REVIEW

Nitric oxide homeostasis as a target for drug additives to cardioplegia

BK Podesser[1,3] and S Hallström[2,3]

[1]The Ludwig Boltzmann Cluster for Cardiovascular Research, Medical University of Vienna, Vienna, Austria
and [2]Institute of Physiological Chemistry, Center for Physiological Medicine, Medical University of Graz, Graz, Austria

The vascular endothelium of the coronary arteries has been identified as the important organ that locally regulates coronary perfusion and cardiac function by paracrine secretion of nitric oxide (NO) and vasoactive peptides. NO is constitutively produced in endothelial cells by endothelial nitric oxide synthase (eNOS). NO derived from this enzyme exerts important biological functions including vasodilatation, scavenging of superoxide and inhibition of platelet aggregation. Routine cardiac surgery or cardiologic interventions lead to a serious temporary or persistent disturbance in NO homeostasis. The clinical consequences are ''endothelial dysfunction'', leading to ''myocardial dysfunction'': no- or low-reflow phenomenon and temporary reduction of myocardial pump function. Uncoupling of eNOS (one electron transfer to molecular oxygen, the second substrate of eNOS) during ischemia-reperfusion due to diminished availability of L-arginine and/or tetrahydrobiopterin is even discussed as one major source of superoxide formation. Therefore maintenance of normal NO homeostasis seems to be an important factor protecting from ischemia/reperfusion (I/R) injury. Both, the clinical situations of cardioplegic arrest as well as hypothermic cardioplegic storage are followed by reperfusion. However, the presently used cardioplegic solutions to arrest and/or store the heart, thereby reducing myocardial oxygen consumption and metabolism, are designed to preserve myocytes mainly and not endothelial cells. This review will focus on possible drug additives to cardioplegia, which may help to maintain normal NO homeostasis after I/R.
British Journal of Pharmacology (2007) 151, 930–940; doi:10.1038/sj.bjp.0707272; published online 8 May 2007

Introduction

Nitric oxide (NO) has been identified as a key regulator of vascular function, representing one of the most powerful vasodilators, antagonizing vasoconstrictors such as endothelin. Consequently, an imbalance in NO production, availability or increased NO degradation on the one hand and increased endothelin production or reduced degradation on the other hand leads to vasoconstriction. In contrary, NO overproduction seen in acute organ rejection or during septic shock leads to vasoplegia. Therefore it is fair to say that NO homeostasis is the precondition for physiologic blood pressures and flow conditions. The action of NO in vascular and cardiovascular regulation is not limited to its vasodilatation properties. NO inhibits platelet aggregation, reduces oxygen consumption, has lusotropic potential, influences directly or indirectly myocardial contractility, scavenges superoxide (O_2^-) (antioxidant properties), prevents leukocyte adhesion and is an antiproliferative and anti-inflammatory mediator.

It is of common knowledge that atherosclerosis affects the major site of NO production, the endothelium. The clinical consequences of atherosclerosis are coronary artery and/or peripheral artery disease, hypertension, stroke, and so on. On the basis of severe atherosclerosis, routine cardiac surgery or cardiologic interventions lead to a serious temporary or persistent disturbance in NO homeostasis. The clinical consequences of persistent impaired NO activity and increased radical oxygen species activity lead to 'endothelial dysfunction' and ultimately to 'myocardial dysfunction': no- or low-reflow phenomenon and temporary reduction of myocardial pump function.

Correspondence: Dr BK Podesser, Ludwig Boltzmann Cluster for Cardiovascular Research, Währinger Gürtel 18-20, Medical University of Vienna, A-1090 Vienna, Austria.
E-mail: b.k.podesser@cardiovascular-research.at or Dr S Hallström, Institute of Physiological Chemistry, Center for Physiological Medicine, Medical, University of Graz, Graz, Austria.
E-mail: seth.hallstroem@meduni-graz.at
[3]These authors contributed equally to this work.

However, solutions to arrest the heart during cardiac surgery, the so-called cardioplegic solutions, have been primarily designed to protect the myocytes from ischemia and the following reperfusion (Bretschneider et al., 1975; Braimbridge et al., 1977; Buckberg 1979; Southard, 1995). Cardiovascular research has recognized rather late that the endothelium of the heart is an important organ that locally regulates coronary perfusion and cardiac function by paracrine secretion of NO and vasoactive peptides (Linz et al., 1992). Therefore cardioplegic solutions in general and storage solutions in cardiac transplantation in particular are now critically discussed with respect to preservation of both myocytes and endothelial integrity and function.

This review will focus on NO homeostasis as a target for drug additives to cardioplegia. The maintenance of NO homeostasis is a complex field and in the case of cardioplegia directly related to prevention of ischemia/reperfusion (I/R) damage. Strategies for additives to cardioplegic solutions include calcium antagonists, scavengers of O_2^- and indirect and direct supplementation of NO. Therefore we briefly address the following topics:

- NO homeostasis in the cardiovascular system
- Disturbed NO homeostasis during I/R
- Clinical state of the art to preserve the heart during cardiac surgery and transplantation
- The endothelium as a target for cardioplegic solutions
- Discussion and outlook

