Organ protection by the noble gas helium
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Citation for published version (APA):
Smit, K. F. (2017). Organ protection by the noble gas helium
Chapter 3

Targets involved in cardioprotection by the non-anesthetic noble gas helium

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Current Drug Targets 2015;16:786-92.
ABSTRACT

Research data from the past decade indicate that noble gases like xenon and helium exert profound cardioprotection when applied before, during or after organ ischemia. Of all noble gases, especially helium has gained interest in the past years because it does not have an anesthetic “side effect” like xenon, allowing application of this specific gas in numerous clinical ischemia/reperfusion situations. Because helium has several unique characteristics and no hemodynamic side effects, helium could be administered in severely ill patients.

Investigations in animals as well as in humans have proven that this noble gas is not completely inert and can induce several biological effects. Though the underlying molecular mechanisms of helium-induced cardiac protection are still not yet fully understood, recently different signaling pathways have been elucidated.
INTRODUCTION

Helium belongs, next to neon, krypton, xenon, and the radioactive radon, to the group of noble gases. Within this group the chemical elements are all characterized by filled valence orbitals, carrying a maximum amount of electrons in the outer shell of the atom. These properties suggests that helium is ‘inert’ and has a very low chemical reactivity. Under standard conditions, the noble gases are odorless, tasteless, colorless monatomic gases.

In contrast to xenon, helium has no anesthetic properties. The application of 80–90 atmospheres of helium pressure was shown to increase the minimum alveolar concentration for volatile anesthetics. This means that helium counteracts the anesthetic effects of other inhalational anesthetics. Helium is therefore defined as a ‘non-immobilizer’, describing a gas that does not induce anesthesia (immobilize) but that probably has other behavioral effects. In the last decade, another property of helium has become center of several experimental and clinical investigations: it has been demonstrated that, although ‘inert’ and non-anesthetic, helium exerts in vivo and in vitro effects on a cellular level. Helium is able to reduce ischemia/reperfusion damage in cardiac tissue. Because helium also has no hemodynamic side effects when clinically applied, it might be useful for organ protection in critically ill patients. This review will summarize and discuss the underling pathways of helium conditioning and will focus on the cellular effects of helium in the heart. Understanding and exploring these effects might finally lead to new strategies of tissue repair that can be employed in clinical myocardial ischemia/reperfusion situations.

Pre- and postconditioning

The first described form of “conditioning” is ischemic pre-conditioning, which was described in animals more than 20 years ago. This phenomenon has been described in most living cells and in all mammalian species. Conditioning the heart can be important in situations where a brief interruption of blood flow to an organ occurs. This is the case during organ transplantation, surgery, acute myocardial infarction and percutaneous coronary interventions (PCI).

Most intensively investigated is myocardial pre-conditioning, which refers to an application of short periods of ischemia before a subsequent longer ischemic period. Preconditioning can be divided into an early (immediate effect, lasting for 2–3 hours) and late phase of pre-conditioning (protection reappears after 24 hours lasting for 2-4 days). Recent research also focused on post-conditioning, which is achieved by application of short repetitive cycles of ischaemia directly at the onset of reperfusion. Another very recent concept of conditioning is remote pre-/per- or post-conditioning, which is defined by the application of sublethal ischemia to an organ that is located distant from the target organ. The obvious advantage of remote conditioning is that no additional manipulation on the threatened organ itself has to be performed.
As applying ischemia to an organ yields a high risk of complications for the patient, the search for alternative options to mimic ischemic conditioning quickly showed that different pharmacologic compounds activating adenosine, muscarinic, α-adrenergic, opioid or bradykinin receptors also contributed to organ protection. Among these substances, halogenated fluorocarbons like isoflurane, desflurane and sevoflurane, as well as the noble gases, are meanwhile well known to induce cardioprotective effects.

Physical, chemical and molecular properties of helium

In order to get a better overview of the unique properties of the noble gas helium we will summarize the most relevant physical, chemical and also molecular effects of noble gases that are supposed to be at least in part responsible for mediating any pharmacological effect. Among all noble gases, helium has a molecular weight of only 4 g/mol and has the lowest melting and boiling points of all elements. In contrast to oxygen, which has a density of 1.43 g/m^3, helium has a very low density of 0.179 g/m^3. The absolute viscosity of helium is 201.8 m poise, which is lower compared to oxygen (viscosity of 211.4 m poise) and normal air (188.5 m poise). Because the flow of a gas depends on the density and viscosity of each element that is present in the respective gas mixture, airflow through the lungs is depending on the gas mixture that is inhaled. Due to the low density of helium, inhalation of this gas reduces work of breathing and helium is hence available for clinical use in patients with obstructive airway diseases. Ventilators that allow administration of helium by invasive and non-invasive ventilation have been invented and helium can be used during heart, vascular or transplantation surgery, all typical clinical ischemia/reperfusion situations occurring on a daily basis.

