The potential use of N-octanoyl-dopamine (NOD) in organ transplantation
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11.1 Summary
11.2 General discussion
11.3 Conclusions and future perspectives
11.1 Summary

As the mismatch between organ supply and demand of donor organs continues to grow [1,2], there is an unmet socio-economical and clinical demand for new strategies to increase the donor organ pool. When it comes to utilization of organs from extended criteria donors (ECD), the question to patients and transplant clinicians is whether this organ will function sufficiently and long enough to provide both a survival benefit and a quality of life advantage for the recipient. As compared to donor organs from optimal donors, those from ECD have in general a shorter overall long-term graft survival [3]. Moreover ECD organs may already have accumulated damage due to aging or disease history, making these organs more prone to loss of function as a consequence of subsequent damage occurring during the transplant process. Thus, with the increasing acceptance of organs from ECD, there is an increasing need for methods to maintain or even improve organ quality in these donors. In particular brain death, ischemia, organ preservation and reperfusion are considered as important factors for functional deterioration of donor organs and therefore represent potential therapeutic targets to improve organ quality [4,5]. The work described in this thesis includes in vivo and in vitro studies to assess the potential benefit of N-octanoyl-dopamine (NOD) in protecting allografts. The results obtained should provide pre-clinical evidence for the efficacy of NOD, either as donor or recipient treatment modality, in transplantation medicine.

Chapter 2 provides an overview of N-acyl-dopamine derivatives (NADD) and related compounds, and their potential use as therapeutics in transplantation medicine. We summarize the available data on biological activities of NADD in transplantation relevant entities, e.g. cold inflicted injury, I/R-injury, immune-modulation and inflammation.

In Chapter 3 we used an in vivo model for I/R-injury to compare if dopamine (DA)- or NOD-treatment results in amelioration of tissue damage and/or improvement of organ function. Moreover the potential involvement of TRPV1 in the renoprotective effect was tested. The main findings of this study are the following. Firstly, in vitro studies revealed that NOD, but not DA, dose-dependently activates TRPV1 receptors. In renal tissue TRPV1 positive nerve fibers and fiber networks showed a peritubular and vascular localization. In addition, TPRV1 was expressed in distinct tubuli in rat renal tissue and on cultured human proximal tubular epithelial cells (PTEC). Secondly, in vitro NOD strongly impaired NF-κB activation of TNF-α stimulated PTEC resulting in a decreased expression of VCAM-1.
Yet, *in vivo* inhibition of NF-κB by NOD was modest and was not paralleled by a decrease in renal expression of NF-κB regulated genes in rats that were subjected to ischemia-induced acute kidney injury (AKI). Monocyte infiltration one day after the onset of AKI was reduced in NOD-treated rats as compared to DA-treated or untreated rats, but was not significantly different at day five anymore. No significant differences in the expression of adhesion molecules and cytokines were observed at any time-point of investigation. Thirdly, AKI was mitigated in NOD-, but not in DA-treated rats. This was reflected by a better tubular epithelial integrity and improved renal function.

In **Chapter 4** we further evaluated the anti-inflammatory effect of NOD on TNF-α stimulated endothelial cells and attempted to elucidate the underlying mechanism. We showed that 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. 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 serine 276. We also showed that *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 and still present. Finally, we provide evidence that redox activity and hydrophobicity are important molecular entities that are required for the anti-inflammatory properties of NOD.

Because NOD contains a polar head group, *i.e.* the redox active catechol moiety, and a highly hydrophobic fatty acid tail, we speculated that NOD might have access to different intracellular compartments where it may impair the redox milieu. Particularly in the ER the change of an oxidative to a reductive environment has major consequences for oxidative protein folding. Impairment of the latter will induce an adaptive response in cells also known as the unfolded protein response (UPR). Indeed, we found in **Chapter 5** that NOD and other redox active NADD transiently activate the UPR. This property seems to be dependent on the redox activity of these compounds. NOD did not affect cell viability, but strongly impaired cell proliferation of HUVEC, most likely by attenuation of cells in the S-G2/M-phase. In concordance to this,
mRNA expression for a number of genes involved in S-G2/M-progression was significantly down-regulated by NOD. Interestingly, long-term NOD-treatment resulted in hypometabolism and thermotolerance, as suggested by a decreased intracellular ATP-concentration, activation of AMPK and increased resistance to cold-inflicted cell injury.