NO homeostasis in the cardiovascular system

NO pathway
NO is constitutively produced under physiologic conditions by the two isoenzymes eNOS (endothelial nitric oxide synthase, NOS-3) and nNOS (neuronal nitric oxide synthase, NOS-1) that are both calcium and calmodulin dependent, and a third enzyme, iNOS (inducible nitric oxide synthase, NOS-2) which is independent of the calcium–calmodulin pathway and not expressed under physiologic circumstances in the endothelium (Balligand and Cannon, 1997). Within the cardiovascular system, eNOS is the most important isoform. eNOS is a Ca^{2+}-, NADPH-, flavin- and biopterin-dependent enzyme, which utilizes the guanido nitrogen atom of L-arginine and incorporates molecular oxygen to generate NO and L-citrulline (Wu, 2002). Thus, NO is concerned with the regulation of vascular tone by inducing relaxation of vascular smooth muscle cells through stimulation of the enzyme-soluble guanylyl cyclase. Activation of soluble guanylate cyclase leads to conversion of magnesium guanosine $3',5'$-triphosphate to the second messenger guanosine $3',5'$-monophosphate guanosine, which stimulates two cyclic guanosine monophosphate (cGMP)-dependent protein kinases (PKG I and PKG II) (Kojda and Kottenberg, 1999). PKG I is the major kinase-mediating vasodilatation and inhibition of platelet aggregation. PKG I acts via a phosphorylization of phospholamban and a phosphorylization of the 1,4,5-inositoltriphosphate receptor-associated cGMP kinase substrate. The activity of cGMP is terminated by a rapid conversion to GMP, catalyzed by various phosphodiesterases (Moncada et al., 1991; Gewaltig and Kojda, 2002).

NO-mediated effects
Produced by endothelial cells, NO is responsible for the following actions:
- vasodilatation (Ignarro et al., 1987)
- inhibition of platelet aggregation (Azuma et al., 1986; Radomski et al., 1987; Kalinowski et al., 2002)
- reduction of oxygen consumption (Loke et al., 1999; Recchia et al., 1999)
- lusitropy (an increase in cardiac compliance) (Paulus and Shah, 1999) and therefore
- a direct- or indirect-mediated increase in myocardial contractility (Brady et al., 1993; Kanai et al., 1997)
- scavenging of O_2^- (antioxidant properties) (Beckman et al., 1990)
- prevention of leukocyte (neutrophil) adhesion (Ma et al., 1993)
- reduction of proliferation and inflammation (Garg and Hassid, 1989; Wright et al., 1992)

After cardioplegic arrest and storage initial reperfusion is a critical step. Better organ preservation with improved cardioplegic solutions focusing on both, myocytes and endothelial cells, are of major interest. Also the first step of many, leading to successful heart transplantation is adequate organ preservation ensuring maintenance of organ viability and function. Thereby, the major challenge is to minimize pathologic damage and impairment of function because during procurement, storage and implantation, the heart is always exposed to I/R.

Disturbed NO homeostasis during I/R

Re-establishing blood flow to ischemic tissues or organs (reperfusion) is an essential step in many surgical procedures (Korthuis et al., 1985), including reperfusion after cardioplegic arrest or transplantation of the heart after organ preservation in cardioplegic solutions. However, reperfusion, especially after prolonged ischemia, leads to changes in vasomotility and an increase in microvascular permeability causing tissue reperfusion edema (Sternbergh et al., 1993). These are constant features of I/R injury. The consequences of such an injury are frequently massive edema formation and tissue destruction (Baue, 1992; Grace, 1994). Constitutive eNOS plays a fundamental role in the pathogenesis of I/R injury. The onset of ischemia leads to elevated intracellular calcium ion concentrations mediated by increased levels of catecholamines. These increased calcium ion concentrations activate eNOS (enhanced generation of NO). Data by Huk et al. (1997) and Hallström et al. (2002) also show that I/R injury is initiated by a massive burst of NO production (measured in vivo with a porphyrinic-based microsensor – Malinski and Taha, 1992) after onset of ischemia, which depletes local L-arginine and/or (6R)-5,6,7,8-tetrahydrobiopterin (BH$_4$) concentrations, followed by high production of O_2^- after reperfusion and consequently

high production of peroxynitrite. It has become clear from studies with purified eNOS that under substrate or cofactor depletion, the enzyme may become 'uncoupled'. In such an uncoupled state, electrons are rather diverted to molecular oxygen rather than to L-arginine, resulting in production of O_2^- (Xia and Zweier, 1997; Vasquez-Vivar et al., 1998) and consequently high production of peroxynitrite. Although the mechanisms underlying endothelial dysfunction are likely to be multifactorial, it is important to note that increased production of oxygen-derived free radicals by an uncoupled eNOS markedly contributes to this phenomenon (Münzel et al., 2005).

The formation of excessive local peroxynitrite concentrations and consequent cleavage products during the initial phase of reperfusion is deleterious. Three of these cleavage products (hydroxyl free radical, nitrogen dioxide free radical and nitronium cation) are among the most reactive and damaging species and may be major contributors to the severe I/R damage (Huk et al., 1997). The pathophysiologic settings mentioned above are associated with endothelial dysfunction. In cardiac transplantation I/R, hypothermia and atherosclerosis are of particular interest. The first two pathologic conditions contribute to endothelial dysfunction during and shortly after transplantation. Atherosclerosis of the coronary arteries of the transplanted organ, to some extent being a result of the endothelial dysfunction set by hypothermia and I/R, is one of the leading causes for deaths years after transplantation and is directly related to the time of ischemia of the allograft (Valantine, 2003; Weis and Cooke, 2003).

