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Review

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Tetrahydrobiopterin attenuates ischemia-reperfusion injury following organ transplantation by targeting the nitric oxide synthase: investigations in an animal model

Abstract: Ischemia-reperfusion injury is a primarily non-allospecific event leading to the depletion of the essential nitric oxide synthase cofactor and potent antioxidant tetrahydrobiopterin. Suboptimal concentrations of tetrahydrobiopterin result in a reduced biosynthesis of nitric oxide leading to vascular endothelial dysfunction. Tetrahydrobiopterin supplementation has been shown to protect from this pathological state in a plethora of cardiovascular diseases including transplant-related ischemia-reperfusion injury. Even though still controversially discussed, there is increasing evidence emerging from both human as well as animal studies that tetrahydrobiopterin-mediated actions rely on its nitric oxide synthase cofactor activity rather than on its antioxidative properties. Herein, we review the current literature regarding the role of tetrahydrobiopterin in ischemia-reperfusion injury including our experience acquired in a murine pancreas transplantation model.

Keywords: animal model; ischemia-reperfusion injury; nitric oxide; organ transplantation; tetrahydrobiopterin.

Enzymes: calpain (EC 3.4.22.52); catalase (EC 1.11.1.6); glutathione peroxidase (EC 1.11.1.9); NADPH oxidase (EC 1.6.3.1); nitric oxide reductase (EC 1.7.99.7); nitric oxide synthase (EC 1.14.13.39); phospholipase A_2; (EC 3.1.1.4); superoxide dismutase (EC 1.15.1.1); xanthine oxidase (EC 1.17.3.2).

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Introduction

"Solid organ transplantation is one of the most remarkable and dramatic therapeutic advances in medicine during the past 60 years. This field has progressed from 'clinical experiment' to routine and reliable practice" [1].

Many obstacles such as development of adequate immunosuppressive drugs, improvements in surgical techniques, as well as progresses in postoperative protocols had to be overcome to establish simultaneous pancreas-kidney transplantation as therapy of choice for patients suffering from diabetes with end-stage renal failure [2–5]. Even though reaching clinical routine during the past two decades, pancreas transplantation shows the highest prevalence of surgical and postoperative complications of all routine solid organ transplants [6].

In addition to immunological factors such as acute and chronic graft rejection, several non-immunological factors including donor risk factors (e.g., hemodynamic instability, vasopressor administration), prolonged resuscitation, prolonged preservation time and especially ischemia-reperfusion injury have been identified to play an important role in the increase of graft pancreatitis [7, 8].

According to the literature, ischemia-reperfusion injury alone has been claimed to be responsible for up to 10% of early graft losses [9].

Ischemia-reperfusion injury

Ischemia-reperfusion injury represents an early occurring, primarily non-allospecific event influencing early graft function and its short-term as well as long-term survival, not only in pancreas transplantation but also in other organ transplants [10]. Briefly, ischemia-reperfusion is characterized by two distinct events namely (i) the ischemic and (ii) the reperfusion/reoxygenation phase [11].

Deprivation of oxygen represents the predominant injury process during the ischemic phase giving rise to a variety of cellular, metabolic and ultrastructural changes, due to decreases in cellular oxidative phosphorylation. This results in a failure to resynthesize energy-rich phosphates, namely adenosine 5′-triphosphate (ATP) and phosphocreatine, altering the active ATP-dependent transmembrane ion transport and by that favoring the intracellular accumulation of calcium, sodium and water [11, 12]. The increased cytosolic calcium concentration itself may activate hydrolases, including phospholipases (especially phospholipase A_2) and proteases (calpains and others), which enhance the injury process by degradation of their substrates, such as by calpain-mediated proteolysis of cytoskeletal proteins [12]. Furthermore, elevated intracellular sodium concentrations may amplify osmotic swelling and thereby contribute to the disruption of the plasma membrane. Both pathways finally induce cellular death, typically represented as a non-apoptotic (necrotic) form of cell death [12].

Additional tissue damage evolves as a result of reperfusion/reoxygenation by restoring blood flow. Although crucial for the survival of an ischemic tissue, reperfusion also enhances the damage initiated by ischemia. This event is also known as ‘reflow paradox’ [13]. The phenomenon is associated with the adhesion of leukocytes to the postcapillary venules and subsequent leukocyte activation, chemotaxis and transmigration. Several adhesion molecules expressed on the surface of leukocytes and/or endothelial cells are involved in this multistep process [13]. Additionally, also complement activation is favored, which alters vascular homeostasis, and especially compromises the blood flow in ischemic organs and significantly enhances tissue damage [14].

