Thiol-based cardioprotection

de Koning, Marie-Sophie

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CHAPTER 8

Discussion and Future Perspectives

GENERAL DISCUSSION

In this thesis, we explored possible new pharmacological strategies to protect the heart against ischemia-reperfusion (I/R) injury. We studied the associations of ketones bodies (KBs) and free thiols with outcomes after ST-segment elevation myocardial infarction (STEMI) and we evaluated the importance of free thiols in heart failure. We further examined the safety and efficacy of the hydrogen sulfide- and thiol-donating compound sodium thiosulfate for the reduction of I/R injury in patients presenting with STEMI.

The main conclusions of this thesis are:
- Circulating KBs at 24 hours after reperfusion are associated with functional outcomes after STEMI;
- Changes in free thiols during the first 24 hours after reperfusion may be associated with functional outcomes after STEMI;
- Free thiols are associated with heart failure severity and outcome;
- Sodium thiosulfate appears safe and is well tolerated in patients presenting with acute coronary syndrome;
- Sodium thiosulfate is safe in patients presenting with STEMI, but does not reduce I/R injury.

Part I. Possible new therapeutic targets for ischemia-reperfusion injury
Ketone bodies

In **Chapter 2**, we were the first to study KBs, metabolic substrates produced by the liver, in human I/R injury. We demonstrated that KBs are elevated in patients presenting with STEMI, compared to known reference values in the general population. Second, we observed that higher levels of circulating KBs at 24 hours after reperfusion were associated with increased infarct size and lower left ventricular ejection fraction (LVEF). Although our results do not prove causality, it is likely that KBs might play a role in I/R injury. Under physiological circumstances, the heart sparingly uses KBs as an energy source (<10% of all ATP production). However, in several other conditions of cardiac disease, including heart failure and arrhythmogenic cardiomyopathy the heart increasingly (to >20%) relies on KBs as a metabolic fuel.[1,2] In our study ketogenesis and cardiac ketone metabolism were not measured, but it is plausible that KBs are produced to a higher extent during I/R injury due to the release of catecholamines and natriuretic peptides,[8] and that higher circulating KBs will also lead to increased cardiac ketone metabolism. Namely, previous studies demonstrated that higher circulating KBs are paralleled by increased cardiac ketone metabolism in healthy hearts,[1] as well as in a wide range of conditions of cardiac stress, including heart failure with both reduced and preserved ejection fraction and arrhythmogenic cardiomyopathy.[9,10] Preclinical data, together with the results of our study suggest that therapeutic ketosis may hold promise as therapeutic target for I/R injury. We know from animal studies that this shift towards ketone utilization is likely to be adaptive, because disruption of the ketone utilization potential was associated with pathologic cardiac remodeling in preclinical models of heart failure.[3,4] Moreover, animal

models of I/R injury demonstrated favorable effects of higher KB concentrations, achieved by supplementation of exogenous ketones or its precursors, or adherence to a ketogenic diet (therapeutic ketosis). These reported beneficial effects included decreased infarct size, improved cardiac function, reductions in oxidative stress and increased ATP production.[5–7]

Clinical research with exogenous ketone supplementation is currently limited to patients with heart failure, with promising results: Infusion of KBs in patients with heart failure with reduced ejection fraction led to beneficial hemodynamic effects, including increased cardiac output, which was already detectable in the physiological concentration ranges of circulating KB levels.[11] It can be hypothesized that, next to hemodynamic benefits due to improved cellular energy metabolism, also other pleiotropic effects that have been attributed to KBs, e.g. beneficial effects on endothelial function, oxidative stress, inflammation and mitochondrial function, might aid the heart during I/R injury.[12]
However, before clinical evaluation of treatment effects of ketone supplementation in human I/R settings, more mechanistic evidence should be collected concerning cardiac KB metabolism and its role in I/R injury. Moreover, larger animal models with KB supplementation in an I/R setting are warranted to increase the likelihood of translational success. Thereafter, randomized clinical trials evaluating treatment effects of KBs in myocardial infarction can be considered, while also taking into account the issues discussed in **Part II** below.