The immediate consequences of I/R and hypothermia are endothelial dysfunction: vasoconstriction due to decreased NO production or bioavailability and dominance of endothelins, a group of vasoactive peptides, produced by the endothelium (Gourine et al., 2001; Laude et al., 2001). This leads to no reflow and finally to organ failure. In the heart, this temporary failure is called 'myocardial stunning'. The term myocardial stunning, as it is used by many authors, is characterized as a mechanical dysfunction that persists after reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary blood flow. This implies that this is a postischemic dysfunction that is fully reversible, no matter how severe or prolonged, and that the dysfunction is not caused by a primary deficit of myocardial perfusion (Bolli, 1990). Figure 1 summarizes the effect of I/R on NO homeostasis and depicts possible targets of therapeutic intervention.

Clinical state of the art to preserve the heart during cardiac surgery and transplantation

Cardioplegic arrest

Till date, the vast majority of heart operations are performed on the arrested heart. Exceptions are so-called off-pump coronary artery revascularizations. The numbers differ largely between countries and centers, in Europe approximately 15% of all coronary bypass operations are performed without cardiac arrest and consequently without the use of the heart–lung machine (HLM). The possible advantage of
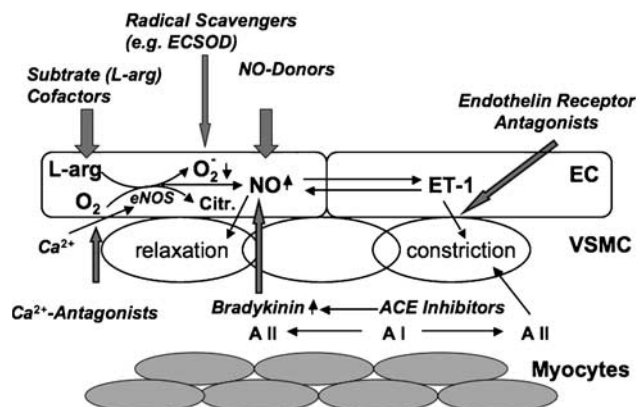

Figure 1 Nitric oxide (NO) homeostasis as a target for drug additives to cardioplegia – possible routes: following reperfusion after cardioplegia, the levels of NO are decreased mainly due to 'uncoupling' of endothelial nitric oxide synthase (eNOS), which leads to enhanced level of superoxide (O_2^-). This results in increased vascular tone due to reduced bioavailability of NO and therefore reduced relaxation as well as enhanced production of the vasoconstrictors endothelin-1 (ET-1) and angiotensin II (A II). This reperfusion injury can be attenuated by enhancing the levels of bioavailable NO with substrates/cofactors of eNOS, NO-donors (feedback inhibition and prevention of 'uncoupling'), bradykinin, blocking ET-I receptors, blocking the receptors (AT-I) or production (ACE- inhibitors) of angiotensin II and free radical scavengers of superoxide such as extracellular superoxide dismutase (ECSOD). Calcium antagonists on the other hand can counteract the initial Ca^{2+} increase at the onset of ischemia and prevent eNOS stimulation. The enhanced (normalized) levels of bioavailable NO, for example, attenuate/prevent inflammation by inhibiting the activation of the proinflammatory transcription factor nuclear factor kappa B (NF-κB) and cell adhesion molecules. As consequence this results in a decreased expression of proinflammatory cytokines and reduced leukocyte (neutrophil) adhesion. The above figure mainly illustrates the possible routes of intervention.

off-pump surgery is its minimal invasiveness, defined in this case not by a limited skin incision but by a reduction in the side effects of the HLM on inflammation. The results so far show that patients with high co-morbidities, such as renal failure, reduced cardiac function or a history of neurological complications benefit most. However, for the majority of patients undergoing coronary revascularization, no difference has been identified between on- and off-pump surgery (Sellke et al., 2005). In all other surgical procedures, especially those, where the heart has to be opened such as in pediatric and valve surgery as well as in cardiac transplantation, cardioplegic arrest is mandatory.

The concept of cardiac arrest has been developed by the demands of cardiac surgeons to replace a calcified, stenotic valve or to perform a perfect communication between two vessels (anastomosis) with small diameters. However, uncoupling the heart from its natural source of oxygen and fuel (carbohydrates and fatty acids) leads to I/R damage and ultimately to myocardial infarction. Therefore, strategies were developed to enable this necessary uncoupling already from the very beginning of cardiac surgery (Melrose et al., 1955; Shumway et al., 1959). Common goal is the reduction of myocardial oxygen consumption either by hypothermia or by uncoupling of cardiac contraction. The former is based on experimental data showing that reduction of myocardial

temperature from 37 to 22°C reduces oxygen consumption by 90% (Buckberg et al., 1977). The latter is based on the fact that 60–70% of cardiac energy goes into contraction. Hyperpolarization by K^+, Mg^+ or Na^+ containing cardioplegic solutions induces cardiac arrest even under normothermic conditions (Bretschneider et al., 1975; Braimbridge et al., 1977). Till date, a combination of these two techniques is the gold standard of myocardial protection for heart procurement. In general, cardiac surgery temperature can vary from cold to tepid or normothermia, according to the patients special needs and the surgeons preferences (Guru et al., 2006).

