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

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CHAPTER 1

General Introduction and aim of the Thesis

Introduction

The number of organs from extended criteria donors used for transplantation is increasing worldwide. Furthermore, the quality of allografts further deteriorates at different stages in the process of transplantation. Therefore, the need for cytoprotective compounds that have the propensity to mitigate organ allograft damage is increasing in parallel. Carbon monoxide and dopamine are two of the most promising cytoprotective molecules. Carbon monoxide (CO), a byproduct of heme degradation, is attracting growing attention from the scientific community as it plays a prominent role as second messenger, regulating a plethora of physiological processes. CO releasing molecules are under evaluation in preclinical models for the management of inflammation, sepsis, ischemia/reperfusion injury, and in particular organ transplantation. Not only CO but also dopamine and dopamine derivatives that are devoid of hemodynamic action are highly interested compounds for implementation in transplantation medicine as they harbor a number of protective properties. These properties seem to be independent of receptor engagement, but may be governed by the unique chemical characteristics of the catechol structure. In this thesis a new class of CO releasing molecules is described and, together with various hydrophobic dopamine derivatives, studied for their cytoprotective properties. The cytoprotective properties studied herein are mainly related to transplantation relevant processes, e.g. prevention of cold inflicted injury and inflammation. This introduction therefore will only describe the current problems in organ transplantation as framework for implementation of these compounds in transplantation medicine. Carbon monoxide and N-acyl dopamines will be introduced in chapter 2 and 3.

1.1 Kidney transplantation and organ quality

General aspects of the organ donor pool

Kidney transplantation is widely accepted as the treatment of choice for patients with end stage renal disease as it improves quality of life and significantly reduces mortality compared to patients remained on dialysis [1]. However, organ shortage is the major hurdle in contemporary transplantation medicine which impedes successful treatment of all patients with end-stage renal failure. In the last decade, median waiting times for organ transplantation have been doubled. As an example, the number of patients registered for kidney transplantation has increased by approximately 260% [2, 3]. Clearly, adequate strategies are warranted to put a halt to the growing imbalance between organ demand and supply. Over the last years a number of such strategies have been implemented to expand both the living and deceased donor pool. Paired kidney exchange programs for blood group incompatible living donor recipient combinations, the use of deceased extended criteria donors (ECD) and even the use of cardiac death donors (DCD) are only few examples of how the practice of organ donation has changed in time.

A significant number of patients in need for renal transplantation are highly sensitized. Because it is difficult to find a cross-match negative cadaveric donor for such patients, they have the least chance to obtain an allograft and consequently the time on the waiting list is high. In 2000 Cecka et al demonstrated that five years graft survival rates for patients receiving sibling, spouse, or living unrelated donor kidneys were well above those for cadaver donor types [4]. In a recent study of Montgomery et al it has been shown that live-donor transplantation after desensitization provides a significant patient survival benefit for patients with HLA sensitization, as compared to waiting for a compatible organ. By 8 years this survival advantage more than doubled [5]. Recent data also revealed that approximately 50% of potential living donors suitable for organ donation do not proceed because of the presence of circulating donor specific ABO-antibodies or human leucocyte antigen antibodies [6]. These studies clearly emphasize that efforts to overcome incompatibility barriers in live-donor renal transplantation have a large impact. Appropriate clinical measures and optimizing desensitization protocols made ABO-incompatible living donor kidney transplantation successful with patient and graft survival rates comparable to ABO-compatible kidney transplantation [7-9] [10]. These accomplishments in the field of living kidney donation go along with a significant increase in the number of assigned living donors [11, 12].

Grafts from deceased donors however, still remain the major part that constitutes the potential donor pool. In an attempt to counteract the increasing disparity between organ supply and demand, and to reduce excessive waiting times, selection criteria for organ donation have been extended. These extended donors, also referred as 'marginal' donors, are classified brain-dead donors with a relative risk (RR) for graft loss of >1.7 when compared with kidneys derived from standard criteria donors. Extended criteria donors (ECD) are defined as donors being either older than 60 years or older than 50 years with any two of the following criteria: 1. hypertension, 2. cerebrovascular cause of death, 3. pre-retrieval serum creatinine of >1.6mg/dl. ECD kidneys are associated with a higher rate of primary non-function, delayed graft function (DGF), rejection, and are more susceptible to organ preservation injury, drug toxicity, and complications associated with post-transplant hypertension [13]. Despite the increased relative risk for graft loss, recipients of renal allografts retrieved from ECD still benefit from transplantation in terms of survival and quality of life as compared to patients remaining on dialysis [14]. Likewise, donation after circulatory death, i.e. deceased cardiac death donors (DCD), has been endorsed by the World Health Organizations in 2011 and is now practiced in 10 of 27 European Union countries. DCD kidneys are comparable to kidneys from brain dead donors with respect to long-term graft survival, although the former display higher rates of primary graft non-function and delayed graft function [15, 16].