The anesthetic potency of a substance is almost linearly correlated with its oil/water partition coefficient. The low fat solubility of helium determines the low partial pressure of helium in the central nervous system. It was shown that helium administrated in supra-atmospheric levels induced tremors and convulsions in rats that had been exposed to 84.6 ± 22.2 atmospheres of helium. These results suggest that helium activates the central nervous system rather than depressing the neural cell activity.

Cardioprotection induced by helium—what do we know?

In 2007, Pagel and co-workers were the first to show that three times 5 minutes inhalation administration of 70% helium before a coronary artery occlusion for 30 minutes followed by 3 hours of reperfusion significantly reduced infarct size in rabbit hearts. This was the first proof that helium is capable of inducing preconditioning of the heart.

In this study, the authors used several different inhibitors in order to block enzymes of the so called “reperfusion injury salvage kinase (RISK) pathway”. They showed that administration of a phosphatidylinositol-3-kinase (PI3K) antagonist, a mitogen/extracellular signal-related kinase 1 (MEK-1) inhibitor and an inhibitor of the 70-kDa ribosomal protein s6 kinase (p70s6kinase) all were capable to block helium induced cardioprotection in the ischemic rat heart.
In an interesting set of subsequent studies, the same authors identified several potential key players in the mechanism underlying helium induced preconditioning. Pro-survival kinases from the RISK pathway inhibit glycogen synthase kinase-3 beta (GSK-3\(\beta\)) activity and stimulate apoptotic protein p53 degradation. To evaluate whether these two pathways would probably also play a role in helium induced cardioprotection, Pagel and co-workers investigated if a pharmacological inhibition of GSK-3\(\beta\) and p53 would lower the threshold of helium-induced protection.\(^{18}\) Employing the mitochondrial permeability transition pore (mPTP) opener atracyloside in their in vivo setting, the authors could show that inhibition of GSK-3\(\beta\) or p53 lowers the threshold of helium-induced preconditioning via a mPTP-dependent mechanism.\(^{18}\)

A similar result was achieved by the use of morphine (0.1 mg/kg) in combination with helium-preconditioning in rabbits.\(^{19}\) These data suggest that helium preconditioning involves an opioid-receptor mediated mechanism. This could have significant implications in the clinical scenario, as morphine is a routinely used opioid analgesic applied in patients with ischemia-reperfusion problems, e.g. in patients with acute myocardial infarction. The above mentioned pro-survival kinases PI3K, ERK1/2 and their downstream targets: endothelial nitric oxide synthase (eNOS), p53 and GSK-3\(\beta\), all have been shown to prevent opening of the mPTP in ischemic preconditioning.\(^{20}\)

Mitochondria are the source of cellular energy deliver and determine the life or death fate of individual cells, whole organs, and finally the entire organism. The three main mitochondrial phenomena: 1) opening of mitochondrial adenosine triphosphate-regulated potassium channel (mitoKATP), 2) generation of a small reactive oxygen species (ROS) burst, and 3) maintenance of the mPTP have been linked to cardioprotective effects, and they are likely not mutually exclusive. The opening of the mPTP causes mitochondrial dysfunction, and preconditioning is considered might be protective by preserving cardiac mitochondrial function, thereby reducing tissue damage.

The opening of the mPTP during reperfusion is enhanced by normalization of acidic pH after the blood flow has been restored.\(^{21}\) In order to extend their finding that helium induces preconditioning via preventing mPTP opening, Pagel and coworkers applied transient alkalosis during the early reperfusion phase and showed that helium-induced cardio-protection was abolished.\(^{22}\) However, the mPTP inhibitor Cyclosporine A could restore the cardioprotection in the presence of alkalosis.\(^{22}\) These data indicate that helium inhibits mPTP opening by maintaining intracellular acidosis during early reperfusion. Regarding the role of ROS and the mitochondrial K-ATP channel in helium preconditioning it was shown that the ROS scavengers N-Acetylcysteine and N-2-mercaptopropionyl glycine or the K-ATP-channel blocker 5-hydroxydecanoate completely abolished helium induced preconditioning in rabbits.\(^{23}\)

In a study of our own laboratory we analyzed cardiac mitochondrial function by measuring the rate of oxygen consumption in isolated mitochondria in rats.\(^{24}\) The rate of oxygen consumption of isolated mitochondria after administration of a complex 2 substrate (state 2), adenosine diphosphate (state 3), and after complete phosphorylation of adenosine diphos-
phosphate to adenosine triphosphate (state 4) was used to elaborate a direct effect of helium conditioning on mitochondrial function. The results showed a mild mitochondrial uncoupling, as helium-induced preconditioning increased state 4 respiration (state 3 respiration was not changed), thus reducing the respiratory control index (state 3/state 4).