Depending on the concentration of Na^+, two types of cardioplegic solutions can be distinguished: intra- and extracellular solutions (Podesser et al., 1996). In Europe, the clinically most relevant intracellular solution is Bretschneider's HTK solution with a Na^+ concentration of 15 mmol/l compared to the extracellular solutions such as St Thomas' cardioplegia (Na^+ 117 mmol/l) or blood cardioplegia according to Buckberg (Na^+ 120 mmol/l). Especially, HTK is also widely accepted as storage solution for cardiac transplantation together with Celsior, a solution designed to combine the general principles of hypothermic organ preservation specific to the heart with the possibilities to use it also during initial donor arrest, poststorage graft reimplantation and early reperfusion (Perrault et al., 2001).

Whenever the aortic root is not only clamped but also dissected and the heart is arrested and excised for organ procurement, it has to be stored. Again, these storage solutions have been designed as cardioplegic solutions for myocyte preservation only. Current clinical routine allows 4–5 h maximum for organ procurement, transport and beginning of reperfusion (Jahania et al., 1999). Experiments conducted with isolated porcine coronary endothelial cells to investigate the effect of cold ischemic storage of these cells in University of Wisconsin solution, another storage solution, mainly used for visceral organs in the US, have shown that a significant decrease in eNOS activity was seen after storage for more than 6 h. There was no significant decrease in cells subjected to cold ischemic storage for 1 h only (Redondo et al., 2000).

However, with an increase in the average life span, cardiac surgeons face an older population of patients undergoing coronary bypass surgery, valve replacement and reconstruction as well as transplantation. This cohort of patients is more likely to have impaired endothelial function. Consequently new strategies have to be developed to maintain operative mortality and morbidity at acceptable levels.

The endothelium as a target for cardioplegic solutions

Additives to cardioplegic solutions influencing NO homeostasis
Radical scavengers. Scavenging of O_2^- may increase NO bioavailability (Table 1). Indeed, in the past three decades, much work has been performed to use superoxide dismutase (SOD) as a therapeutic agent. Most efforts focused on cytosolic SOD, although it is not normally found in extracellular sites. Despite some success in experimental

studies, there has been little success in clinical applications. The variable effects of cytosolic SOD may be related to the limited cell penetration of the exogenous-applied cytosolic enzyme.

New approaches focus on therapeutic equivalents of extracellular superoxide dismutase (ECSOD), which is electrostatically bound to heparan sulfate proteoglycans in the glycocalyx of various cell types (for example, endothelial cells). The ECSOD is the major SOD in extracellular fluids. ECSOD has a highly hydrophylic positively charged, carboxyl terminal that imparts the high heparin affinity, which allows the enzyme to be bound to heparin sulfate proteoglycans on endothelial surfaces (Sandstrom et al., 1992). Recombinant human ECSOD type C has been shown to have cardioprotective effects in isolated rat heart subjected to I/R (Sjöquist et al., 1991) as well as in isolated cold-arrested rat hearts (Hatori et al., 1992). More recently a human chimeric SOD, a product of a fusion gene encoding a mutant manganese SOD and the carboxy terminal 26-aminoacid tail from ECSOD, has shown to improve myocardial preservation of isolated rabbit hearts subjected to warm and cold ischemia (Nelson et al., 2002).

Calcium antagonists. A number of studies have reported that administration of diltiazem before or during I/R reduces infarct size and protects against reperfusion injury (Klein et al., 1984; Seitelberger et al., 1994; Tadokoro et al., 1996). The beneficial effects of diltiazem on ischemic myocardium are generally thought to be due to coronary artery vasodilatation and a decrease of heart rate as well as cardiac contractility (Ferrari and Visioli, 1991). The net result of these effects is a reduction in the imbalance between oxygen supply and demand in acutely ischemic myocardium, through a simultaneous increase in collateral blood flow (mediated by coronary vasodilatation) and a reduction in oxygen consumption (secondary to a decrease in heart rate, contractility and afterload).

It has become apparent that free oxygen radical generation and calcium overload contribute to the development of reperfusion injury (Hess and Manson, 1984; Ferrari, 1996). We and others have shown data that suggest that calcium antagonists protect the lipid fraction within the cell membrane against the toxic effects of free oxygen radicals (Koller and Bergmann, 1989; Kröner et al., 2002). Lipid peroxidation is considered a major mechanism of oxygen radical toxicity, thereby altering membrane permeability (Hess and Manson, 1984; Ambrosio et al., 1991). Calcium antagonists administered before ischemia may help maintaining NO homeostasis by preventing activation of eNOS and thereby preventing 'uncoupling'.

Angiotensin converting enzyme inhibitors and bradykinin. There have been numerous experimental (Kitakaze et al., 1995; Zahler et al., 1999; Korn et al., 2002) and clinical reports (GISSI-3 APPI study Group, 1996; Lazar et al, 1998) on the beneficial action of angiotensin converting enzyme (ACE) inhibitors in the setting of I/R. The concept behind the use of ACE inhibitors is well understood: ACE is also known as kininase II. This dual enzymatic position explains its dual action: on the one hand, the conversion of angiotensin I (AT I) to

Table 1 Additives to cardioplegic solutions influencing NO homeostasis (most relevant experimental and clinical studies)

