In vitro studies on the cytoprotective properties of Carbon monoxide releasing molecules and N-acyl dopamine derivatives

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Chapter 10

Summary, general discussion and future perspectives

Summary

The introduction of new powerful immunosuppressive agent in clinical practice did parallel a significant reduction of acute rejection episodes and improved short-term graft survival. Yet, late graft loss did not change equally. Chronic allograft nephropathy, interstitial fibrosis and tubular atrophy (IF/TA) continues to be a major cause for late graft loss and readmission onto the waiting lists. Now that important risk factors for chronic allograft nephropathy, IF/TA have been identified, i.e. brain death, ischemia-reperfusion injury, cold static organ preservation and calcineurin inhibitors, new therapeutic modalities are warranted to counteract the gradual decline in long term graft survival. The studies performed in the first part of this thesis describe a new group of compounds able to release CO through an enzyme-triggered mechanism, while in the second part N-acyl dopamines, in particular N-octanoyl-dopamine, have been tested for their cytoprotective properties and the mechanisms by which they convey protection. As elaborated in **chapter 2** and **chapter 3** CO releasing molecules (CORMs) and N-acyl dopamines are promising compounds for use in transplantation medicine as they have the ability to protect cells against the deleterious event of static cold preservation and are able to inhibit tissue inflammation.

The introduction of CO-releasing molecules (CORMs) by Motterlini significantly paved the way in the field of CO research and contributed to a large understanding of how CO may exert its salutary effects in a number of pathological conditions [103, 105, 106]. While initially a variety of organic compounds were explored for their potential use as CORMs, e.g. haloalkanes, aldehydes, oxalates and silacarboxylic acids, it turned out that their CO release rate and toxicity profile did not justify further development [131]. Due to the strong coordinating properties of transition metals with CO attention subsequently shifted to the use of metal carbonyl complexes as potential CORMs. The first transition metal carbonyls which were explored as CORMs were simple transition metal carbonyls that were soluble only in organic solvents [105]. Subsequently, novel compounds were developed, which displayed different rates of CO release and were more compatible with biological systems [125]. Most of the published CORMs used in biological studies either spontaneously release CO when dissolved in aqueous solutions, e.g. CORM-3 [125], or require special physical or chemical stimuli to favour CO dissociation from these complexes [205-209]. It must be emphasized however, that also the use of CORMs does not preclude CO delivery to other tissues than the target tissue as it freely diffuses in the body once released. Yet, tissue specific delivery of CO might be more desirable to avoid or limit potential adverse effects. One possibility to overcome this hurdle is allowing CORMs to release CO

only intracellularly, which ideally should be triggered by cell-specific enzymes [210]. In **Chapter 4** we introduce so called enzyme-triggered carbon monoxide releasing molecules (ET-CORM) as bioactive compounds able to release CO in biological systems. These molecules are hydrolyzed by tissue esterases allowing delivering CO only intracellular. We have demonstrated that structural alterations of ET-CORMs significantly affect their biological activity. Our data also indicate that different ET-CORMs behave differently in various cell types (epithelial vs. endothelial). In general cytotoxicity of 2-cyclohexenone and 1,3-cyclohexanedione-derived ET-CORMs was more pronounced in HUVEC compared to PTEC and was dependent on the position and type of the ester (acyloxy) substituent(s) (acetate > pivalate > palmitate). Protection against hypothermic preservation injury was only observed for 2-cyclohexenone derived ET-CORMs and was not mediated by the ET-CORM decomposition product 2-cyclohexenone itself. Structural requirements for protection by these ET-CORMs were different for HUVEC and PTEC. For the former ET-CORM the decomposition product 2-cyclohexenone also inhibited VCAM-1 expression.