Another important pathophysiological process during reperfusion and reoxygenation of an ischemic organ is an increased production of toxic reactive oxygen species (ROS) including superoxide anions, hydroxyl radicals, hypochlorous acid, hydrogen peroxide and the nitric oxide (NO)-derived peroxynitrite [15]. They constitute a group of oxygen-atom containing molecules, which are highly reactive due to the presence of unpaired valence shell electrons [16]. Under physiological conditions different enzymes such as superoxide dismutase, catalase and glutathione peroxidase represent an endogenous protective mechanism with the ability to scavenge and reduce accruing ROS formation [17]. However, during ischemia and reperfusion, endogenous protective systems are overwhelmed by the excessive amount of ROS. This excess may also result in a depletion/consumption of antioxidants (e.g., vitamin C, vitamin E), which are able to scavenge oxygen radicals non-enzymatically under physiological conditions [18]. ROS provoke a variety of harmful effects on the cells including DNA strand breakage due to modifications of the desoxyribose, and further oxidation of polydesaturated fatty acids in lipids, and oxidation of amino acids in proteins, as well as oxidative inactivation of specific enzymes by oxidation of cofactors, and finally they may induce cell death [17].

In addition to this, a variety of potential sources for ROS production have been identified so far; for example, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system located in the phagocyte membrane or the malfunctioning mitochondrial electron transport chain seem to play a pivotal role. Also, free metal ions may facilitate the production of toxic radicals [18]. Furthermore, the nitric oxide synthase (NOS) enzyme family has been identified as a potential source for ROS [17].

Nitric oxide synthases

NO is a gaseous mediator produced by a variety of mammalian cells. It plays a key role in neurotransmission, control of blood pressure and vessel homeostasis and also in cellular defense mechanisms. In addition to being derived from nitrite by certain reducing systems such as the respiratory chain or xanthine oxidase, or being set free from NO donors such as *S*-nitrosoglutathione or nitroglycerine, endogenous NO is primarily generated by a group of enzymes called NOSs [19, 20].

So far, three distinct isoforms of NOS are known. Neuronal NOS (nNOS/NOSI) and endothelial NOS (eNOS/NOSIII) are generally referred to as constitutively expressed and calcium-dependent, whereas inducible NOS (iNOS/NOSII) is expressed at high levels only after

induction by cytokines or other inflammatory agents, and its activity is calcium-independent. Although all three isoforms are characterized by regions of high homology, each isoform exhibits distinctive features, which reflect their specific in vivo functions [21].

nNOS is the largest protein of the three isoforms. It is constitutively expressed in the central and peripheral nervous system (CNS and PNS), but it is also present in skeletal muscle, macula densa and placenta. In the CNS, NO produced by nNOS has been found to subserve many different functions such as learning, feeding, waking and sleeping, neurosecretion and behavior [22–24]. In the PNS, NO derived from nNOS acts as a neurotransmitter of the nitrergic nerves, which receive electrical signals from the CNS via parasympathetic preganglionic fibers and ganglia. NO is involved in the regulation of smooth muscle cell of the blood vessels and cardiac myocytes, the gastrointestinal tract, the penile corpora cavernosa, the urethra and the prostate [25, 26]. In a mouse model harboring a targeted disruption of nNOS, lack of nNOS leads to the development of enlarged stomachs, with hypertrophy of the pyloric sphincter and the circular muscle layer [27].

eNOS is mainly expressed in vascular endothelial cells. Other relevant sources for this isoform include cardiac myocytes and cardiac conduction tissue [28–31]. Although often referred to as constitutively expressed, hormones such as catecholamines and vasopressin, autacoids such as bradykinin and histamine, platelet-derived mediators such as serotonin and ADP, or mechanical forces such as blood flow or shear stress lead to an activation of the enzyme by an increase of intracellular calcium. Thus, either by influx of extracellular calcium or by release from intracellular calcium stores, production of NO can be induced. NO also represents a crucial factor for the normal functioning of the cardiovascular system. NO produced by eNOS relaxes the vasculature and inhibits adhesion and aggregation of platelets [32]. Furthermore, NO inhibits adhesion of leucocytes and macrophages to the endothelium, and migration and proliferation of smooth muscle cells [33].

