Helium-induced cardioprotection: in sickness and in health, for better or for worse?

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CHAPTER

General discussion

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Conditioning of the heart by helium

In rats, fifteen minutes of helium postconditioning (PostC) reduced myocardial infarct size in comparison to ischemia/reperfusion, as we showed in this thesis in chapter 4, 5, 6 and 7. These findings are in line with results from studies done in our and other laboratories, showing that helium induces early preconditioning (EPC) in rabbits and rats\textsuperscript{1-7}. In all of these studies it was demonstrated that application of short cycles of helium before the index ischemia reduces infarct size. Experiments with sevoflurane-induced preconditioning (PC) in guinea pigs showed that 2 cycles of 5 minutes of 1.4 vol% sevoflurane decreased infarct size to a greater extend than 1 cycle of 15 minutes of 2.8 vol% sevoflurane\textsuperscript{8}.

These data show that the way the conditioning protocol is carried out is quite important. While PC has been induced mostly by repetitive short cycles, this is different for PostC. Sevoflurane-induced PostC in rats was achieved by both a short episode of inhalation for 2 minutes\textsuperscript{9} but also after prolonged administration of 15 minutes\textsuperscript{10}. In this thesis, the 15-minute helium PostC protocol proved to be effective. On the contrary, prolonged administration of helium during reperfusion did not induce protection. This is very different from the expectations one could have from a pharmacological point of view; it sounds logic that a stronger protection arises after continued inhalation of a protective substance. With helium PostC this was not the case, as infarct sizes returned to control levels after 30 and 60 minutes of helium PostC (chapter 4). A similar observation was done in chapter 3 with sevoflurane. While 3 five-minute cycles of sevoflurane reduced infarct size, continuous administration with sevoflurane did not exert myocardial protection.

As prolonged administration of helium did not result in enlarged protection, it was hypothesized that multiple stimuli might still be protective. Pagel and colleagues\textsuperscript{2} earlier investigated the application of 1, 3 and 5 five-minute cycles of helium prior to index ischemia, and found that infarct sizes were reduced from 44% in control to 35%, 25% and 20% respectively. These findings were confirmed by the same group in another study\textsuperscript{6}. Apparently, more cycles of PC increase the level of protection. This phenomenon was applied in the Zucker rat in chapter 6; both 3 and 6 five-minute cycles were used to protect the diabetic heart, unfortunately without success. It seems that simply repeating ‘parts’ of one type of conditioning is not strong enough to convey protection in the diseased heart.

A combination of PC and PostC resulted in increased protection in rats and rabbits\textsuperscript{9,11,12}. In rats it was shown that sevoflurane EPC combined with sevoflurane PostC reduced infarct size in a stronger way than sevoflurane EPC alone\textsuperscript{9}. In this study, sevoflurane-induced EPC was applied by 2 five-minute cycles of sevoflurane with a
10-minute washout before ischemia; sevoflurane-induced PostC was executed by a two-minute episode at the onset of reperfusion\(^9\). We hypothesized that a combination of different conditioning protocols would therefore be needed to induce infarct size reduction in the hypertensive myocardium in chapter 7. We combined helium PostC with helium LPC and also applied a triple intervention by the combination of LPC and EPC plus PostC. In healthy animals this did not enhance protection in comparison to PostC alone, but all interventions did reduce infarct size in comparison to control. In the hypertensive animals, only the triple intervention reduced infarct size, suggesting an increased threshold to protection in the diseased myocardium.

The percentage of the helium gas administered in the studies of this thesis also deserves some attention; 70% in the animal studies versus 79% in the clinical studies. From a clinical point of view, 79% helium in a mixture with 21% oxygen is the maximum amount of helium that should be administered, unless it is desirable to administer a hypoxic gas mixture. As it is often preferential to increase the administered level of oxygen to patients somewhat above 21%, the 70% helium percentage use in the animals seems to be justified. There is only one argument against the use of 70% helium and it is of pharmacologic nature. One could argue that a more pronounced effect of helium conditioning could be found when a stronger dose is used.

