Helium-induced cardioprotection: in sickness and in health, for better or for worse?
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Citation for published version (APA):
Oei, G. T. M. L. (2013). Helium-induced cardioprotection: in sickness and in health, for better or for worse?

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General introduction and outline of this thesis

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GENERAL INTRODUCTION

According to the World Health Organization (WHO; www.who.org) an estimated 17.3 million people died in 2008 from cardiovascular disease - about 30% of all deaths – and this number will increase to an estimated 23.3 million people per year in 2030. Of these deaths, an estimated 7.3 million were attributed to coronary heart disease. Coronary heart disease is a disease of the blood vessels supplying the heart. The severity and extension of this disease is highly influenced by co-existing risk factors such as hypertension, obesity, hypercholesterolemia, hyperglycemia, insulin resistance, atherosclerosis and heart failure, but also by sex and age of the patient.

In coronary artery disease, vascular injury, lipid deposition, thrombus formation and inflammation eventually lead to a partial or total obstruction of a coronary artery, resulting in ischemia of the downstream myocardial tissue. As myocardial ischemia is reflected by an imbalance between oxygen supply and demand, reperfusion of threatened tissue is pivotal. Reperfusion is done by fibrinolytics or percutaneous coronary intervention (PCI), but restoration of blood flow to compromised tissue is also performed by coronary artery bypass graft surgery (CABG).

Time course of reperfusion and windows of protection

In general, the time to intervention (reperfusion) after ischemia is of vital importance to the amount of myocardium that can be salvaged. Already in 1977 it was shown that cellular damage after an ischemic episode develops according to a wave front pattern, and timely reperfusion is essential. However, while ischemia itself causes substantial tissue damage, reperfusion also contributes to the final size of the infarcted area, a phenomenon called: “reperfusion injury”. In an experimental study in mice undergoing regional ischemia it was shown that both duration of ischemia as well as reperfusion contributed to the final extent of infarct size. In this study it was demonstrated that extension of index ischemia resulted in larger infarct sizes; mice exposed to 120 minutes of reperfusion had an infarct size of 18% after 30 minutes of ischemia, which was increased to 69% after 60 minutes of ischemia. Simultaneously, an increase of reperfusion time after an equal index ischemia extended the infarcted area. To illustrate: after 30 minutes of ischemia, infarct size increased from 18% to 69% when reperfusion time was prolonged from 120 to 240 minutes. Similarly, an increase of reperfusion time from 120 to 240 minutes increased infarct size from 40% to 72% after 45 minutes of ischemia.

Taken together, the final extent of the infarct size thus consists out of ischemic cell damage and reperfusion injury, as shown in Figure 1. Nonetheless, the very existence
of reperfusion injury has been debated vigorously as some argued that reperfusion only hastened the initial injury after ischemia and others considered it as a contributor to de novo injury\(^6,5\).

**Ischemic pre- and postconditioning**

Regardless of its definition, the amount of research conducted to attenuate or even eradicate ischemia/reperfusion injury is vast. In 1986, Murry and colleagues first presented the preconditioning phenomenon\(^6\). In dogs exposed to 40 minutes of coronary artery occlusion and 4 days of reperfusion it was shown that precedence of index ischemia by 4

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**Figure 1.** In the upper graph, the extent of cell death is depicted on the y-axis and time is depicted on the x-axis. Underneath the graph an overview of the different conditioning protocols is shown for each category: ischemic and anesthetic conditioning. I-EPC = ischemic early preconditioning, I-LPC = ischemic late preconditioning, I-PostC = ischemic postconditioning, A = anesthetic.
cycles of 5 min of coronary occlusion interspersed with 5 min of reperfusion significantly reduced infarct size from 29% in controls to 7% in treated animals without a difference in collateral blood flow. Moreover, they also showed that once the index ischemia was prolonged, 3 hours instead of 40 minutes, preconditioning could not exert protection anymore. Apparently, cardioprotective effects of preconditioning are abrogated or cannot be induced when the index ischemia is too long, i.e. the initial damage is too big. These findings emphasize the importance of timely reperfusion.