Platelet activation and adherence to the endothelium are prominent features of the inflammatory cascade and may occur in donors with severe traumatic brain injury [6,7]. In addition, platelet activation may occur in recipients as a consequence of cold ischemia/reperfusion-injury [8]. Because platelets express different α- and β-adrenergic and dopaminergic receptors [9,10], 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 it has been shown that dopamine is an ADP-dependent platelet agonist [11,12] acting via D2-like, but not D1-like, receptors [13]. In keeping with the redox active catechol structures of NOD and DA we compared in Chapter 6 to what extent NOD differs from DA in terms of platelet activation. It was found that, in contrast to DA, NOD causes inhibition of platelet function. Redox activity seemed to be required for the inhibitory effect of NOD, since N-octanoyl-tyramine (NOT) did not affect platelet function. Redox activity was likely required intracellularly since acetylation of the redox active moieties within NOD (A-NOD) did not abrogate inhibition of platelet function. Based on our results and previous observations that DA is a co-activator of platelet function, we hypothesize that DA, through a receptor-mediated mechanism, increases platelet function in a co-agonistic fashion [13], while the hydrophobic tail of NOD enables it to pass the plasma membrane more easily to elicit its inhibitory effects most likely via intracellular redox active processes.

As shown in the Chapters 3 and 4 NOD inhibits NF-κB activation. Because the pro-inflammatory transcription factor NF-κB is also essential for T cell activation in Chapter 7 we tested the hypothesis that NOD might affect T cell activation. Indeed, we found that NOD transiently suppressed T cell proliferation and activation, albeit that early T cell activation events, e.g. CD3 capping or initial cytokine production, were not affected. Importantly, NOD showed a strong synergy with CNI to inhibit T cell activation, suggesting that lower CNI dosages might be required for effective immunosuppression when used in combination with NOD and thereby lowering CNI related side effects.
For implementation of NOD in relevant transplantation models, as described in Chapter 8, we first established a rat orthotopic aortic transplantation model (as a model for chronic rejection) combined with simultaneous implantation of osmotic single- or double-minipumps. Double-pump implanted rats displayed similar post-operative weight gain and physical activity indicating similar levels of discomfort compared to single-pump implanted rats. However double-pump implanted rats had an increased risk for pump-related complications, e.g. pump disconnection and leaky catheters.

In Chapter 9 we applied the double-pump implantation model described above to test if long-term (4 weeks) NOD-treatment affects transplant vasculopathy (TV) in this aorta transplant model. We observed that NOD attenuates the development of TV as assessed by reduced neointima (NI) formation and a lower degree of αSMA positive neointimal cells 4 weeks after transplantation. Although there was also a trend towards a lower degree of graft infiltrating T cells after 2 weeks, this did not reach statistical significance. In vitro, NOD inhibited smooth muscle cell (SMC) proliferation by a G1-cell cycle arrest and prevented TNF-α-induced SMC apoptosis. Yet in vivo, the proliferation rate of neointimal SMC (as quantified by Ki67 staining) was not significantly influenced by NOD.

Using the same in vivo model, in Chapter 10 we tested the effect of vanin inhibition on TV development. The vanin inhibitor RR6 was administered orally and this treatment significantly inhibited serum vanin activity as only a rest pantetheinase activity of 5% was detected in RR6-treated rats. Similar to NOD, RR6-treatment also resulted in less NI formation 4 weeks after aortic transplantation. This reduction in NI information was not accompanied by a reduction in the degree of graft infiltrating T cells or macrophages 2 weeks post-transplantation. Unlike NOD, RR6 could only inhibit PDGF-induced SMC proliferation but was not effective when SMC proliferation was triggered by a cocktail of insulin, apo-transferin, hydrocortisone, insulin-like growth factor 1 (IGF-1) and fibroblast growth factor (FGF). In vivo, neither the relative contribution of neointimal αSMA+ cells nor their proliferation rate was influenced by RR6.
11.2 General discussion

The studies presented in this thesis suggest that the clinical application of NOD might have multiple beneficial effects on damaging events which either occur in the donor during organ preservation or in the recipient after organ transplantation. Clinically, the beneficial effect of donor dopamine-treatment has already been demonstrated in renal and heart allograft recipients [14,15]. The observation that the salutary effect of donor dopamine-treatment in renal allograft recipients is more pronounced when cold ischemia time is long [15], and the observation that dopamine is able to protect a wide variety of cells against cold inflicted injury [16,17], have nourished the assumption that donor dopamine-treatment predominantly affords protection to renal allografts by preventing tissue damage that occurs during organ preservation. It should however be emphasized that donor dopamine-treatment also improves transplantation outcome of heart allograft recipients [14], even though cold ischemia time of heart allografts is short and mostly in the range of 4 to 6 hours. This therefore raises the question as to whether other protective mechanisms might also be involved in the overall protective properties of dopamine.

The use of dopamine in donor management has raised some concerns amongst clinicians as even in low-dose dopamine-treatment of brain dead donors may cause hypertension or tachycardia in the minority of these donors. Moreover, it has been suggested that the use of inotropes may cause necrosis of cardiomyocytes as a consequence of increased intracellular calcium and opening of the mitochondrial permeability transition pore [18,19]. Because the salutary effect of donor dopamine-treatment on transplant outcome is independent of its hemodynamic properties, this underscores the need for new compounds with improved protective properties that are devoid of hemodynamic activity. Although only tested in pre-clinical studies thus far, NOD seems to fulfill these requirements.