In contrast to the other isoforms, iNOS is not constitutively expressed, but it is synthesized de novo in a number of cell types such as macrophages, natural killer cells and neutrophils, and also in smooth muscle cells, cardiomyocytes and microglia under inflammatory conditions. Even though iNOS can bind calmodulin, this protein is Ca^{2+}-independent and permanently active, and it can act as a high-output system generating large amounts of NO, which is required for killing of bacteria, viruses, parasites and fungi. The most relevant triggers for iNOS expression are endotoxins and proinflammatory cytokines [34, 35]. NO and its derivatives exert its antimicrobial effect by causing DNA/RNA damage, inhibiting protein synthesis, altering proteins by *S*-nitrosylation, ADP-ribosylation or tyrosine nitration, and further by inactivating enzymes through disruption of Fe-S clusters or heme groups, or oxidizing membrane lipids [36]. However, the effectiveness of NO as an antipathogen agent depends on its local concentration and redox environment, as well as the pathogens themselves. Many pathogens possess mechanisms to protect themselves from nitrosative stress or may also develop tolerance to NO and its derivatives. For instance, *Escherichia coli* has been found to be protected from NO-induced growth inhibition by overexpressing cytochrome *bd* oxidase, whereas *Neisseria meningitidis* and *Salmonella enterica* protect themselves by expressing nitric oxide reductase [37, 38].

Nevertheless, under normal physiological conditions iNOS is only slightly expressed or is absent and seems to have no impact on the cardiovascular system. This conclusion is supported by the lack of a phenotype of uninfected iNOS knockout mice [39].

What isoforms have in common is that they are capable of synthesizing NO in a two-step oxidation of L-arginine to L-citrulline. The initial hydroxylation of L-arginine leads to the formation of N^G-hydroxy-L-arginine, which can also act as a substrate for NOS. This is followed by oxidation of the intermediate, using a single electron from NADPH to form L-citrulline and NO [40, 41].

To fulfill this function it is crucial that the enzyme constitutes a stable homodimer. Each monomer can be functionally and structurally divided into two major domains: a C-terminal reductase domain and an N-terminal oxygenase domain [42]. Furthermore, the five cofactors, NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme and tetrahydrobiopterin, are essential for the appropriate functioning of the enzyme [41]. The reductase domain contains binding sites for one molecule each of NADPH, FAD and FMN, whereas the oxygenase domain binds the other two cofactors heme and tetrahydrobiopterin, as well as the substrate L-arginine. Calmodulin (CaM), which has a key role in stabilizing the structure and activation of the enzyme, binds at the interface between the two regions.

NADPH serves as an electron donor to the reductase domain. Electrons proceed via FAD and FMN redox carriers to the oxygenase domain of the other monomer. There they interact with the heme iron and tetrahydrobiopterin at the active site to catalyze the production of NO [43].

The reductase and oxygenase domains of NOS are distinct catalytic units, which together provide the complete machinery required for NO production [21].

In the past decade, in particular, tetrahydrobiopterin emerged as a crucial factor for normal functioning of all three NOS isoforms [44].

Tetrahydrobiopterin and endothelial dysfunction

Tetrahydrobiopterin is an essential cofactor for the catalytic activity of all NOS isoforms. As such, tetrahydrobiopterin has far reaching effects on both function and structure of these enzymes. It has the ability to shift NOS heme iron to a high spin state, increase substrate affinity for arginine, and furthermore it stabilizes the active dimeric form of the enzyme [45]. Additionally, NOS-bound tetrahydrobiopterin may also act as a redox-active cofactor and it may also neutralize free radicals during regular NO biosynthesis by NOS [46].

Increasing evidence suggests that optimal intracellular tetrahydrobiopterin concentrations are essential for the normal functioning of eNOS and endothelial cells [47].

Indeed, oxidative stress, induced by ischemia-reperfusion injury and many other vascular disease states, has been shown to cause depletion of intracellular tetrahydrobiopterin levels under a critical threshold value, by decreasing expression of GTPCH-1, depleting NADPH and by oxidation of the highly redox-sensitive tetrahydrobiopterin to the inactive 7,8-dihydrobiopterin [48]. In endothelial cells these suboptimal concentrations of tetrahydrobiopterin lead to the so-called "uncoupling" of the NOS enzyme. This means that the enzymatic reaction becomes uncoupled from NADPH consumption, and electron flow is directed towards formation of superoxide anion and hydrogen peroxide rather than NO. Furthermore, superoxide anions subsequently react with NO synthesized by adequate functioning NOS and form the highly cytotoxic peroxynitrite, which subsequently causes severe oxidative damage to proteins, lipids and DNA, as well as consumption of reducing agents such as tetrahydrobiopterin itself [49, 50].

