,000 events were acquired from each sample. The proportion of active fibrinogen receptor as well as P-selectin and LAMP3 surface expression is presented as mean fluorescence intensity (MFI).

Whole blood platelet aggregometry

The effect of DA and NOD on platelet aggregation was investigated by whole blood impedance aggregometry using a multiple platelet function analyzer (Multiplate Instruments, Dynabyte GmbH, Munich, Germany). Hirudin anticoagulated blood of 6 healthy volunteers was treated either with DA or NOD solely or in combination with classical platelet agonists 0.75 µM U46619 (thromboxane A₂ analog) and 0.5 µM ADP (Sigma Aldrich, Munich, Germany; Tocris Bioscience, Wiesbaden-Nordenstadt, Germany) according to Multiplate standard protocols. Aggregation was recorded for 6 min and maximum aggregation is given in arbitrary units (AU).
cAMP assay
For the determination of intracellular cAMP levels, samples of 200 µl platelet suspension in HBS buffer were incubated with either 10 µM PGI₂, 100 µM NOD, 5 µM ADP or in combination at 37°C for 15 min. Platelets were lysed with lysis reagent provided by the assay supplier and samples were immediately frozen in liquid nitrogen. After thawing, the samples were centrifuged and supernatants were used to measure cAMP (Biotrak cAMP ELISA; Amersham Pharmacia Biotech, Freiburg im Breisgau, Germany).

Statistical analysis
All laboratory assays were performed on blood samples of 6 healthy individuals. Statistical significance was calculated on the basis of two-sided t-tests for paired samples using SPSS 12.0 (SPSS, Chicago, USA) statistical software.
Results

Effect of NOD on fibrinogen receptor activation and platelet degranulation

Platelet activation was investigated in PRP of 6 healthy individuals by flow cytometry. 100 µM DA or 100 µM NOD alone did not significantly affect binding of the anti-CD62P, anti-CD63 and Pac-1 antibodies to platelets (Figure 1). Agonist induced activation was clearly noticed for 5 µM U46619 (Figure 1) and ADP (data not shown) reflected by a significant MFI increase for CD63, CD62P and Pac-1. In the presence of U46619, DA significantly enhanced the expression of all three antigens on platelets. In contrast, addition of equimolar concentrations of NOD significantly inhibited the expression of all three antigens.

Effect of NOD on platelet aggregation

Since NOD significantly impaired platelet degranulation and fibrinogen receptor activation, we further tested the effects of NOD on whole blood aggregation. For all 6 healthy volunteers neither 100 µM DA nor 100 µM NOD alone affected platelet aggregation (Figure 2A). Under conditions of agonist-induced platelet activation NOD significantly lowered the platelet aggregation response to U46619 (Figure 2A). Similar results were obtained when ADP was used as agonist (data not shown). Lower NOD-concentrations, i.e. 1 and 10 µM, did not significantly inhibit platelet aggregation (Figure 2B) and were also not able to lower the expression of CD63, CD62P and Pac-1 (data not shown).
Figure 1: Effects of DA and NOD on platelet fibrinogen receptor activation and platelet degranulation.

A: Fibrinogen receptor activation was measured by surface binding of FITC-labeled Pac-1 antibody by flow cytometry in PRP of six healthy individuals. Representative result of platelet co-stimulation with the classical platelet agonist U46619 and the catecholamine DA showed increase in Pac-1 binding, whereas the presence of the DA derivate NOD inhibited the U-induced Pac-1 binding.

B: Statistical analysis of A. DA and the DA derivative NOD had no effect on fibrinogen receptor activation. Co-stimulation of platelets with 5 µM U46619 and 100 µM DA resulted in significant increase of Pac-1 binding. In the presence of the DA derivative 100 µM NOD, U46619-induced Pac-1 binding was significantly inhibited.

Platelet degranulation was investigated by measuring surface expression of CD62P and CD63 by flow cytometry in PRP of six healthy individuals (middle and right bars). 100 µM DA and 100 µM NOD had no effect on platelet degranulation. Co-stimulation of platelets with 5 µM U46619 and 100 µM DA resulted in significant increase of CD62P and CD63 expression. In the presence of the DA derivative 100 µM NOD, U46619-induced CD62P and CD63 expression were significantly inhibited (*p<0.05, **p<0.01, ***p<0.001).
The effect of NOD on cAMP production

To determine the molecular mechanism of the inhibitory effect of NOD on platelet activation, we measured intracellular cAMP concentrations in platelets. Prostacyclin I$_2$ (PGI$_2$; 10 µM) was used as positive control. As expected, PGI$_2$-treatment of PRP from all 6 healthy individuals led to a significant increase in intracellular cAMP, when compared to untreated samples. This was also observed when platelets were stimulated with ADP/PGI$_2$ (Table 1). Addition of NOD to unstimulated or ADP stimulated PRP samples did not alter intracellular cAMP-concentrations, suggesting that the inhibitory effect of NOD was not mediated via cAMP (Table 1).