**Mechanisms of helium-induced conditioning**

The signaling pathways that are supposed to be involved in helium-induced EPC are set out in chapter 2. Simplified, the so-called survival kinases and mitochondria were suggested as major players. Survival kinases are proteins in the cell that function as switches; they can switch on and off and thereby influence the next protein in the pathway. By posttranslational modification such as phosphorylation they turn to the active or passive state and subsequently influence another protein. A whole set of signaling kinases ultimately affects an end target, which could be any organelle or receptor within a cell. An example of an end target that determines whether cell survival or death takes place, are the mitochondria.

By use of blockers directed against specific kinases, phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), 70-kDa ribosomal protein s6 kinase (p70s6K) and glycogen synthase kinase-3beta (GSK-3beta) were found to be involved in helium EPC\(^1,2\). Beside these kinases, three mitochondrial channels were also investigated and found to be involved in helium EPC; the mitochondrial permeability transition pore (mPTP)\(^2\), the mitochondrial adenosine triphosphate-regulated potassium channel (mK\(_{\text{ATP}}\))\(^3\) and the mitochondrial calcium-sensitive potassium channel (mK\(_{\text{Ca}}\))\(^7\). Stress-induced formation of the mPTP in particular is thought to play an important role
in helium conditioning. Cardioprotective effects of helium EPC were abrogated after simultaneous maintenance of a metabolic alkalosis during reperfusion, a condition that causes opening of the mPTP. Protection was restored again after co-administration of the mPTP formation inhibitor cyclosporin A.

In this thesis, both the involvement of pro-survival kinases and mitochondria were investigated. In chapter 6 we investigated phosphorylation of Akt, ERK1/2, and GSK-3beta, all proteins that belong to the group of survival kinases and of which ERK1/2 and GSK-3beta were found to be involved in helium-induced early preconditioning. In chapter 6 we demonstrated that Akt and ERK1/2 were not involved in helium EPC, as phosphorylation levels were not higher in comparison to control animals. Nevertheless, a reduced phosphorylation of GSK-3beta was found during the index ischemia in animals that were exposed to helium EPC. In contrast, in chapter 7 we found no differences in GSK-3beta phosphorylation level after helium PostC in comparison to control.

Taken together it seems that involvement of the above-mentioned survival kinases have only been shown in experiments that were performed with non-specific blockers. The more specific investigations on a biochemical level of these proteins could not confirm these data. In chapter 6 it is proposed that helium EPC causes mild mitochondrial uncoupling during the index ischemia, a state that prevents mPTP opening. This was shown by measurement of different respiratory states of the isolated mitochondria and calculated as the respiratory control index.

Additional to the investigations on the effect of helium conditioning on mitochondria and survival kinases, we also investigated other mechanisms of helium conditioning. In chapter 4 we looked at the effect of helium postconditioning on the protein- and mRNA levels of inflammatory cytokines in the myocardium. Both the myocardium at risk -i.e. downstream of the ligated coronary artery- and adjacent healthy tissue were investigated, but in neither type of tissue we found signs of involvement of innate immunity in helium conditioning. This implies that helium conditioning does not exert its protective effects by attenuating the hyper acute cytokine burst. Instead, we suggest in chapter 5 that helium PostC affects a whole set of genes that are involved in cell death and cell survival, all related to necrosis, apoptosis and/or autophagy. We show that helium conditioning induces a relative up-regulation of multiple genes that are employed in autophagy and against apoptosis. It is possible that a short episode of helium inhalation during reperfusion results in a higher level of autophagy, which enables cells to get rid of damaged proteins and organelles that accumulate during ischemia and the first minutes of reperfusion. For cellular survival it is of vital importance that these products can be disposed outside of the cell. The combined stimulation of autophagic cell processes and anti-apoptotic pathways could have resulted in the reduction of cell damage that has been observed in all studies of this thesis.
Limitations of experimental models and translation to the clinic