According to the original ‘conditioning’ experiments in animal behavior, preconditioning of the heart is based on the idea that a ‘memory’ for ischemic circumstances is created, thereby rendering increased tolerance to future prolonged ischemic episodes. Preconditioning-induced cardioprotection is therefore not to be confused with cardioprotective ‘treatments’. An example is the use of ACE-inhibitors in patients with hypertension and myocardial infarction; ACE-inhibitors have to be taken on a chronic, daily basis and lower the blood pressure but also protect the heart against ischemia/reperfusion injury. The difference between conditioning and a treatment is that discontinuation of the treatment (i.e. one stops taking the pills) results in a loss of the effects (the blood pressure rises, the protection is abrogated). In preconditioning this is typically not the case; preconditioning results in a rapid development of the protective state, and lasts for 1-2 hours (early preconditioning, EPC). Preconditioning also has a late phase (late preconditioning, LPC), in which protection does not start until 24 hours after the initial stimulus, and lasts for approximately 72 hours.

The preconditioning stimulus is thus biphasic and applied before the index ischemia. Although preconditioning proved to be a very effective method to reduce infarct size, its clinical applicability is limited as occurrence of an ischemic episode is not predictable in many clinical situations. Postconditioning seemed to solve this problem; it was protective in dogs exposed to 60 min of ischemia and 3 hours of reperfusion. In this study, application of 3 cycles of 30 seconds of reperfusion interspersed by 30 second cycles of reocclusion at the onset of reperfusion reduced infarct size from 25% in controls to 14% in treated animals. Infarct size in the postconditioning group was moreover comparable to the preconditioning group, showing the great potential of the postconditioning stimulus. This study first of all showed the great effectiveness of postconditioning, but secondly proved the very existence of reperfusion injury: when a treatment administered at reperfusion successfully lowers infarct size, some kind of damage should occur in this time window.

Nevertheless, successful application of postconditioning strongly depends on the duration of the postconditioning cycles and the time point of administration. In the first experiments that were done to investigate the concept of postconditioning, five-minute cycles of ischemia followed by reperfusion were used, comparable to the first
preconditioning protocols. To the investigator’s surprise this did not work and it took another 11 years before it was shown that short cycles of 30 seconds were effective in infarct size reduction. The first minute of reperfusion proved to be very critical as well, as delay of the first occlusion in the postconditioning protocol until one minute after onset of reperfusion abrogated cardioprotection in comparison to the application of the first stimulus at 30 seconds after the onset of reperfusion. Apparently, critical events take place during the first minute(s) of reperfusion.

Other ways of “conditioning”-induced cardioprotection

Conditioning agents are numerous and comprise a wide array of stimuli. A protective effect of a potential detrimental stimulus was first described when the phenomenon of ischemic preconditioning was discovered. Since then, other potential dangerous circumstances were used as conditioning stimuli; short episodes of those circumstances, such as heat-stress, hypothermia, lipopolysaccharide exposure and hypoxia exerted protective effects. They can therefore be categorized as “stress-induced cardioprotection”.

“Anesthetic conditioning” refers to the use of volatile anesthetics as conditioning agents, a technique investigated since the 1990s. The volatile anesthetics such as sevoflurane induce preconditioning and postconditioning. Additionally, the anesthetic noble gas xenon also induces pre- and postconditioning. The non-anesthetic noble gas helium is chemically similar to xenon as they both belong to the family of noble gases. In 2007 it was first shown that helium gas is able to induce preconditioning. The advantages of helium are numerous: (1) it is much cheaper than xenon and readily available, (2) it does not have cardiovascular effects, (3) it has beneficial physical properties such as low density and viscosity, (4) it does not induce anesthesia and (5) it can therefore be used in various clinical settings. This makes it a perfect candidate for use in the clinical setting as a conditioning agent.

The mechanisms underlying anesthetic preconditioning have been extensively investigated. Generally said, the preconditioning stimulus triggers a cascade of events, starting with alteration of multiple protein kinases, and signal transduction through G-protein coupled receptors, eventually leading to a set of end-effectors, among which are sarcolemmal and mitochondrial $K_{ATP}$ channels and the mitochondrial permeability transition pore. Eventually, all pathways and its end-effectors result in cell death or survival. For an overview of ischemic, and anesthetic conditioning protocols in relation to ischemia/reperfusion injury, also see Figure 1.
CHAPTER 1