Additives	Results	References
Radical scavengers	ECSOD type C is cardioprotective in isolated cold-arrested rat hearts; human chimeric SOD improves myocardial preservation in the rabbit heart	Sjöquist et al. (1991), Hatori et al. (1992), Nelson et al. (2002)
Calcium antagonists	Diltiazem protects lipid fraction within the cell membrane Against the toxic effects of free oxygen radicals Diltiazem reduces infarct size and protects against reperfusion injury	Koller and Bergmann (1989), Kröner et al. (2002) Tadokoro et al. (1996), Klein et al. (1984)
ACE inhibitors	Quinaprilat improves postischemic systolic and diastolic function, coronary perfusion and energy phosphates in the healthy rabbit and the failing rat heart undergoing I/R Captopril and TCV-116 reduce chronic allograft rejection	Korn et al. (2002), Podesser et al. (2002) Furukawa et al. (1996)
Bradykinin	Bradykinin preconditioning improves postischemic left ventricular and microvascular function	Feng et al. (2000, 2006)
Endothelin receptor antagonists	Bosentan preserves endothelial and cardiac contractile function during I/R in isolated rat and mouse hearts via a NO-mediated mechanism TBC-3214Na improves postischemic systolic and diastolic function, in the failing rat heart undergoing I/R	Gonon et al. (2004) Trescher et al. (2006)
Substrates and cofactors of eNOS	L-arginine cardioplegia supplementation reduces troponin T and CK-MB levels in CABG L-arginine reduces cytokine release and wedge pressure in CABG BH$_4$ is cardioprotective in cold rat heart preservation	Carrier et al. (2002), Kiziltepe et al. (2004) Colagrande et al. (2006) Yamashiro et al. (2003)
NO-releasing compounds	S-nitrosoglutathione monoethyl ester shows protective effects during cardioplegic arrest in isolated rat hearts S-NO-HSA improves functional and metabolic outcome after cold organ storage in the isolated rabbit heart (6 h) and in orthotopically transplanted pig hearts (4h)	Konorev et al. (1996) Semsroth et al. (2005) Gottardi et al. (2004)

Abbreviations: ACE, angiotensin converting enzyme; BH$_4$, (6R)-5,6,7,8-tetrahydrobiopterin; CABG, coronary artery bypass grafting; CK-MB, creatinine kinase-myocardial band; ECSOD, extracellular superoxide dismutase; eNOS, endothelial nitric oxide synthase; I/R, ischemia/reperfusion; NO, nitric oxide; TBC-3214Na, selective endothelin A-receptor blocker; TCV-116, selective angiotensin II-receptor blocker; S-NO-HSA, S-nitroso human serum albumin; SOD, superoxide dismutase.

angiotensin II is blocked, leading directly to vasorelaxation; on the other hand, the breakdown of bradykinin is blocked, leading to an increase in NO with consequent vasodilatation (Hartmann, 1995). The cardioprotective effect of ACE inhibitors could be abolished by the presence of icatibant (HOE 140), a specific bradykinin-B2 receptor antagonist (Linz et al., 1992).

Similarly, both captopril and lisinopril were able to enhance subthreshold preconditioning by augmenting bradykinin levels, an effect also eliminated by icatibant (Morris and Yellon, 1997). However, the ability of ACE inhibitors to confer protection in the absence of a preconditioning stimulus is more controversial (see Review from Baxter and Ebrahim, 2002). Experimental studies have used bradykinin preconditioning before ischemic arrest with cold crystalloid cardioplegia (Feng et al., 2005, 2006). In the cardiovascular system, the action of bradykinin is vasodilatation, mediated in several vascular beds by the release of NO and prostacyclin (Hatta et al., 1997; Wirth et al., 1997). Pretreating the heart with bradykinin before crystalloid cardioplegic ischemia significantly improved postischemic left ventricular and microvascular function, suggesting that pharmacological bradykinin may be an important addition to our current methods of myocardial and microvascular protection with hyperkalemic cardioplegia (Feng et al., 2000).

In two recent studies we have shown that preischemic ACE inhibition can significantly improve myocardial function in the healthy and in the failing heart, undergoing I/R compared to cardioplegia alone. The treated hearts showed an improvement in postischemic systolic and diastolic function, coronary perfusion as well as higher levels of high-energy phosphates (Korn et al., 2002; Podesser et al., 2002). The direct use of ACE inhibitors in the setting of heart transplantation is rare. Sasayama and co-workers report on the effect of captopril and a selective angiotensin II (AT II)-receptor blocker in a murine model of graft sclerosis. Both, the ACE inhibitor and the AT II receptor blocker were able to reduce chronic allograft rejection compared to controls (Furukawa et al., 1996).

Endothelin receptor antagonists. Another endothelium-derived substance is the potent vasoconstrictor endothelin 1 (ET-1) (Yanagisawa et al., 1988). ET-1 mediates vasoconstriction by binding to the endothelin type A and B receptors located on the smooth muscle cells. ET-1 may also mediate

NO-dependent vasodilatation by activation of the endothelin type B receptor located on the vascular endothelium (de Nucci *et al.*, 1988; Arai *et al.*, 1990; Sakurai *et al.*, 1990). NO not only counteracts the vasoconstrictor effect (Kourembanas *et al.*, 1991) but also inhibits the release of ET-1 from endothelial cells (Boulanger and Lüscher, 1990). The production and release of ET-1 is increased during ischemia and contributes to I/R-injury. Selective ET_A and mixed ET_A/ET_B receptor agonists have been shown to be cardioprotective in I/R. For instance, the dual ET_A/ET_B receptor agonist bosentan has been shown to preserve endothelial and cardiac contractile function during I/R in isolated rat and mouse hearts via a mechanism depending on endothelial NO production (Gonon *et al.*, 2004). Similarly, we have studied the selective ET_A-receptor blocker TBC1421 as an adjunct to blood cardioplegia in healthy and failing rat hearts in the setting of acute and chronic ischemia. Interestingly, cardiac function was preserved in both, the acute and the chronic treatment group, whereas preservation of coronary relaxation was seen only in the acute group (Trescher *et al.*, 2006). These additional cardioprotective effects of ET-receptor blockers are especially important, as chronic treatment with ET-receptor blockers is currently only used in the clinical situation of severe pulmonary hypertension.