Further in vitro studies presented in **Chapter 5** confirm that the type and the position of the ester determine CO release and thus the biological activity. In particular, esterase-triggered CO release from the pivalates (R = *t*-Bu) and palmitates (R = $C_{15}H_{31}$) was slower than from the acetates (R = Me) and this was in accordance with the observed biological effect. In addition ET-CORMs with the ester in the outer position were more active than the same with the ester in the inner position of the 2-cyclohexenone moiety. Although both studies indicate a clear structure-activity relation of ET-CORMs, our data do not unambiguously demonstrate that their biological effect directly relates to their CO releasing properties. It might be that differences in cellular uptake also may explain the differences in biological effect amongst the ET-CORMs. Yet, it would be expected that the more lipophilic compounds would have a better cellular uptake and thus a better hydrolysis and a biological effect. Against this assumption are the experiments presented in **Chapter 5** where we showed that the more lipophilic compounds barely release CO and do not exert a significant biological effect. It thus seems that the efficiency of ET-CORM hydrolysis is the main determinant for their biological effect. Based on the geometry of the chemical structure of pivalate- and acetate- containing ET-CORMs it is conceivable that the former might be less accessible for hydrolysis by esterase. This is in line with previous publications [213, 214] that have demonstrated differences in hydrolysis efficiency of acetate and pivalate containing pro-drugs and our own data with respect to CO release as measured by Mb assay or headspace gas chromatography [137, 237, 238, 365]. It should be emphasized that CO release from ET-CORMs is a two-step process in which first the ester functional group is hydrolyzed followed by oxidation of the resulting dienol-$Fe(CO)_3$ moiety. Hence the ease by which the dienol-$Fe(CO)_3$

moiety becomes oxidized will influence the rate of CO release from ET-CORMs. Since the ease of oxidation seems to depend on the position of the ester functional group, this may underlie the differences in both CO release and biological effect of the 2-cyclohexenone-derived ET-CORMs *rac*-**1** and *rac*-**4**. Indeed we could demonstrate in **Chapter 5** that CO release from *rac*-**4** is significantly higher as compared to *rac*-**1**. In **Chapter 6** we further demonstrated that the differences in biological activity between *rac*-**1** and *rac*-**4** remained even when both compounds were made as cyclodextrin formulation. This also strongly supports the notion that differences in cellular uptake between *rac*-**1** and *rac*-**4** unlikely were responsible for the differences in their biological effects. Importantly, we demonstrate that ET-CORMs have potent anti-inflammatory actions and are able to inhibit TNF-induced VCAM-1 expression. Inhibition of VCAM-1 expression is mostly accompanied by the induction of HO-1, albeit that a causal relation between both phenomena has not been studied. Nonetheless, our data are compatible with inhibition of the NFκB and activation of Nrf2 pathways respectively to explain the influence of ET-CORMs on VCAM-1 and HO-1 expression. In addition, their ability to release CO intracellularly makes them more potent compounds, as they exert their biological effect at a low µM concentration range in comparison with other CORMs. In particular, our group has shown that CORM-3 is able to inhibit TNF-a mediated VAM-1 induction first at a 0,5mM [221] concentration. In the studies presented in this thesis we demonstrated that ET-CORMs are able to inhibit VCAM-1 at a µM range concentration [238, 365]. Interestingly, rac-4 shown to be active at a 1µM.

A consequence of static cold preservation is the release of heme from heme containing proteins. This causes an increase in oxidative stress and consequently damage to the cells. As proposed by Nakao et al [39, 199], CO confers its protective effect against hypothermia mediated cell damage by binding to cytochrome P450 heme proteins thereby stabilizing the heme interaction. It was therefore unexpected that ET-CORMs are able to protect against cold-inflicted injury as they concomitantly release iron, which plays a central role in the pathophysiology of cold preservation injury [38, 366]. However upon hydrolysis of ET-CORMs, iron and CO are released in a molar ratio of 1 to 3 in favor of CO. Moreover, based on enzyme thermodynamics it can be argued that only a small proportion of ET-CORMs will be hydrolysed at low temperatures. Because already small quantities of CO are able to protect cells against cold inflicted injury, under low temperatures hydrolysis of a small amount of ET-CORMs might generate sufficient amounts of CO to stabilize cytochrome P450 heme proteins, without causing too much harm to cells through iron release.

Although ET-CORMs compromise a promising new group of CO releasing molecules able to release CO intracellular through a more controlled mechanism, the clinical applicability still requires more pre-clinical testing particularly in *in vivo* models.