Free thiols
In **Chapter 3** we investigated the role of free thiols, strong antioxidants and markers of oxidative stress, in I/R injury. In 378 patients presenting with STEMI, we demonstrated that a larger decline in free thiols during the first 24 hours after reperfusion, indicative of increased oxidative stress, was associated with larger infarct size and lower LVEF in univariable and age- and sex-adjusted analyses. Unfortunately, effects in multivariate analyses were diluted. One explanation for this can be that our study included also patients presenting with already partial or complete restoration of coronary flow at presentation with STEMI (i.e. TIMI 2 or 3 flow). However, the largest release in reactive oxygen species and thus shifts in free thiol concentrations is expected immediately after reperfusion.[13] Clinically, this coincides with the opening of the (almost) completely occluded coronary artery, i.e. TIMI flow 0-1 at the start of the primary percutaneous coronary intervention (PCI). This was however the case in only 62.1% of the included patients. When focusing our analysis on this subpopulation, significant associations between free thiols and functional outcomes were observed, in contrast to patients with TIMI 2-3 flow where associations were absent. Therefore, our results should be validated in larger cohorts of patients presenting with STEMI with occluded coronary arteries. Especially since other studies showed robust associations between oxidative stress markers and disease severity and outcomes in patients with myocardial infarction.[14,15]

As a logical consequence of these observed associations, multiple trials tried to target oxidative stress during acute myocardial infarction. These trials tested the administration of vitamin C (Vit C), deferoxamine (DFO) and allopurinol, among many others. Results were inconclusive or neutral.[16–18] However, compared to other antioxidants, free thiols not only act as simple antioxidants or reactive oxygen species (ROS) scavengers, but have been implicated in a broader concept of the recently defined 'redox species interactome' (RSI). The RSI is a complex network of biochemical reactions, through which cells can sense their environment and adjust their metabolic machinery to enable cellular homeostasis.[19] Messengers of this RSI include ROS, reactive nitrogen and sulfur species, and gasotransmitters including hydrogen sulfide (H_2S). Their targets are metal centres and protein thiols. Protein thiols, including free thiols, are considered to act as central hubs of inter-organ redox communication. They transduce a variety of redox-regulated events, mainly via oxidative protein modification, covering both short-term (e.g. alteration of protein structure and activity) and longer-term (e.g. regulation of gene expression) biological adaptions, taking both place in the intracellular and extracellular space. Although there is still a lot to discover in redox biology, free thiols might proof to be a more central therapeutic target for oxidative stress-associated diseases, including cardiovascular diseases.[20]

In **Chapter 4** of this thesis, we demonstrated robust associations between lower levels of circulating free thiols, indicative of higher oxidative stress, and more advanced heart failure and adverse clinical outcomes in patients with new-onset or worsening heart failure. Associations of free thiols with clinical outcomes were consistent across predefined subgroups (for example for heart failure with reduced, mid-range and preserved ejection fraction). These results may imply that free thiols can be used as a risk stratification tool in patients with heart failure. Moreover, our results also shed light on the potential for future development of redox-targeted therapeutics in the context of the cardiovascular disease continuum. The cardiovascular disease continuum is a framework that considers cardiovascular disease as a chain of events, initiated by a myriad of risk factors and progressing through numerous physiological pathways towards end-stage heart disease.[21] Previous studies have demonstrated associations between free thiols and disease severity in early stages of this continuum, i.e. stable atherosclerotic coronary artery disease,[15,22] and our data shows associations in later phases of this continuum, i.e. myocardial infarction (**Chapter 3**) and heart failure (**Chapter 4**). It is hypothesized that intervention anywhere along the chain of events could disrupt the pathophysiological process and confer cardioprotection. Oxidative stress has been identified as an important pathophysiological mediator in this continuum,[21] and because free thiols are centrally involved in redox biology and oxidative stress, therapeutic modulation of free thiols may hold promise. However, first more mechanistic studies are warranted, investigating whether free thiols are merely markers of oxidative stress or also important mediators in disease progression. Subsequently, before designing treatment studies, also more mechanistic research should be performed to optimize patient selection, timing of administration and specific (sub)cellular localizations of thiol modulation (for example modulation of free thiols vs intracellular thiols). Literature namely suggests that

modulation of thiol-based proteins in all patients with oxidative stress-associated diseases, may potentially disturb physiological redox signaling, with harmful consequences.[20,23] Therefore, targeted redox approaches should be developed according to strict protocols, and should probably only be reserved for patients with poor redox status.