Infarct size reduction after helium preconditioning was blocked by Iberotoxin, a mitochondrial calcium sensitive potassium (mKCa) channel blocker. In a study investigating the differences between young and aged myocardium, infarct size reduction by helium was completely abolished by the protein kinase A (PKA) blocker H-89. In the same study it could be shown that also activation of mKCa channels by NS1619 reduced infarct size in young and aged rats. Interestingly, the adenylyl cyclase activator forskolin (in a concentration of 300 μg/kg) reduced infarct size only in young animals. A higher dose of forskolin (1000 μg/kg), however, reduced infarct size also in aged rats. It is therefore suggested that helium preconditioning involves and is partly mediated by activation of PKA and that changes in PKA regulation could explain the age-dependent loss of tissue protection by preconditioning.

Next to the above-mentioned enzymes, also nitric oxide synthase (NOS) has been implicated in helium-induced preconditioning in rabbits. The non-selective NOS inhibitor N-nitro-L-arginine methyl ester was infused during the helium preconditioning protocol and completely abrogated cardioprotection. In contrast, the selective inducible NOS inhibitor or a selective neuronal NOS inhibitor did not affect helium-induced preconditioning. These data show that NO might be generated by NOS during helium preconditioning.

Regarding the use of different helium concentration in rats, Huhn et al. induced late preconditioning by a 15 minute administration of 70%, 50%, 30%, and 10% helium in a time window 24 hours before ischemia/reperfusion. A helium concentration of 30% was still protective but a concentration of 10% was not, indicating that there is a threshold for a certain concentration of the noble gas. This threshold is quite low, allowing also inhaling a significant amount of oxygen, a fact that might play a critical role when the noble gas is used in clinical ischemia-reperfusion situations. In additional experiments, Huhn et al. showed that the COX-2 inhibitor NS-398 blocked helium induced late preconditioning. Most of the described mechanisms have been investigated by different pharmacological interventions rather than by direct biochemical methods. How helium might affect these pro-survival kinases and thus mediate cardioprotection is yet completely unknown.

In a different approach, not using any pharmacological blockade but rather a gene expression screening along with histology scores, we got more detailed information on target genes that are regulated by helium induced postconditioning. Rats were subjected to 25 min of ischemia and 5, 15, or 30 min of helium postconditioning. Semi-quantitative histological analysis revealed that 15 min of helium postconditioning reduced the extent of ischaemia/reperfusion-induced cell damage, but this effect was not observed after 5 and 30 min of helium postconditioning. The protective 15 min helium-postconditioning stimulus resulted in up-regulation of 17 of 23 genes involved in necrosis, and in 18 of 25 genes involved in pro-apoptosis.
On the other hand, 4 of 23 (necrosis) and 7 of 25 genes (pro-apoptosis) were down-regulated. From the anti-apoptotic genes, 9 of 11 and from genes involved in autophagy, 24 of 32 were up-regulated after helium postconditioning. Taken together, these data indicate that helium postconditioning at least partly is mediated by alterations in the cell death program.

Interestingly, other noble gases were proven to disrupt conformational changes of the proteins urate oxidase, an intracellular globular protein composed of large hydrophobic cavities and Annexin V, a protein with a hydrophilic pore inside known to bind to cell membranes by a calcium-dependent action. Different cell membrane receptors, e.g. the N-methyl-D-aspartate receptors, the two-pore domain potassium channel TREK-1, the plasmalemmal ATP-sensitive potassium (K$_{ATP}$) channel, the nicotinic acetylcholine receptor as well as the 5-hydroxytryptamine type 3 receptor, have been shown to be affected by noble gases. It has to be mentioned that these investigations were mostly performed in neuronal cells and for different noble gases than helium. That these cellular effects are also involved in cardiac protection by helium is therefore speculation. However, it is worth mentioning that they clearly link noble gas action to membrane structures.