Substrates and cofactors of eNOS

L-Arginine.   The most widely investigated additive to cardioplegia concerning NO homeostasis is L-arginine. The NOS substrate L-arginine has been reported to be effective as a supplement to cardioplegia in a dose range between 2 and 5 mmol/l in numerous experimental studies. The critical timing of L-arginine in cardioplegic arrest and reperfusion seems to be important. It has been shown in isolated working rat hearts that L-arginine given during initial reperfusion is deleterious to recovery of myocardial function. However, given before cardioplegic arrest, it exerts its beneficial effects (Engelman *et al.*, 1996). Furthermore, different effects of L-arginine after cardioplegic arrest under moderate and deep hypothermia in isolated working rat hearts have been reported (Amrani *et al.*, 1997). In this study, L-arginine as an additive to St Thomas' No 1 cardioplegic solution was effective only after moderate hypothermic ischemia (1 h; 20°C) and not after deep hypothermic ischemia (4 h; 4°C). Temperature therefore seems to be another important factor. The different approaches include supplementation of L-arginine during warm blood cardioplegia (Hayashida *et al.*, 2000), supplementation of L-arginine directly to different cardioplegic solutions before short- (Amrani *et al.*, 1997) and long-term ischemia in isolated working rat hearts (Desrois *et al.*, 2000), or in a heterotopic rat heart transplantation model (Caus *et al.*, 2003). In a recent paper on cardiac allograft preservation in an orthotropic cardiac transplant pig model using intermittent perfusion of donor-shed blood supplemented with L-arginine the question of the dose is addressed. The low dose group (L-arginine: 2.5 mmol/l) in this study showed a greater ability to wean-off cardiopulmonary bypass had an improved recovery of left ventricular function and a decreased release of inflammatory cytokines compared to control. In contrast, the high dose group (L-arginine: 5.0 mmol/l) resulted in severe endothelial injury in terms of uniform ischemic contracture and no recovery of ventricular function after 5 h of global ischemia (Ramzy *et al.*, 2005).

Recently, the first clinical studies about the use of L-arginine in addition to cardioplegia demonstrated efficacy in reducing postoperative troponin T and creatinine kinase-myocardial band levels in coronary bypass graft operations (Carrier *et al.*, 2002; Kiziltepe *et al.*, 2004). A more recent study also showed reduced cytokine release (significant for interleukin 6) and myocardial damage (troponin T) in coronary artery bypass patients due to L-arginine cardioplegia supplementation. Wedge pressure as a clinical surrogate for ventricular volume load and intensive care unit stay was also significantly reduced in the treatment group (Colagrande *et al.*, 2006). All these studies have in common that high doses of L-arginine added to the first 500 ml solution, that is, 7.5 g L-arginine in 500 ml of cardioplegia (Carrier *et al.*, 2002; Colagrande *et al.*, 2006) were used with best results when warm (33°C) cardioplegia was infused. Lower doses are safe but inefficient (Carrier *et al.*, 1998). According to the results of experimental work, all the above clinical approaches were preformed by addition of L-arginine to cardioplegia before reperfusion.

Cofactors of eNOS. BH_4 is an absolute cofactor required for NO synthase and is thus a critical determinant of NO production. Activation of purified eNOS in the presence of suboptimal levels of BH_4 results in uncoupling. The concomitant addition of L-arginine and BH_4 abolishes O_2^- generation by eNOS (Vasquez-Vivar *et al.*, 1998).

Furthermore, in human aortic endothelial cells exposed to prolonged stretching, inhibition of BH_4 synthesis has been shown to stimulate O_2^- production (Hishikawa and Lüscher, 1997). Effects of BH_4 (20 μmol/l) supplementation have been studied in an *in vitro* human ventricular heart cell model of simulated I/R cellular injury, as assessed by means of trypan blue uptake, was significantly prevented (Verma *et al.*, 2002). Very recently, cardioprotective effects of BH_4 in cold heart preservation (4°C) have been shown by Yamashiro *et al.* (2003). BH_4 improved the contractile and metabolic abnormalities in the reperfused cold preserved rat hearts that were subjected to normothermic ischemia. Additionally BH_4 significantly alleviated ischemic contracture during ischemia and restored the perfusate levels of nitrite plus nitrate.

The cardioprotective effect of BH_4 implies that BH_4 could be a novel therapeutic option. Although not discussed by the authors it is known that BH_4 is a very unstable compound, which is rapidly oxidized to dihydrobiopterin. Therefore, the compound in solution is presumably dihydrobiopterin, which is intracellulary converted to BH_4 by dihydrobiopterin reductase with NADPH. In part, this possibility is discussed by Hasegawa *et al.* (2005).