Part II. Safety and efficacy of sodium thiosulfate in acute myocardial infarction

The aim of **Part II** of this thesis was to study the safety and efficacy of STS, a strong antioxidant and H_2S- and thiol-donating compound, in acute myocardial infarction. In **Chapter 5**, we demonstrated STS to be safe and well tolerated in patients presenting with acute coronary syndrome. Subsequently, we designed a proof-of-principle trial to test the hypothesis that STS reduces I/R injury in patients presenting with a first STEMI (**Chapter 6**): The **G**roningen **I**ntervention study for the **P**reservation of Cardiac Function with **S**odium thiosulfate after STEMI (GIPS-IV trial). This trial was a double-blind placebo controlled multicenter trial, with the primary objective to test if STS reduces infarct size at 4 months follow-up, as determined by cardiac magnetic resonance imaging. We studied an accurate number of patients and used the primary outcome in line with recommendations for early assessment of potential cardioprotective therapies.[24] Unfortunately, the main finding of this thesis was that STS does not reduce myocardial infarct size after STEMI (**Chapter 7**). Furthermore, the administration of STS did not lead to a reduction in enzymatic infarct size, nor improvement in LVEF or NT-proBNP levels. Hence, we could not substantiate cardioprotective effects from STS in this thesis.

There are some possible explanations for this lack of clinical benefit, which can be divided to the following categories: (I) Translational failure; (II) Compound/intervention related explanations; (III) Patient selection, and (IV) Study design and outcome related explanations.

Translational failure

Before we undertook the GIPS-IV trial many preclinical studies were available showing promising cardioprotective results of H_2S or an H_2S-donating compound. These studies included cellular studies, as well as studies undertaken in rodent, canine or porcine animal models. Meta-analysis showed robust infarct sparing effects independent of the type of H_2S-donor used.[25] STS specifically was investigated in multiple rodent models of I/R injury, with a wide range of signaling changes observed, including antioxidant, anti-inflammatory and anti-apoptotic effects, and preserved mitochondrial activity.[26–29] However, like GIPS-IV, many preceding studies also lacked to demonstrate clinical benefit in patients with myocardial I/R injury (myocardial infarction), despite promising preclinical data. The difficulty in translation of preclinical results to treatment of patients may be a broader and more general problem.[30] The first explanation is that physiology of rodents is substantially different from humans. Fors larger animal models, the differences are (much) smaller. Therefore, possible new compound should also be tested in larger animal models. These studies are already conducted in case of

H_2S,[31,32] however not with STS in settings of I/R injury. Second, most animal studies investigate compounds in healthy animal models, although in clinical practice, diabetes, atherosclerosis, and hypertension often coincide with acute myocardial infarction. STS was tested in preclinical I/R models with comorbidities and the results were not solely positive.[33,34] Another utmost important issue are the interactions with concomitant medications, for example antiplatelet therapies, that are administered early after clinical myocardial infarction and may influence cardioprotective effects in humans, but play no role in animal models. Also, different and often short I/R protocols were used across different research groups, limiting preclinical validation of results. Lastly, many potential cardioprotective compounds were tested in a setting in which they were administered before the induction of ischemia, whereas in clinical practice this is not possible and compounds can ideally be administered only shortly before reperfusion. To increase the likelihood of translational success, new recommendations for pre-clinical studies were composed.[35] Harmonization of protocols and extensive external validation of preclinical studies before clinical evaluation might increase the chance of translational success.

Compound/intervention related explanations

Myocardial I/R injury is a complex phenomenon, which involves, but is not limited to the following underlying mechanisms: oxidative stress, inflammation, apoptosis, metabolic changes and microvascular dysfunction. After multiple translational failures involving therapies that targeted only one of those underlying mechanisms, nowadays the recommendation is to investigate therapies targeting multiple mechanisms at once.[36] Since H_2S is a compound with pleiotropic effects, that plays a central role in the physiology of many tissue processes (including the RSI), it qualifies well for a translational attempt. Of many H_2S-donors, STS was especially promising, due its additional antioxidant properties and established clinical safety profile.