As a consequence, the uncoupled enzyme itself becomes a ROS source and thereby causes, sustains and enhances endothelial dysfunction [51, 52].

The concept of endothelial dysfunction, defined as a functional but not yet macroscopically visible pathological state of the endothelium, evolved over the past years as a result of numerous studies on diseased arteries both in experimental animals and in patients with vascular disease [53].

Even though the molecular basis of endothelial dysfunction is not completely understood, numerous studies indicate the depletion of intracellular tetrahydrobiopterin levels as a central mechanism. The loss of NO due to NOS uncoupling induces a proinflammatory state, manifested as inappropriate vasoconstriction, platelet aggregation, leucocyte adhesion and smooth muscle cell proliferation [54–56].

Various in vivo experiments using either genetically modified mouse strains or inhibiting different enzymes of the tetrahydrobiopterin metabolism showed that eNOS function is directly related to eNOS-tetrahydrobiopterin stoichiometry [48]. In addition, recent human data strengthened these results, demonstrating a considerable amelioration of different vascular diseases such as arterial hypertension, atherosclerosis and hyperlipidemia after exogenous tetrahydrobiopterin application [48, 49, 57–59]. However, also a crucial involvement of the neuronal isoform in physiological regulation of vascular tone by directly influencing the vascular smooth muscle tone has been discussed in several in vitro as well as in vivo studies [60, 61].

Despite this controversy on the underlying mechanism of action, tetrahydrobiopterin has also gained interest as a possible therapeutic option in solid organ transplantation. Reduction of lung edema and oxygen-derived free radical injury in lung grafts transplanted into pigs [62] as well as a protective action in a porcine cardiac ischemia model [63], more recently in a rat Langendorff model, and in a rat kidney clamping model could be observed [64, 65].

So far, three possible mechanisms can be suggested: (i) recoupling of NOS isoforms in order to switch off a major ROS producer as well as (ii) the antioxidative capacity of tetrahydrobiopterin, which would be able to neutralize occurring free radicals. Finally, (iii) synergisms between these two mechanisms cannot be ruled out.

In previous investigations performed by our group, we were able to show that ischemia-reperfusion injury associated graft pancreatitis was successfully attenuated by single-shot donor therapy with 50 mg/kg b.w. tetrahydrobiopterin in a murine heterotopic pancreas transplantation model [66, 67]. Furthermore, in a recent study we compared tetrahydrobiopterin treatment to treatment with the antioxidants ascorbic acid and folic acid, and to the pterin derivate tetrahydroneopterin, which resembles all characteristics of tetrahydrobiopterin except for NOS cofactor activity. Herein, we observed that only tetrahydrobiopterin protected the grafts from lethal ischemia-reperfusion injury, suggesting a crucial role of one or more NOS isoforms in the pathogenesis of ischemia-reperfusion [68].

Many investigations using NOS inhibitors, for example, L-NAME have been performed. However, L-NAME is a rather unspecific NOS inhibitor with equal efficacy on nNOS [69]. By contrast, to address the NOS enzyme finally responsible for the beneficial effects of tetrahydrobiopterin, we are currently performing investigations using knockout mice lacking either one of the NOS enzymes. This investigation should allow a clear differentiation between the different isoforms and clarify their role in the pathogenesis of ischemia-reperfusion injury at least in our model.

Conclusion

The strong correlation between tetrahydrobiopterin depletion and acute as well as chronic vascular diseases, including transplantation, has been described in a variety of publications. In this context, the NOS cofactor activity of tetrahydrobiopterin seems to play a major role rather than its antioxidative effect. Even though over the years the eNOS isoform was the main focus of attention of ischemia-reperfusion injury related research, recent findings attribute an important role in vascular hemostasis also to the neuronal isoform. Which isoform comes into question as a target of exogenous tetrahydrobiopterin supplementation is yet to be discovered.

Both oral as well as intravenous application of tetrahydrobiopterin has been shown to be safe in humans [70, 71]. Oral supplementation is the standard treatment of tetrahydrobiopterin deficient phenylketonuria [72]. In addition, successful treatment of arterial hypertension with tetrahydrobiopterin tablets has already been described [73].

Therefore, clinical application of tetrahydrobiopterin is feasible and should be considered as a further therapeutic option in the prevention of ischemia-reperfusion injury following solid organ transplantation. Supporting results in more stringent large animal models of organ transplantation and unraveling the target of this treatment will eventually pave the way into clinical trials.

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