NO-releasing compounds.   In models of myocardial I/R injury, damage can also be reduced by addition of exogenous NO either as authentic NO gas (Johnson *et al.*, 1991) or NO-donating compounds (Siegfried *et al.*, 1992; Bilinska *et al.*, 1996). Similarly, in the setting of organ transplantation, improved organ function has been observed when exogen-

ous NO-donating agents have been included in the hypothermic storage solution in a heart model (Pinsky et al., 1994). Exogenous NO exhibits a negative feedback on the production of NO by eNOS (product inhibition of the enzyme; Griscavage et al., 1995) and thereby can prevent uncoupling of the enzyme in I/R. NO donors used in storage solutions to date have been glyceryl trinitrate (Bhabra et al., 1997), sodium nitroprusside (SNP) (Yamashita et al., 1996) and diazeniumdiolates (Du et al., 1998). In the NO donor SNP, a molecule of NO is coordinated to iron metal forming the square bipyramidal complex with five cyanide anions. Interactions of SNP with reducing agents lead to the formation of NO. In the vascular system, NO release by SNP requires the presence of vascular tissue (Bates et al., 1991; Marks et al., 1995). NO formation from SNP is accompanied by cyanide release (Bates et al., 1991), which can be toxic to the vascular cells. It has also been reported that SNP could be involved in the formation of O_2^- anions and in consequent peroxynitrite. Due to these toxic effects of SNP, different metallonitrosyl complexes have recently been developed as NO delivery agents such as nitrosyl ruthenium complexes (Wang et al., 2000; Sauaia et al., 2003; Bonventura et al., 2004; Oliveira et al., 2004). These agents have not been used as drug additives to cardioplegic solutions until now.

A new class of compounds with special features is S-nitrosothiols. Low molecular weight S-nitrosothiols such as S-nitrosoglutathione and S-nitrosocysteine have a short half-life in a range of a few seconds, whereas high molecular weight S-nitrosothiols such as S-nitrosoalbumin show a prolonged half-life between 15–20 min in vivo (Hallström et al., 2002). It has been shown that (a) S-nitroso-N-acetyl-DL-penicillamine induces late preconditioning against myocar-

dial stunning and infarction in conscious rabbits (Takano et al., 1998), (b) S-nitrosoglutathione monoethyl ester has protective effects in isolated rat hearts during cardioplegic ischemic arrest (Konorev et al., 1996), (c) S-nitrosoglutathione inhibits platelet activity during coronary angioplasty (Langford et al., 1994) and suppresses increased NOS activity after canine cardiopulmonary bypass (Mayers et al., 1999). In addition, studies have shown that synthetic compounds such as furoxans require thiols for their thiol-mediated generation of NO (Feelisch et al., 1992).

Concerning NO homeostasis and cardioplegia, we are currently working with a new NO-Donor, S-nitroso human serum albumin (S-NO-HSA). This special high molecular weight S-nitrosothiol has an exact equimolar S-nitrosation and high S-nitrosograde due to a defined preprocessing (Hallström et al., 2002). In an experimental setting of 2 h of hindlimb ischemia and reperfusion, Hallström et al. (2002) could show that a supplementation of NO with S-NO-HSA before ischemia or before reperfusion preserves the function of eNOS, stabilizes the basal production of NO, decreases production of reactive oxygen species, and therefore has beneficial effects in reduction of I/R injury. In a second study, simulating clinical cold organ procurement and storage, hearts were reperfused after 6 h of ischemia on an isolated, erythrocyte-perfused working heart model. We could not only show a statistical significant beneficial hemodynamic and metabolic effect of S-NO-HSA compared to control but also compared to supplementation with L-arginine (Semsroth et al., 2005). In Figure 2, we have included data from these experiments. In a third group of experiments, orthotopically transplanted pig hearts underwent 4 h of ischemia followed by 3 h of reperfusion. We observed
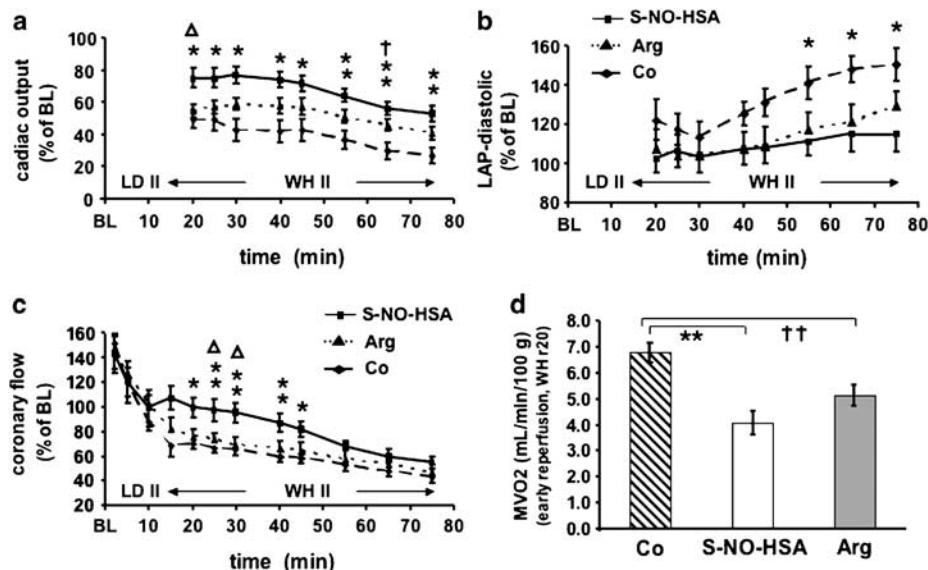