In the second part of this thesis we focused on N-acyl dopamine derivatives, and particular on N-octanoyl dopamine (NOD). Since the initial study published in 2009 by our group on the salutary effect of donor dopamine treatment on transplantation outcome of renal allograft recipients [45], a series of further studies confirmed the central role of dopamine in transplantation medicine and proposed possible mechanisms to explain these effects [151, 367-369]. Based on these findings we developed a non-hemodynamic dopamine derivative, i.e. N-octanoyl dopamine (NOD), to avoid adverse blood pressure and cardiac effects when applied to brain dead donors. Due to its increased hydrophobicity NOD is taken up by cells much better as compared to dopamine and displays superior protective effects in terms of prolonged hypothermic preservation [162], inhibition of platelet activation [309] inhibition of NF-κB [194] and acute kidney injury [178]. In **Chapter 7** we tested if NOD is able to modulate cellular immunity for potential use as a T cell suppressive agent. Indeed we found that NOD transiently suppressed T cell proliferation and activation. Notably NOD showed a strong synergy with CNI to inhibit T cell activation, providing a possible rational for using combinations of CNI with NOD to lower the nephrotoxic effect of CNI without impairing effective immunosuppression. Most of the protective effects that have been described for NOD are mediated by the aromatic structure, and thus, to its redox reactivity [162, 178, 194, 309, 370]. The catechol structure is widely distributed in nature and contains two important chemical entities; its ability to act as reductant and to chelate iron [310, 371]. Because of its increased hydrophobicity we speculated that NOD would be able to cross intracellular membranes and severely change the redox milieu within subcellular compartments. Indeed, as presented in **Chapter 8** we found that NOD and all redox active tested N-acyl dopamine derivatives (NADD) induce the unfolded protein response possibly through oxidation of the catechol structure and thus in turn donation of reduction equivalents. NOD did not affect cell viability but strongly impaired cell proliferation, most likely by attenuation of cells in the S-G2/M-phase. 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. Activation of the UPR via NOD compromises a rather intriguing finding as UPR supposed to activate apoptosis under ER stress conditions. It should be emphasized however that the UPR first induces a series of adaptive events, e.g. increasing the ER-associated degradation system to get rid of inappropriately folded proteins and decreasing de novo protein synthesis to minimize protein load in the ER. Apoptosis mostly occurs if all these protective measures

fail and the cell is beyond repair. Based on the assumption that UPR activation might be integral to long-term survival in the state of cold torpor in hibernating animals [303, 372], apart from its direct chemical properties NOD may convey protection against cold inflicted cell injury by UPR activation. Also its anti-inflammatory effect may, at least partly underlie UPR activation. A recent review nicely highlighted that even though ER stress triggers activation of NF-κB in the early phase, UPR activation has the potential to inhibit NF-κB activation in the later phase [373, 374]. C/EBP is a family of transcription factors required for development and responses to injury in various tissues and organs. Interestingly, C/EBPβ is also induced by ER stress and is able to inhibit NFκB activation through a direct p65 subunit interaction [375-377]. Unpublished data from our group support that NOD treatment is associated with an upregulation of C/EBPβ at the mRNA level and a concomitant decrease in the cytoplasmic fraction of CEBP/β, suggesting sequestration to the nucleus. The latter however has not unequivocally been demonstrated.

In **Chapter 9** we finally studied the molecular entities within N-acyl dopamines that are responsible for their TRPV1 activating and anti-inflammatory properties. The main findings of this study are the following. Firstly, the propensity of NADD to activate TRPV1 most likely relies on their ability to interact with the anchor residues Y511 and S512 on the TRPV1 protein. This might be accomplished by pi-stacking of the aromatic rings of Y511 and NADD and further stabilization via hydrogen bridges. Secondly, adequate projection of the hydorphobic tail of NADD seems to be required for effective TRPV1 activation. Thirdly, the anti-inflammatory effects of NADDs dependent on the ability of these compounds to donate reduction equivalence in different cellular compartments. Therefore, a balance is needed between cellular uptake and redox activity of the compounds.

Conclusion and future perspectives

Although ET-CORMs are emerging as an attractive system to controlled, enzyme-triggered CO release, there are some issues that have to be studied in more detailed in the future. Firstly, thus far ET-CORMs have been tested *in vitro*. Depending on the mode of application they may strongly differ in toxicity and their anti-inflammatory properties as compared to the *in vitro* results. In addition, the fate of released iron and α,β-unsaturated ketones needs to be addressed to exclude accumulation of these by-products in critical organs such as liver. Hence pharmacokinetic studies are needed to assess and improve their biocompatibility and pharmacokinetic profile.