In this thesis well-established in vivo and in vitro models have been used to investigate helium conditioning. Animal models in particular always pose ethical and moral dilemmas for the involved research team as well as the scientific community in general. However, we still need animal models to keep up the scientific solutions and developments in medicine. An example of a disease that cannot be investigated without the use of animals is coronary heart disease, for which we used the myocardial regional ischemia/reperfusion model. Although well established and widely used, there are some disadvantages with this model. The most profound problem with this model is the origin and development of the phenomenon myocardial ischemia. In humans, risk factors such as obesity, diabetes and hypertension influence the severity and time frame of disease development and consequently also affect the window of protection.

In general, five objections can be distinguished when considering these types of animal experiments and might explain why translation of successful experimental therapies to the clinic is scarce: (1) patient populations are heterogeneous while experimental studies are often conducted in male subjects receiving standardized diets and live in controlled environments; (2) the duration of index ischemia is relatively shorter in animal experiments compared to clinical situations, which enlarges the possibility of salvaging strategies; (3) experimental animals are young and healthy whereas patients are old with comorbidities; (4) patients often use multiple pharmacologic treatments a priori that affect the final myocardial injury; (5) in animal studies function of other organs than the heart remain uninvestigated while treatments could be detrimental for these other organs and preclude clinical use. In the last years, several reviews on this issue have been written and set out the problems researchers were faced with when testing new therapeutics in clinical trials, such as ischemic and anesthetic conditioning. A recent review article addressed this issue and analyzed 16 ischemic PC, 12 ischemic PostC, 25 remote PC and 13 pharmacologic conditioning trials. Of the 66 clinical trials that have been conducted, not a single one was large enough and could show a profound benefit of cardiac conditioning. It has to be noted that in clinical trials, infarct size measurement is still a subject of debate, as it is difficult to find a marker that shows minimal natural variability. This is the reason why experimental studies have an advantage; infarct size measurement by TTC-staining is a very reliable method.

A small step towards the translation of helium as an organ protective agent to the clinic has been made in this thesis. In chapter 8 and 9 human male and female volunteers were used to investigate helium in humans in vivo. In both studies, a relatively small number of healthy, young subjects were used and exposed to helium inhalation. Despite
their contribution to a better understanding of the effects of helium in vivo, these studies clearly targeted a non-realistic population considering the lack of comorbidity and young age. Nevertheless, these studies are a crucial first step towards larger clinical trials in which human subjects are used.

**Future research**

This thesis has answered questions but inevitably also raised questions and left some problems unsolved. In conjunction with the last paragraph, it should be emphasized that a clinical trial with sufficient patient should be performed in order to find out whether helium can be used as an adjunct therapy to reperfusion therapy and in which modality it should be used. This thesis has provided answers considering timing and duration of the helium conditioning protocol in rats, but it is unknown whether these protocols can be applied in patients in the same form.

Before such a clinical trial would be conducted it would be wise though, to further investigate certain issues and questions that were raised in this thesis. These issues all relate to the underlying mechanism of helium conditioning and our insufficient understanding of its cardioprotective effects. One of the most important points comprises pathways that are involved in prevention of apoptosis and execution of autophagy. As mitochondria are likely to be important players in helium conditioning as well, one could imagine that the clearing of defective mitochondria from cells in order to keep it healthy and functional might play a role.

To conclude, helium-induced conditioning seems to be for the better, but so far this only holds true for healthy animals and healthy volunteers. It remains a question whether helium-induced conditioning can also be applied in sickness, i.e. in individuals with concomitant disease or comorbidity. Future research should therefore include investigations in experimental models of disease and eventually the target patient population, i.e. the diabetic-, hypertensive- or obese aged patient.
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