Figure 2: Effect of DA and NOD on whole blood platelet aggregation. Whole blood platelet aggregation was measured in six healthy individuals.

A: 100 µM DA and 100 µM NOD caused no significant platelet aggregation when compared to untreated whole blood samples. Co-stimulation of whole blood with 0.75 µM U46619 and 100 µM DA had no significant effect compared to U46619 alone. In the presence of 100 µM NOD, U46619-induced platelet aggregation was significantly inhibited.

B: The inhibitory effect of NOD on whole platelet aggregation was found at 100 µM concentration (*p=0.009), but not at lower concentrations (1 µM and 10 µM).
**Structural requirements for inhibition of platelet function by NOD**

To further investigate the molecular entities of NOD that convey the inhibitory effect on platelet function, we tested structurally different NOD-like compounds (Figure 3A). While DA and NOD strongly differ in the hydrophobicity, their redox activity is largely similar. In contrast, NOT strongly differs in redox activity with that of DA or NOD but its hydrophobicity is similar to NOD [20]. To assess if redox activity was required intra- or extracellular, the hydroxy moieties of NOD were acetylated (A-NOD) to prevent oxidation. Deacetylation of A-NOD can occur intracellular by virtue of esterase activity. Similar as shown in figure 1B, DA co-activates U46619-induced platelet degranulation whereas NOD caused significant inhibition of CD62P expression on platelets. The inhibitory effect on CD62P expression was completely lost when equimolar concentrations of NOT were used, suggesting that the redox moieties within NOD might be instrumental for inhibition of platelet function. Although inhibition of CD62P was slightly reduced for A-NOD this still reached statistical significance indicating that redox activity was likely to be required intracellular for mediating inhibition of CD62P expression (Figure 3B).

<table>
<thead>
<tr>
<th>cAMP level&lt;sup&gt;a&lt;/sup&gt;</th>
<th>medium</th>
<th>PGI&lt;sub&gt;2&lt;/sub&gt;</th>
<th>NOD</th>
<th>ADP</th>
<th>ADP/PGI&lt;sub&gt;2&lt;/sub&gt;</th>
<th>ADP/NOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>2.54</td>
<td>30.4</td>
<td>5.27</td>
<td>2.24</td>
<td>21.81</td>
<td>1.95</td>
</tr>
<tr>
<td>± SD</td>
<td>± 1.07</td>
<td>± 6.51</td>
<td>± 3.21</td>
<td>± 0.52</td>
<td>± 6.56</td>
<td>± 0.83</td>
</tr>
<tr>
<td>p-value&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
<td>n.s.</td>
<td>n.s.</td>
<td>0.007</td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: The effect of NOD on cAMP production.
<sup>a</sup>pmol cAMP/10<sup>6</sup> platelets. <sup>b</sup>t-test: condition vs. untreated (medium); n.s., not significant.
N-octanoyl-dopamine is an inhibitor of platelet function

Figure 3: Effect of different DA derivates on platelet degranulation.
The effect of different DA derivatives was measured by flow cytometry in PRP of six healthy individuals. A: Molecular structure of the DA, NOD, NOT and A-NOD is shown. Compared to DA, NOD possesses a long hydrophobic tail. According to less hydroxy groups, NOT is conducting less redox potential compared to NOD and DA, while having the same hydrophobicity as NOD. A-NOD also possesses the hydrophobic tail, but for redox activity it needs the presence of intracellular esterases. B: Compared to U46619-stimulation the addition of 100 µM DA led to a significant increase of CD62P expression, whereas 100 µM NOD and 100 µM A-NOD caused significant inhibition. The addition of NOT had no effect on U46619-induced CD62P expression.
Discussion

In the present study we performed \textit{in vitro} analyses on blood samples of six healthy individuals in order to prove hypotheses regarding the effect of structural modifications of DA. The main findings are the following: Firstly, in contrast to DA, NOD caused significant inhibition of platelet function. Secondly, redox activity seemed to be required for the inhibitory effect of NOD since NOT did not affect platelet function. Thirdly, A-NOD also inhibited platelet activation, presumably, through deacetylation by intracellular esterases indicating that redox activity is required intracellular. Based on our results and previous observations that DA is a co-activator of platelet function [17] we confirmed that DA increases platelet function through a receptor-mediated mechanism, while the hydrophobic tail of NOD enables it to pass the plasma membrane more easily and to elicit its inhibitory effects most likely via intracellular redox active processes.