Figure 2 S-nitroso human serum albumin (S-NO-HSA) attenuates ischemia/reperfusion injury after prolonged cardioplegic arrest in isolated rabbit hearts: in an isolated erythrocyte-perfused rabbit working heart model after assessment of hemodynamic baseline values hearts were randomly assigned to receive S-NO-HSA (0.2 μmol/100ml, n = 8), L-arginine (10 mmol/100 ml, n = 8) or albumin (control; 0.2 μmol/100 ml, n = 8). After 20 min of infusion, the hearts were arrested and stored in Celsior solution (4°C) enriched with respective drugs for 6 h, followed by 75 min of reperfusion. Postischemic recovery of hemodynamic parameters are shown. (a) Time course of cardiac output; *P<0.05, **P<0.01 S-NO-HSA versus control. ΔP<0.05 S-NO-HSA versus L-arginine; †P<0.05 L-arginine versus control. (b) Time course of coronary flow; *P<0.05, **P<0.01 S-NO-HSA versus control. ΔP<0.05 S-NO-HSA versus L-arginine. (c) Time course of left atrial pressure; *P<0.05 S-NO-HSA versus control. (d) Myocardial oxygen consumption; **P<0.01 S-NO-HSA versus control. ††P<0.01 L-arginine versus control. (This figure is from Semsroth et al., 2005: J Heart Lung Transplant 24: 2226–2234© Elsevier Publishing Group with permission.)

beneficial hemodynamic and metabolic effects of S-NO-HSA compared to control (Dworschak *et al.*, 2004; Gottardi *et al.*, 2004).

Discussion and outlook

Still the supplementation of NO is controversial since there are studies that report negative effects. This might be caused by either different experimental settings, differences in NO donors or different methods of supplementation. In a review, Bolli (2001) concludes that endogenous and exogenous NO is beneficial in protecting the unstressed heart, not preconditioned against damage occurring during I/R. NO plays a critical bifunctional role in late preconditioning, a preconditioning phase that appears 12–24 h after an ischemic stimulus and persists for 72 h. In the latter situation, enhanced production of NO by eNOS is essential to trigger enhanced production of NO by iNOS that is required to mediate the anti-stunning and anti-infarct actions of late preconditioning.

As noted by Vasquez-Vivar *et al.* (1998) and Huk *et al.* (1997), the production of NO depends on sufficient supply of its substrate L-arginine and cofactors as BH_4. A relative deficiency of L-arginine leads to an 'uncoupling' of NOS activity with the production of O_2^- anion instead of NO. It is important to note that if iNOS is expressed *in vivo* (for example, in sepsis), being independent of calcium and calmodulin, then there is a much higher production of NO. Therefore, iNOS is even more predisposed to deplete substrates and cofactors and to predominantly produce O_2^-. This circumstance might also explain the deleterious effects of iNOS induction in many experimental settings as for example in a model of transgenic mice transfected with iNOS under the control of a cardiac-specific promotor leading to cardiomyopathy, bradyarrhythmia and sudden cardiac death (Mungrue *et al.*, 2002). Therefore, preservation of NO production during organ storage by either supplementation of L-arginine as the physiologic substrate of eNOS or supplementation of NO via the use of a NO-donor may be beneficial for the subsequent heart transplantation.

In the same review as mentioned above, Bolli (2001) examined the role of NO in modulating the severity of I/R injury. Seventy-three percent of the reviewed studies showed that NO either endogenous or exogenous exert a beneficial effect on myocardial protection against infarction or stunning. Caus *et al.* (2003) have shown in a heterotopic heart transplant model in the rat with 3 h of ischemia that adding L-arginine to their storage solution had a highly significant beneficial effect on graft function after early reperfusion (1 h after aortic declamping).

Similarly, Schwarzacher *et al.* (1997) reported that in an experimental model of balloon angioplasty, administration of L-arginine at the site of previous vascular damage resulted in a reduction of endothelial dysfunction and improvement of NO generation. This promising study was performed before the era of drug-eluting stents (DES). The possible clinical implications of simultaneous cardioprotective drug delivery and balloon angioplasty and stenting have been overruled by the development of DES. However, with the current critical discussion on DES and the possible risk of late stent thrombosis (Pfisterer *et al.*, 2006), the approach with simultaneous cardioprotective drug delivery and balloon angioplasty could see an experimental and clinical revival.

Reduced NO bioavailability not only has effects on the endothelium but also on the sarcolemmal membrane of the cardiac myocytes. Xu *et al.* (2003) could show that sarcolemmal-associated NOS isoforms, nNOS and eNOS, may serve to modulate oxidative stress during ischemia in cardiac muscle and thereby regulate the function of key membrane enzymes including $(Na^+ + K^+)$-ATPase with a resulting prevention of calcium overload. Pretreatment with a NO-donor NOC-7 (1-hydroxy-2-oxo-3-(*N*-3-methyl-amino-propyl)-3-methyl-1-triazene) markedly protected both, sarcolemmal NOS isoforms as well as the function of the $(Na^+ + K^+)$-ATPase during ischemia. The protection was also facilitated by the radical scavenging properties of NO released by NOC-7.

In summary, protection of the endothelium and maintenance of NO homeostasis has been recognized as important concept for myocardial protection and is currently evaluated experimentally and clinically. This review has focused on possible drug additives to cardioplegia, which can influence the impairment of NO bioavailability.

Acknowledgements

Conflict of interest

The authors state no conflict of interest.

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