### A role for membrane proteins “caveolins” in helium induced cardioprotection

As helium is an inert gas with a lower activity to react with other compounds or receptors, but nevertheless has been show to induce biological changes, membrane structures and membrane proteins might be important in helium-induced cardioprotection.

Caveolae, also named “little caves”, are cholesterol- and sphingolipid-enriched invaginations of the plasma membrane. These little caves are considered a subset of lipid rafts. The important structural proteins that are essential for the formation of Caveolae are called Caveolin. Caveolins exist in three isoforms. Caveolins are critically involved in ischemic as well as isoflurane-induced conditioning. Caveolins have scaffolding domains that anchor and regulate proteins. The isoforms Caveolin-1 and -2 have been shown to be expressed in multiple cell types, while caveolin-3 is mostly expressed in striated (skeletal and cardiac) muscle and certain smooth muscle cells. Caveolins regulate multiple cellular processes, including vesicular transport, cholesterol and calcium homeostasis, as well as inter- and intracellular signal transduction. Most importantly, caveolins have recently been detected in mitochondria. Under the intracellular control of Caveolins different signaling molecules are recruited to the Caveolae, where they induce a direct temporal and spatial regulation of signal transduction.

Recent evidence from research shows that several signaling molecules might exist as multiprotein complexes. These complexes are called “signalosomes”. They continuously change their form and dissociate under basal or stimulated conditions. Caveolins are suggested to play a significant role in the dynamics of these multiprotein complexes. Specifically, in regard to signaling molecules involved in cardiac protection, many G–protein coupled receptors (GPCR) including opioid and adenosine receptors localize to caveolae. Additionally, many
of the signaling molecules involved in cardiac protection, including the G-alpha subunit of heterotrimeric G-proteins, Src kinases, PI3K, eNOS, PKC isoforms and ERK, all have been linked to cardioprotection and are known to bind the scaffolding domain of caveolin.\textsuperscript{51-52} Additionally, caveolins have recently been shown to modulate mitochondrial function.\textsuperscript{51-52}

First investigations aiming to elucidate a possible connection of helium with caveolins in fact showed that helium inhalation affects caveolin-1/3 expression in mice heart.\textsuperscript{53} In this study, mice inhaled either helium (70\%) or oxygen (30\%) for 20 min. Thirty minutes or 24 hours after inhalation hearts were excised and blood was withdrawn in order to obtain serum from the mice. Results showed that helium inhalation decreased caveolin-1 and caveolin-3 expression in the heart after 24 hours. This was confirmed by analyses of the buoyant caveolin enriched fractions also showing lower caveolin levels. In addition, caveolin-1/-3 levels were elevated in serum of mice. These results suggest that circulating factors in the bloodstream may be part of the mechanism that mediates protection in different forms of conditioning. The summarized experimental data for helium induced conditioning are depicted in figure 1.

**Helium in diseased animal models**

A limitation of most of the above described studies is that they were performed in healthy animal models. However, it has been shown that different co-morbidities like, diabetes, aging and hypertension block conditioning.\textsuperscript{54} This fact clearly indicates the need of further research in diseased models that more reliably reflect the real patient. Only translation of experimental data in diseased animals enables us to develop clinical strategies of tissue repair in patients suffering from ischemia/reperfusion situations of the heart. Regarding such investigations, only a few studies have been performed in animals. In one study, healthy Wistar Kyoto rats and spontaneous hypertensive rats were subjected to 25 min of myocardial ischaemia and 120 min reperfusion. The animals inhaled 70\% helium for 15 min after the index ischaemia (helium post-conditioning), combined with 15 min helium inhalation 24 hours prior to index ischaemia (helium late pre-conditioning) or a triple intervention containing 3 additional cycles of 5 min helium inhalation shortly before ischemia (helium early preconditioning).\textsuperscript{55} In hypertensive animals, the helium triple intervention by pre-/post- and late conditioning was able to induce cardioprotection, a single intervention by helium postconditioning alone was however not protective.\textsuperscript{55} Investigations on the phosphorylation of GSK-3\(\beta\) and PKC-\(\varepsilon\) in this experimental setting revealed that helium conditioning does not involve GSK-3\(\beta\) and PKC-\(\varepsilon\) related mechanisms.\textsuperscript{55}

In pre-diabetic Zucker obese rats,\textsuperscript{56} helium induced cardioprotection was blocked. In this study in Zucker lean rats but not in Zucker obese rats helium was capable to induce mild mitochondrial uncoupling.\textsuperscript{56} Moreover, myocardial ERK\(1/2\) and Akt phosphorylation were not affected by helium in both animals types. However, GSK-3\(\beta\) phosphorylation was reduced in the heart only in Zucker lean animals but not in the obese rats.\textsuperscript{56}
In the senescent myocardium, Heinen et al. demonstrated that administration of helium in a preconditioning manner did not lower infarct size. Moreover, the above described mild mitochondrial uncoupling after helium inhalation could only be found in young but not in old animals. In a second study in aged animals infarct size was reduced by activation of mKCa channels using in young and aged rats.
Helium used in clinical situations: are we yet so far?