Cellular changes during reperfusion

At the onset of reperfusion, many cellular, molecular and metabolic processes take place simultaneously, which result directly from the events that take place during an ischemic episode. Ischemia results in depletion of ATP and high-energy phosphates, which cease aerobic metabolism. Consequently, the cell is bound to switch to anaerobic glycolysis. The concomitant decrease of pH will deactivate troponin C and cause Ca\(^{2+}\) overload. Simultaneously, activation of intracellular proteases, e.g. calpain results in hypercontracture, necrosis and activation of apoptotic cascades as well as opening of the mitochondrial permeability transition pore. Prompt return of blood flow immediately increases oxygen levels and restores substrates essential to generation of ATP. While this situation is beneficial for cell survival, the quick reestablishment of normo-pH abolishes the low-pH induced inhibition of mitochondrial permeability transition, hypercontracture and calpain activity, resulting in cell damage. The recovery of pH also leads to changes in membrane ion transportation, resulting in Ca\(^{2+}\) overload. Furthermore, the sudden recovery of aerobic metabolism by activation of xanthine oxidase induces a reactive oxygen species (ROS) burst. ROS induce damage in proteins, DNA and cellular structures such as organelles, and evoke an inflammatory response.

The interplay of the inflammatory response with the endothelium and the effects on the vascular bed play an important role during reperfusion. Shortly after the onset of reperfusion, the cardiac release of cytokines activates and recruits neutrophils. Neutrophils in turn produce ROS, proteases and cytokines, which damage the endothelium. The damaged endothelium now increases its production of ROS, cytokines and adhesion molecules, which further enhances endothelial dysfunction: intercellular tight junctions are compromised and lead to increased vascular permeability, facilitating leukocyte influx. Once migrated to tissue, leukocytes will produce more ROS and proteases, leading to oxidative stress and degradation of the extracellular matrix.

The endothelium

The endothelium is ‘at rest’ under normal circumstances and serves dilator, anti-platelet and anti-neutrophil functions. Ischemia/reperfusion poses a stress signal for the endothelium and causes its activation. Activated or dysfunctional endothelium is characterized by a pro-thrombotic and pro-inflammatory state as it increases the adhesion of neutrophils and platelets. Ischemia/reperfusion is also associated with a decrease in availability of endothelial mediators that are associated with vasodilation. Prolonged vasoconstriction after reperfusion results in the amplification of expression of adhesion molecules and production of inflammatory cytokines, both of which account
for the recruitment of inflammatory cells. These inflammatory cells in turn produce more cytokines, further stimulating inflammation and tissue damage.\(^{27}\)

The association of endothelial dysfunction with cardiovascular disease is well known. Endothelial dysfunction in cardiovascular disease is associated with 2 hallmarks: (i) diminished nitric oxide production from vascular endothelium, resulting in prolonged vasoconstriction, (ii) the systemic character; endothelial dysfunction can be measured in different vascular beds and cardiovascular events may occur distant from the diseased endothelial bed.\(^{28}\)

**Conditioning of the diseased heart**

One of the challenges we face in the translation of experimental data of myocardial conditioning to clinical practice is the presence of co-existing disease in the target patient group. Whereas most conditioning techniques were first investigated in the laboratory in healthy animals with isolated myocardial infarction, development and extension of cardiovascular disease in humans depends on co-existing risk factors. Chronic diseases such as hypertension, obesity, hypercholesterolemia, hyperglycemia, insulin resistance, atherosclerosis and heart failure are important in the development and severity of coronary artery disease. These risk factors alter the possibilities to protect the heart by pre- and postconditioning. In addition, sex and aging might have an influence on the protective strategies.\(^{31}\)

Chronic hypertension leads to left ventricular hypertrophy and is an independent risk factor for cardiovascular heart disease.\(^{32}\) In hypertrophic myocardium cell signaling is altered, making the heart more susceptible to ischemic injury, possibly by abrogating the cardioprotective effects of conditioning. Ischemic postconditioning was indeed abrogated in spontaneously hypertensive rats (SHR).\(^{34}\) Alteration of cell signaling seems to be one underlying cause of the abrogated protection in diabetic hearts.\(^{35}\) New therapies to render the diabetic heart more susceptible to cardiac conditioning are therefore targeted on protein kinases that are altered by the presence of diabetes and are supposed to be involved in the conditioning-induced survival pathways.\(^{35,36}\)

Another way to protect the diseased myocardium is the administration of multiple protective stimuli, as a combination of separate protocols might induce larger protection together than they do separately. The combination of ischemic late preconditioning with early preconditioning reduced infarct size in rabbits in a stronger fashion than each stimulus alone.\(^{37,38}\) A stronger reduction of infarct size was also found after the combination of ischemic late preconditioning with sevoflurane-induced early preconditioning.\(^{38}\) As the threshold of conditioning seems to be higher in diseased subjects, this approach might be a solution for patients with comorbidities such as
hypertension and diabetes.