Since the amine side chain of catecholamines is at large responsible for receptor binding and specificity, it is likely that conjugation of a large fatty acid, e.g. octanoic acid, to the amine side chain will impair receptor engagement. Thus far receptor binding studies for NOD have not been performed and no formal proof exists that NOD cannot bind to catecholaminergic receptors. However, experimental evidence indicates that in vivo NOD neither influence blood pressure [20] nor heart rate [unpublished data], and in vitro does not increase cAMP-concentrations in cardiomyocytes [16]. As yet, there is also
no data available on how NOD is taken-up by the cells i.e. this can occur via dopamine transporters, other transporters or via interaction of the hydrophobic tail with the cell membrane. Irrespective of this, experimental studies revealed that intracellular uptake and organel distribution of NOD is better as compared to dopamine, although this has only been studied in endothelial cells [20].

The ortho dihydroxy benzene (catechol) moiety is widely distributed in nature and contains two important chemical entities. Its ability to act as a reductant, which for example contributes to the antioxidative properties of certain bioflavonoids [21] and its ability to chelate iron, which can provide iron chelating properties to certain bacterial siderophores [22], may explain its widely endogeneous usage in nature.

Homeostasis of the redox milieu and availability of iron are important factors that determine the outcome of biological processes. While iron is mainly used as a co-factor in many physiological systems, e.g. heme containing enzymes, redox reactions are an integral part of cell physiology in terms of detoxification of radicals, activation of enzymes and chromatin remodeling. Most of the effects which have been described for NOD show a strict dependency for a redox active (ortho- or para-positioned dihydroxy) aromatic structure, indicating that perhaps impairment of the redox milieu or the availability of iron could be in part responsible for the observed biological activity of NOD. In particular its strong redox activity can explain the inhibitory effect on the activation of redox-dependent transcription factors, e.g. NF-κB and AP-1.

The endoplasmic reticulum (ER) has a highly oxidative milieu that supports appropriate protein folding. By increasing reduction equivalents in the ER, this will have strong repercussions for oxidative protein folding and will lead to an increase of inappropriately folded proteins. Inasmuch it is of pivotal importance for cell viability to restore homeostasis in the ER, as the amount of unfolded proteins start to increase, a set of reactions are initiated, also referred to as unfolded protein response (UPR). When it comes to induction of the UPR, cell adaptation and apoptosis are two sites of the same coin. Whenever the cell is beyond repair it goes into apoptosis as this may have certain advantages above keeping damaged cells within tissues. Adaptation, on the other hand, increases the amount of chaperone proteins to assist in protein folding, increases the ER-associated degradation system to get rid of inappropriately folded proteins and decreases de novo protein synthesis to minimize protein load in the ER. Interestingly, the adaptive arm of the UPR shows a substantial degree of crosstalk with different signaling pathways. While the early phase of the UPR may
trigger the NF-κB pathway, there is growing evidence that the UPR also has the potential to inhibit NF-κB activation [23]. CCAT/enhancer binding protein (C/EBP) is induced by ER stress in several cell types [24-26]. Its overexpression in monocytes impairs p65 phosphorylation and thereby NF-κB transactivation [27]. Under normal conditions TNF-receptor associated factor type 1 (TNFR1), TNFR1-associated death domain (TRADD), TRAF2, receptor interacting protein (RIP) and IKK are essential to promote TNF-α-induced NF-κB activation [28]. Upon activation of the UPR sensor IRE1α, TRAF2 is actively degraded resulting in impaired IKK activation. Down-regulation of TRAF2 by ER stress has also been reported for other cell types [25,29]. In keeping with the observation that NOD inhibits the expression of a number of NF-κB regulated genes and transiently activates the UPR, it remains to be assessed to what extent induction of the UPR contributes to the anti-inflammatory properties of NOD. Also the cytoprotective properties of NOD with respect to TNF-α-induced apoptosis in SMC, as shown in Chapter 9, could be linked to UPR activation causing impaired TNF-α signaling.

NOD and dopamine share a number of biological activities, albeit that for dopamine substantial higher concentrations are required. NOD differs however from dopamine in its ability to activate TRPV1. In analogy to other TRPV1 agonists NOD is able to improve renal function after ischemia-induced AKI. Although we did not provide evidence for a causal relation between TRPV1 activation and improvement of renal function in the setting of AKI, ongoing studies have already shown that NOD is not able to improve renal function in rats that lack functional TRPV1 receptors [Pallavi et al., manuscript in preparation]. The structural similarities between NOD and capsaicin, the classical TRPV1 agonist, may explain why NOD, but not DA, is able to activate TRPV1. In the model of how capsaicin interacts with TRPV1 it is postulated that the aromatic ring in capsaicin aligns with the aromatic ring of tyrosine 511 (Tyr511) in TRPV1, an interaction also known as π-stacking. Because this interaction has low affinity, further stabilization is required for appropriate TRPV1 activation. This occurs via hydrogen bridging and via hydrophobic interactions of the fatty acid of capsaicin that protrudes in the hydrophobic core of TRPV1 (Figure 1) [30]. For NOD a similar scenario can be visualized, yet because dopamine lacks a fatty acid tail it cannot interact with the hydrophobic core of TRPV1.