Even though helium has been applied successfully in patients with respiratory diseases for decades, the enormous amount of research on helium induced cardiac protection could not yet be translated to the clinical situation. Regarding clinical studies, so far only one study in patients that were subjected to a coronary artery bypass grafting surgery exists. These patients inhaled helium (70%) for 3 x 5 minutes before start of the cardiopulmonary bypass (helium pre-conditioning) or at the point when the coronary artery reperfusion was started by declamping of the aorta. Surprisingly, both applied conditioning protocols alone, and even the combination of pre- and postconditioning did not show protective effects on the post-operative troponin release.

Regarding protection of other organs than the heart by helium, more progress was made in human tissue. Helium protects the human endothelium against ischemia/reperfusion damage in a forearm blood flow model of healthy volunteers. After a 20 minute forearm ischemia/reperfusion period endothelial function was measured by response to acetylcholine. Venous occlusion plethysmography measurement of the forearm blood flow showed that inhalation of 3 x 5 minutes of 79% helium inhalation prevented post-ischemic endothelial dysfunction. However, helium preconditioning did not affect plasma levels of cytokines, adhesion molecules, or microparticles. In this study, the endothelium was also protected 24 hours after the helium inhalation, suggesting a late endothelial preconditioning effect on the endothelium by helium inhalation. Interestingly, in this volunteer study an involvement of NOS, which was previously found by Pagel and co-workers in rabbits, could not be confirmed, once again showing differing results of helium induced organ protection in animal and human tissue.

Another study in human healthy volunteers inhaling 50% helium before, during and after ischemia used post-ischemic reactive hyperemia to determine endothelial function. In this study endothelial function was measured before and after 15 min of forearm ischaemia. There we no changes in the post-occlusive hyperemic reaction detected, but the authors found an increased level of the pro-inflammatory marker CD11b and ICAM-1 on leukocytes. In contrast, the expression of the pro-coagulant markers CD42b and PSGL-1 on platelets was decreased. These data suggest a mild anti-inflammatory property of helium in humans.

In a study of Oei et al., male healthy volunteers inhaled 30 minutes if heliox (79% He / 21%O2) or air respectively. In this study in a whole blood ex vivo model the effect of helium on the responsiveness of the human immune response was tested. Blood was withdrawn at different time points after helium inhalation and then incubated with lipopolysaccharide (LPS), lipoteichoic acid, T-cell stimuli anti-CD3/ anti-CD28 or pure media for 0, 2, 4 and 24 hours. In this study, tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta), interleukin-6 (IL-6), interleukin-8 (IL-8), interferon-gamma and interleukin-2 (IL-2) were analysed by cytometric bead arrays. Helium inhalation did not affect levels of TNF-alpha, IL-1beta, IL-6, IL-8, IFN-gamma and IL-2 in comparison to air inhalation. Additionally, a group of volunteers inhaling helium for the extended period of 60 min did not show differences in cytokine produc-
tion after LPS stimulation of whole blood. Thus, in contrast to the study of Lucchinetti and co-workers, in the study of Oei et al. helium had no effect on the responsiveness of the innate and early adaptive immune system.\textsuperscript{60}

**SUMMARY AND CONCLUSION:**

Taking together, cardioprotective and anesthetic properties of gases often coexist, as is the case for the volatile anesthetics and xenon but not for helium making it unique between these substances.

This suggests that gaseous properties and protective effects are connected and share parts of a common pharmacologic mechanism. So far, a complete overview of the underlying molecular mechanisms of preconditioning by gaseous agents still cannot be drawn. However, molecular targets like the mitochondrial permeability transition pore (mPTP), mitogen activated protein kinases (MAPK), protein kinase C, B and for helium A, the RISK pathway (AKT/PI,K) and Caveolins are reported to be involved.

Summarizing all experimental data published so far, we suggest that the noble gas helium might become a very promising agent not only in patients with ventilation disorders but also in those patients undergoing critical acute ischemic events of the heart. However, this review clearly demonstrates the need to translate the experimental data to the clinical situation, as studies on cardioprotection by helium in patients are very limited.
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