Risk factors for graft failure in kidney transplantation

In keeping with the current organ shortage, efforts to improve donor organ use should not only be made on organ supply, but also on improving long term graft survival to prevent re-entry of patients on the waiting list. Despite enormous improvements in one-year allograft survival [17], the rate of chronic graft loss has not improved in step. Many factors may contribute to chronic allograft dysfunction and eventually to graft loss [18, 19]. In general both alloantigen-dependent and independent factors account for chronic allograft loss. Indisputably, allografts with a history of acute rejection, either as result of HLA-mismatches or inadequate immunosuppression, are more prone to chronic rejection and allograft loss [20]. Yet allograft quality can also be compromised by a plethora of non-immunological factors such as inadequate renal mass, hypertension, calcineurin inhibitor nephrotoxicity, viral infections, recurrent or de novo glomerular disease and tissue injury as a consequence of organ procurement, preservation and transplantation [21-23].

Tissue injury is inevitable in the transplantation process and occurs at various stages [24]. Brain death [25, 26], cold ischemia time [27] and ischemia/reperfusion injury [28, 29] are considered to be the major determinants of pre-transplantation injury, undoubtedly impairing organ quality. Brain dead patients mostly suffer from neurogenic shock and hypotension, which in turn may result in organ hypoperfusion [30]. Based on numerous experimental studies, there is now also general consensus that brain death induces progressive immune activation, reflected by complement activation, up-regulation of cell adhesion molecules and an increased number of infiltrating leukocytes in end organs [26, 31]. Ischemia/reperfusion (I/R) injury will further damage the graft and may contribute to delayed graft function and chronic allograft dysfunction. I/R injury is more pronounced when cold ischemia time prolongs [29]. The U.S Renal System Registry data suggested that the risk for delayed graft function increases with 23% for every 6 hours of cold ischemia [32]. Even though prolonged cold preservation impairs allograft quality, static cold preservation is the most widely used modality for organ preservation and has contributed to the success of organ transplantation by making organ allocation possible.

Pathophysiology of cold-ischemia reperfusion injury

Because deprivation of oxygen and nutrients switch cell metabolism from an aerobic to anaerobic state only minimal amounts high-energy phosphates (ATP) are generated. Hypothermia is an important principle of organ preservation because at low temperature cell metabolism is retarded, and thus the demand for ATP is decreased. Hypothermic preservation has been the golden standard for organ preservation since many years, but also has several limitations [33, 34]. Even though cell's metabolic activity is strongly diminished at low temperatures, also at low temperatures cells are in need for ATP to maintain ion homeostasis via the Na^+/K^+ ATPase membrane pumps. If ATP is insufficiently available, Na^+ starts to accumulate intracellular thereby increasing osmotic pressure with cellular edema as final consequence. ATP is not only being consumed but also broken down to hypoxanthine. Because hypoxanthine cannot be further metabolized in the absence of oxygen it will accumulate intracellular, and upon oxygen supply during reperfusion reactive oxygen species (ROS) are generated through the action of xanthine oxidase [33, 35, 36]. At the same time antioxidant defense mechanisms are not replenished during ischemia [36], making the cell highly vulnerable to oxidative stress when oxygen supply is restored. It should be emphasized that an intracellular redox imbalance will increases intracellular $[Ca^{+2}]$ [37], which will further exhaust ATP concentration as $[Ca^{+2}]$ homeostasis is also ATP dependent. Mitochondrial ROS production in conjunction with an increased mitochondrial $[Ca^{+2}]$ will result in mitochondrial

permeability transition (MPT) thereby dissipating the mitochondrial membrane potential. ROS are also generated due to the increase of free labile iron [38] (Figure 1).