Even though the use of NOD was primarily envisaged as a donor preconditioning modality, this thesis also demonstrates its potential use in allograft recipients. Acute vascular rejection and chronic rejection, i.e. interstitial fibrosis and tubular
atrophy (IFTA), are the main causes for renal graft failure and loss [32,33]. IFTA is a clinicopathological entity characterized by fibrosclerosis of the different renal structures leading to progressive decline of renal function after kidney transplantation [34]. Although the molecular mechanisms that underlie the pathophysiology of IFTA remain far from clear, it seems to result from the concerted action of immunological and non-immunological factors [35]. The use of calcineurin inhibitors (CNI) are amongst others identified as a non-immunologic risk factor, associated with nephrotoxic [36] and neurotoxic [37] side effects. Attempts to minimize CNI usage or wean patients off CNI have shown that improvement in renal function is often obtainable but only at the expense of increased alloimmune reactions [38]. Developing a calcineurin inhibitor-based long-term maintenance immunosuppressive drug regimen with improved long-term tolerability is therefore a highly desirable endeavor. NOD shows two important properties in this context. Firstly, *in vitro* NOD transiently inhibits T cell activation and acts synergistic to CNI to suppress T cell proliferation (*Chapter 7*). Hence, lower CNI-concentrations might be used and thus reducing adverse effects of CNI. Secondly, NOD inhibits neointima formation in a model of aorta transplantation (*Chapter 9*). The mechanism hereof is however not completely delineated but might involve inhibition of SMC proliferation.

**Figure 1: Structure of capsaicin binding at the TRPV1-receptor.** Interaction of capsaicin (ball and stick model) with TM regions of the TRPV1 monomer. Note the hydrogen bond of capsaicin with serine 512 (Ser512), the π-stacking with tyrosine 511 (Tyr511) and the hydrogen bonds with Tyr511 and lysine 571 (Lys571) of the TRPV1 receptor. Hydrophobic tail of capsaicin follows TM4 into the core of TRPV1. *Graph adapted from [31].*
It is worthwhile to mention that NOD induces vanin (\textit{Vnn1}) expression in endothelial cells as demonstrated in gene array data (~two-fold induction [unpublished data]) and yet inhibition of vanin by RR6 also attenuates TV development (\textit{Chapter 10}). Although the gene array data need to be confirmed in independent experiments, it seems that NOD and RR6 do not prevent neointima formation via the same mechanism of action and therefore could be used as co-treatment for reasons of potential synergy.
11.3 Conclusions and future perspectives

Although the data presented in this thesis are promising and show therapeutic efficacy in transplantation relevant models, there are a number of issues that need further attention before implementation of NOD in human transplantation medicine can be considered.

Firstly, no pharmacokinetic studies have been conducted and therefore it is not clear how much NOD is present in the circulation or within tissues. This is important to know since high concentrations of NOD might also be harmful to the tissue. In collaboration with the Department of Radio-Chemistry (Medical Faculty Mannheim, Heidelberg University, Germany) we have recently synthesized radio fluorinated NOD (\(^{18}\text{F}\)-NOD) in which the \(^{18}\text{F}\) is directly conjugated to the aromatic ring of NOD. Data from in vitro studies revealed that NOD and \(^{18}\text{F}\)-NOD behave similarly in all tested assays. Hence the use of \(^{18}\text{F}\)-NOD may give further information on the pharmacokinetic behavior of NOD.

Secondly, it should also be mentioned that hydrophobicity of NOD makes intravenous application more complicated. In the experiments described in this thesis we made use of NOD emulsions with Tween80 as detergent. Hydrophobic drugs can also be applied intravenously as cyclodextrin formulation. In aqueous medium, the exterior surface of cyclodextrins is hydrophilic while the interior core is hydrophobic. This allows for the formation of guest-host relationships with small hydrophobic molecules. Alternatively, an extensive structure activity study may further help understanding how structural alterations of NOD affect efficacy of protection and may provide new protective compounds with lower hydrophobicity.

Thirdly, this thesis provides a number of putative mechanisms by which NOD might convey its protective properties. Yet, it is not clear which of these mechanisms are predominant in vivo and thus have most therapeutic relevance. Clearly further experiments are warranted to address these issues.
References