Secondly, to avoid toxic adverse effects it would be preferable to obtain tissue specific CO-release. For ET-CORMs, selective CO release and thus tissue targeting would strongly depend on differences in tissue expression of esterases between different organs as well as between healthy and disease states. Even though our study indicates that ET-CORMs evoke different responses in endothelial and epithelial cells they are far from being tissue specific. Nonetheless the oxydiene-$Fe(CO)_3$ may still serve as CO delivery platform. When coupled to specific ligands that are selectively taken-up by cell via specific cellular transporters this platform could potentially lead to tissue specific CO delivery. Also the use of other enzymes with a more restricted expression pattern could be used for activation of ET-CORMs. Recently the viability of the latter approach was demonstrated through the synthesis of compounds consisting of an η^4 -oxydiene-$Fe(CO)_3$ moiety connected to a penicillin G amidase (PGA)-cleavable unit [378].

In this thesis ET-CORMs are presented as pharmaceutical candidates in the field of transplantation. As already mentioned pharmacological intervention can be done in the donor, during allograft preservation or in the recipient. We favor the application of these molecules during cold preservation to limit damage associated with prolonged preservation. Yet, based on the strong anti-inflammatory properties of ET-CORMs, and the strong inflammatory cascade that is initiated upon reperfusion, application of ET-CORMs to the recipient could also be envisaged. In contrast, ET-CORMs-application to the donor may raise some concerns. Even though we could consider this application as a precodioting strategy [372, 379] to increase HO-1 expression [238] and to induce the UPR [380], the strong vasodilatation caused by CO [81, 107] may worsen hemodynamic instability of brain dead donors.

N-acyl dopamine derivatives and especially NOD have emerged in the last years as promising compounds in the field of transplantation. However, even though the efficacy of NOD has already

been shown in *in vivo* models of heart transplantation [152] and acute kidney injury [178], its clinical application cannot be yet considered. First of all, pharmacokinetic and biocompatibility studies are still lacking. Thus data related to absorption, distribution and metabolism (ADME) are still missing. In addition, due to its hydrophobicity its LogP value is relative high making intravenous application problematic. This problem can be overcome by making NOD emulsion. These emulsions have been used in animal models and clearly showed the efficacy of NOD to improve AKI. It should be emphasized however that emulsions require tensides which may have adverse effects on their own. Finally, even though a series of potential targets and mechanism by which NOD confer its protective effects have been proposed and described in this thesis, it is not clear if *in vivo* the same mechanisms will be relevant. To this point, we unexpectedly found that in acute kidney injury the anti-inflammatory properties of NOD are modest in contrast to our *in vitro* data. NOD was able to mitigate acute kidney injury and improve renal function possibly due to TRPV1 activation. One explanation could be that the high *in vitro* intracellular concentrations cannot be achieved in vivo. These discrepancies between the *in vitro* and *in vivo* properties have to be studied and better elucidated in future.

The use of NOD was primarly envisioned as a donor preconditions modality based on the beneficial effects of dopamine-treated donors. NOD is endowed with properties that are not present in dopamine, such as its TRPV1 activating propensity. It remains to be assessed if NOD could act *in vivo* as a vasodilator of the intestinal vascular bed thereby improving mesenteric perfusion and thus reducing mesenteric ischemia. , possibly through TRPV1 acivation. Based on the notion that intestinal ischemia leads to loss of gut barrier function, which in turn may cause activation of the gut associated lymphoid tissue (GALT) [381-383], an intriguing question is as to whether improvement in mesenteric perfusion at the time of brain dead would mitigate immune activation in end-organs. Since the intestine is heavily innervated by TRPV1 sensory nerves and preliminary in vitro data have shown that NOD is able to induce vasodilation of mesenteric arteries, the beneficial effect of donor NOD treatment in experimental models might well be mediated via improvement of mesenteric perfusion. However, the role of mesenteric perfusion in brain death induced immune activation has not been thoroughly addressed and thus requires further detailed *in vivo* experiments.