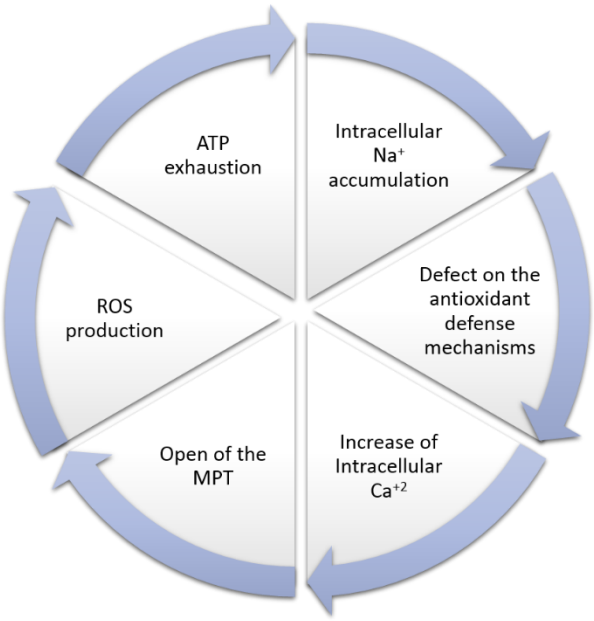

Figure 1: *Pathophysiology of cold-ischemia reperfusion injury. MPT: mitochondrial permeability transition pore.*

Nakao *et al.* have postulated that cytochrome P450 heme proteins are degraded during hypothermic organ preservation. This causes a critical damaging increased level of intracellular free heme which subsequently leads to further oxidative injury [39]. In the presence of increased free iron even low amounts of H_2O_2 can elicit the production of high amounts of free radicals through the classical Fenton reaction. In the Fenton reaction Fe^{+2} reacts with H_2O_2, producing Fe^{+3}, hydroxyl radical and hydroxide ion. Fe^{+2} may be regenerated when Fe^{+3} accepts an electron from O2° (2).

Strategies to improve donor organ allograft quality

Since it is now generally accepted that cold preservation and ischemia/reperfusion injury are important risk factors for primary non function, delayed graft function and chronic dysfunction of kidney grafts, many strategies have been developed to ameliorate tissue injury. These strategies include management of the donor, preconditioning of the kidney, improvement of organ preservation techniques and re-conditioning of the allograft before implantation. In addition to these strategies, which are already partly implemented in transplantation medicine,

also experimental studies on post-conditioning therapies involving the recipient are emerging [40]. These concepts will not be discussed in this paragraph.

Donor management and drug intervention: Early and adequate donor management is of utmost importance not only for maintaining donor organ quality but also for increasing the number of retrievable organs from potential donors. In March 2001, a conference consensus meeting to maximize the number of organs recovered and transplanted from deceased donors has recommended that 4-drug hormonal resuscitation (T3/T4, methylprednisolone, insulin, and arginine vasopressin) of donors with a left ventricular ejection fraction of <45%, and/or unstable hemodynamics, should be initiated. The conference further recommended that the United Network for Organ Sharing (UNOS) should follow the conference guidelines, a recommendation that was implemented by UNOS [41, 42]. Recent data from UNOS have indicated that, when 3-drug hormonal replacement (T3/T4, methylprednisolone, vasopressin) is administered, there has been a 22.5% greater number of organs transplanted from such donors than from donors without this treatment [43]. The increased probability of an organ being transplanted has been estimated to be between 2.8 and 7.3% for all major vascular organs grafted. A second UNOS study indicated that graft survival of patients whose donors have received hormonal therapy was significantly greater than those whose donors had not (89.9 vs 83.9%). In particular, early heart graft dysfunction occurred in only 5.6% of patients when the organ came from a hormone-resuscitated donor, but in 11.6% of patients when the donor had received no hormonal therapy. Furthermore, if the donor had received hormonal replacement therapy, significantly improved one-year kidney and heart graft survivals were demonstrated, although not for recipients of liver grafts [44]. More recently the use of dopamine on potential donors has been revisited by Schnuelle *et al.* , showing in a prospective, randomized, multicenter study that low-dose dopamine treatment of brain death donors has a salutary effect on early renal graft function in the recipient [45]. This will be discussed in more detail in chapter 3 of this thesis.