Cyclic nucleotide signalling is the main inhibitory pathway in platelets causing activation of cyclic nucleotide-dependent protein kinases and in turn phosphorylation of major components of the platelet activation pathways [21]. Both $\alpha_{2A}$-adrenergic and D2-like receptors act through an inhibitory $G_i$-protein coupled receptor signaling cascade, causing inhibition of cAMP synthesis. Despite the fact that NOD does not increase blood pressure \textit{in vivo} [20], there is no formal proof that NOD does not bind to adrenergic or dopaminergic receptors which might increase cAMP concentrations [22,23]. In fact, unpublished data on neonatal cardiomyocytes suggest that NOD is a partial $\beta$-adrenergic receptor agonist that slightly increases cAMP in these cells. Involvement of cAMP as underlying mechanism for the inhibitory action of NOD on platelet activation could however be excluded in the present study as it did not change intracellular cAMP-concentrations.

Reactive oxygen species (ROS) exert critical regulatory functions within the vascular wall and are considered as relevant mediators for platelet activation and thrombus formation [24-26]. ROS can modulate platelet function by decreasing NO bioavailability [27]; a direct role of ROS in the control of platelet functions has also been suggested [28,29]. Therefore, our findings that the inhibitory effects of NOD on platelet function are most likely conferred by its redox activity were not surprising as other studies using antioxidants also have shown inhibitory effects on platelet aggregation [30]. Nonetheless, Begonja and colleagues have reported that inhibition of agonist-induced ROS production mainly affects GPIIb/IIIa activation and has no effect on $\alpha$- and dense granule secretion [28]. These data
N-octanoyl-dopamine is an inhibitor of platelet function

clearly differ from our present findings in that the redox active compound NOD significantly inhibited GPIIb/IIIa activation, granule release and aggregation. Most if not all studies involving the antiplatelet properties of redox active compounds only investigated platelet aggregation and GPIIb/IIIa activation, but not CD63 or CD62P surface expression.

In line with previously published data on the antiplatelet properties of flavonoids [31], it seems that the catechol structure within NOD is of instrumental importance for its inhibitory effect on platelet function. The catechol structure can be masked by acetylation of the hydroxy moieties, which, by virtue of intracellular esterase activity, are converted to the catechol structure. Since A-NOD inhibited agonist-induced CD62P expression our data indicate that NOD inhibits platelet function via impairing intracellular redox dependent processes. The exact mechanism needs further investigation and we can not exclude that conversion of A-NOD to NOD also occurs extracellular as a consequence of released esterase activity.

It is noteworthy to mention that the redox activity of DA does not differ considerably from that of NOD. Yet both agents have opposing effects on platelet function. We assume that the redox activity of DA becomes overruled by specific receptor binding and subsequent outside-in signaling via the G[i]/cAMP pathway. Therefore, the co-agonistic effect of DA is receptor-mediated, whereas the inhibitory NOD effect is mediated by an increased intracellular redox activity.

There is a growing body of evidence indicating that donor dopamine has a salutary effect on transplantation outcome [5-8]. However, in brain dead donors catecholamine clearance is changed and hence low-dose dopamine-treatment could result in tachycardia and hypertension in approximately 15% of the brain dead donors [32]. In vitro studies already have shown that NOD is 40x more effective than DA in protecting human umbilical vein endothelial cells (HUVEC) against cold preservation injury [20]. In the present study we have demonstrated that NOD inhibits platelet function. This property might add to the overall postulated beneficial effect of NOD in settings of organ transplantation. Platelets are clearly part of the recipient response to transplanted organs [33] and may play an important role in the manifestation of ischemia/reperfusion (I/R)-injury [34]. The investigation of biopsies from donors during organ procurement documented that activated platelets are attached to vascular endothelial cells and leukocytes, which might add to acute antibody or cell-mediated rejection [35]. While specific inhibition of platelet effects is of general interest, there is a need for therapeutic concepts, established for clinical use in transplantation. Since NOD is devoid of
hemodynamic properties, and in keeping with the cytoprotective properties of NOD, this makes NOD an attractive candidate compound for donor- or organ conditioning prior to organ transplantation. Further clinical exploration of its use is therefore warranted.

Acknowledgements
We wish to thank Gabriele Rink for expert technical assistance.
References


Chapter 6

N-octanoyl-dopamine is an inhibitor of platelet function