AIMS OF THIS THESIS

The aim of this thesis was to investigate new possible conditioning agents characterized by the ability to reduce infarct size. Our first aim was to investigate whether hypoxia could induce preconditioning in vivo. Due to the limited applicability of hypoxic gas mixtures in clinical practice, we thereafter focused on helium gas as a potential cardioprotective agent. From literature it was already known that the anesthetic noble gas xenon induces preconditioning and postconditioning. We hypothesized that the non-anesthetic noble gas helium can also induce cardioprotection. Therefore, the second aim of this thesis was to find out the correct duration and time point of administration of helium as a conditioning agent, and the underlying mechanisms of cardioprotection. As comorbidity is a huge compromising factor in patients, the third aim of this thesis was to search for ways to protect the diseased myocardium against ischemia/reperfusion injury by the use of helium conditioning.

OUTLINE OF THIS THESIS

Part I (chapters 1-2) is the main introduction of this thesis and comprises chapter 1 with the general introduction and chapter 2 with an introduction to the primary conditioning agent that was investigated in this manuscript: helium gas.

In Chapter 2 the main characteristics of helium gas are described. The biological effects of helium gas on different organs are explained as well as its effects on different molecular pathways.

Part II (chapter 3-7) of this thesis completely consists of animal studies and is split in 2 sections; section A (chapter 3-5) has been conducted in healthy animals and section B (chapter 6-7) in diseased animals.

In Chapter 3 we investigate whether hypoxia can induce cardioprotection. Three different oxygen concentrations are investigated and compared to anesthetic induced
preconditioning by sevoflurane. Additionally we investigate the difference between continuous and short administration of sevoflurane. This chapter basically emphasizes on the intensity and duration of a preconditioning stimulus and the correct timing.

In **Chapter 4**, three different helium postconditioning protocols are investigated in order to determine the correct duration of the stimulus. This study was also designed to investigate the involvement of the innate immune response in the myocardium during ischemia and early reperfusion, as it studies the influence of helium postconditioning on the magnitude of the hyper acute cytokine burst during early reperfusion.

In **Chapter 5** infarct size measurement and histology is used to investigate the extent of cardiomyocyte damage after ischemia/reperfusion. By use of a PCR array of cell death pathways, it is investigated which cell death- and cell survival pathways are triggered after helium postconditioning, focusing on necrosis, apoptosis and autophagy.

Using diabetic and hypertensive rats, **chapters 6 and 7** focus on the differences between ‘healthy’ and ‘diseased’ myocardium. These studies have been designed to investigate differences in cell signaling between healthy and diseased myocardium and the possibilities of protecting the diseased heart against ischemia/reperfusion injury by conditioning.

In **Chapter 6** we investigate whether the diabetic Zucker rat can be protected against ischemia/reperfusion injury by helium-induced pre- and postconditioning. Moreover, we study the involvement of the pro-survival kinases of the reperfusion injury signaling kinases (RISK)-pathway by analyzing extracellular signal-regulated kinases 1/2 (ERK1/2) and Akt/Protein kinase B in helium induced preconditioning. To assess whether attenuated disruption of mitochondrial function plays a role in helium-induced conditioning, we investigate phosphorylation of glycogen synthase kinase-3beta (GSK-3beta) and mitochondrial uncoupling.

In **Chapter 7** we evaluate the use of multiple helium conditioning stimuli to protect the hypertensive myocardium. Additionally we investigate the roles of GSK-3beta and protein kinase C- epsilon (PKC-epsilon) in helium-induced conditioning.

**Part III (chapter 8-9)** of this thesis has been conducted in healthy volunteers.

**Chapter 8** focuses on possible endothelial protection by helium conditioning in a model of forearm ischemia/reperfusion. Forearm endothelial function is measured as
the vasodilatory response to acetylcholine and nitroprusside. As endothelium exposed to damage becomes activated and secretes markers in the circulation, plasma levels of cytokines, adhesion molecules and microparticles were also investigated. In Chapter 9 the safety of helium regarding the immune response is investigated. This study was designed to rule out the possibility that helium inhalation depresses the capacity of the innate immune system to respond to pathogens.
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