Donor management and donor ventilation: Ventilation protocols have been also standardized, yet if how ventilation strategies affect organ quality of BD donors have not been thoroughly studied in experimental models. In a randomized control trial Mascia et al [46] showed that the use of a lung protective strategy (with tidal volumes of 6-8 mL/kg of predicted body weight, positive end-expiration pressure (PEEP) of 8-10 cm H_2O) in potential organ brain dead donors increased the number of eligible and harvested lungs compared with a conventional strategy. Nonetheless current guidelines for potential organ donors recommend ventilation with higher levels of low tidal volume (10-15 mL/kg of measured body weight). (United Network

for Organ Sharing, http://www.unos.org/docs/Critical_Pathway.pdf, Canadian Council for Donation and Transplantation http://www.ccdt.ca/english/publications/background-pdfs/Organ-Donor-Guidelines.pdf).

Organ preservation: After removal from the deceased donor, kidneys are usually placed in a bag of preservation solution in a box of ice, keeping the temperature of the organ around 4°C. While such static cold storage has the advantage of being simple and facilitating easy transport of the kidney from donor hospital to recipient hospital, it has been argued that the kidney may be better preserved if it is placed on a machine where cold preservation solution is pumped through it, flushing out the small capillaries and the accumulating metabolic products. Particular attention has focused on cold machine perfusion of kidneys from DCD donors, which potentially have most to gain from improved storage. However, two recent randomized controlled trials using the same machines have produced contrary results, so the true value of cold machine perfusion remains to be determined [47, 48].

Organ reconditioning: It has been suggested that major parts of the post-preservation injury occur at the time of warm reperfusion but not during ischemic storage. The concept of organ re-conditioning is based on the concept that deleterious priming of the graft during ischemia can be abrogated before transplantation by dynamic revitalization techniques at the end of cold storage. This can be done by either hypothermic machine perfusion [49, 50] or gaseous oxygen persufflation [51]. Owing to its simplicity and ease of application, oxygen persufflation appears to be a particularly convenient approach. Even though the optimal treatment interval for "hypothermic re-conditioning" after conventional cold storage has been experimentally established [52], oxygen persufflation is still in its infancy and not widely used.

Aim of the study

The focus of this thesis is mainly on two classes of bioactive molecules, i.e. Enzyme triggered CO-releasing molecules (ET-CORMs) and N-acyl dopamines (NADDs), which may have a potential use in transplantation medicine, either as in terms of donor conditioning or recipient treatment. In **Chapter 2** I will therefore first discuss the beneficial effects of carbon monoxide and how this might be used in transplantation medicine. In **Chapter 3** the same will be done for N-acyl dopamines. In the experimental parts of my thesis (**chapters 4** to **8**) in vitro experiments are performed, which show how chemical characteristics of these bioactive molecules influence biological responses in terms of cytoprotection, inflammation and acquired immunity. In **Chapter 4** we tested 9 structurally different ET-CORMs in respect to cytotoxicity, their ability to protect against hypothermic preservation damage and their ability to inhibit VCAM-1 expression on cultured human umbilical vein endothelial cells (HUVEC) and renal proximal tubular epithelial cells (PTEC) by using a structure-activity approach. To refine, our finding that the type and the position of the ester moiety play a central role in their biological effect in **Chapter 5** we synthesized and tested as well the biological properties of acyloxy-diene-$Fe(CO)_3$ complexes with different lengths of the aliphatic ester side chain R. Because these complexes are not water soluble, phosphoryloxy-substituted η4-acyloxy-cyclohexadiene-Fe(CO)3 complexes were subsequent developed and tested if these water soluble compounds still have the properties to act as ET-CORMs. In **Chapter 6** we subsequently focused on three of these ET-CORMs and show that a different design of ET-CORMs results in quantitative differences in biological activities in terms of toxicity and inflammation. In **Chapter 7** and **Chapter 8** the focus of our in vitro studies was on N-octanoyl dopamine and assessed its influence on T-lymphocyte activation (**Chapter 7**) and how it may convey cytoprotection in human endothelial cells (**Chapter 8**). In **Chapter 9** we show that different chemical moieties within N-acyl dopamines are responsible for the anti-inflammatory properties and the property to activate transient receptor potential vanilloid type 1 (TRPV1) channels.