An issue which might have led to negative results concerns dosing. From safety and efficacy data derived from other (non-cardiovascular) indications, we assumed that 12.5 grams was the adequate dosage for cardioprotection. We performed a dose-escalating pilot study in patients with acute coronary syndrome (**Chapter 5**), including 18 patients with unstable angina or non-ST-segment elevation myocardial infarction. In this study we observed that 2x15 grams was well tolerated and side effects (nausea and vomiting) were scarce. Due to limited patient numbers, we did not investigate efficacy markers. For the GIPS-IV trial we chose to administer 12.5 grams twice, which is the regular dosage for other indications. It is possible that this dosage was too low to reduce infarct size. Moreover, although different studies suggest that, especially under ischemic conditions, STS is able to release H_2S, the amount released in the heart specifically remains unknown and is difficult to establish.[37] But even in the hypothetical scenario that the dosage of STS was too low, it is unlikely that higher dosages of STS would have been feasible to apply, since in the GIPS-IV trial a substantial amount of participants experienced emetogenic effects. Also, administration of higher dosages means extra intravenous volume supplementation, which can be unfavorable

in patients presenting with STEMI. Therefore, other H_2S-donating compounds should be considered for clinical evaluation when higher dosages would be considered. However, other H_2S-donating compounds are still in the phase of pre-clinical testing and evaluating their cardioprotective potential would require extra approval.

Another potential explanation for the lack of clinical benefit involves the timing of the intervention. In our study we considered it important to administer STS timely before reperfusion, which is certainly a strength of our study. Therefore we started the first dose of study medication before coronary angiography, at the arrival of the patient to the catheterization laboratory. The second dose was administered six hours after the first dose, limiting the biological effects of STS to the first twelve hours after reperfusion. Although we know that the most detrimental effects of I/R injury occur within the first minutes to hours, some aspects of I/R injury last 48-72 hours (inflammatory response).[38] More extended treatment might thus be warranted. However, for logistical reasons this approach was beforehand founded as less feasible in our study (due to early transfer of STEMI patients to other referring hospitals, which were not allowed to administer study medication). Future studies might overcome this issue with prolonged treatment by oral H_2S supplements.

Patient selection

The most important limitation of our trial was that the overall median infarct size was 6.6% [IQR 2.6% to 12.1%], which was relatively small. In the preceding GIPS-III trial the median infarct size was 7.1% [IQR 2.4% to 13.7%], with 24% of the patients having an infarct size <2%. When designing the trial, we targeted to exclude those patients with very small infarctions by applying the inclusion criterium "the presence of ongoing ST-segment deviation and/or symptoms at presentation with STEMI", by which we aimed to only select patients with TIMI 0 or 1 flow at study enrollment. Nevertheless, this exclusion criterium did not lead to larger infarct sizes, compared with GIPS-III. One possible explanation is a 20 minutes shorter time to reperfusion in GIPS-IV compared with GIPS-III, likely due to shorter patient delay and/or logistical advances in the local STEMI network. Especially in comparison with other countries and recent studies around the globe, our time to reperfusion is relatively short, leading to small infarctions.[39,40] Also, the inclusion of patients with TIMI 2-3 flow did possibly lead to a population with smaller myocardial infarction and thus little room for substantial myocardial salvage. We cannot exclude that interventions with STS/H_2S would reduce infarct size in a population at higher risk for larger myocardial infarction.

Study design and outcome related explanations

As aforementioned in the patient selection-section we did not succeed in selecting patients at high risk for large infarction and development of heart failure. Nevertheless, we used a state of the art design for our trial. As primary outcome, we measured infarct size by cardiac magnetic resonance imaging, which is the recommended endpoint for early clinical trials, due to its proven link with clinical outcomes (heart failure and mortality) and good reproducibility.[24,41] Although some reviewers questioned the timing of our primary endpoint assessment (at 4

months follow-up), consensus about optimal timing of infarct size assessment is lacking.[24] However, infarct size assessment after the acute phase is more feasible, due to substantial changes in edema and infarct size during the first 10 days after myocardial infarction. At 4 months or later, myocardial infarct size is considered to be stable, whereas remodeling parameters might continue to change.[42,43] Taken together, in our opinion we chose the correct outcome to detect any clinical benefit. All our secondary outcomes were neutral as well. This substantiates the results of our primary endpoint.

FUTURE PERSPECTIVES

The results of previous cardioprotection trials, as well as the results of the GIPS-IV study, underscore that targeting I/R injury is very complex and challenging. The fact that we did not demonstrate clinical benefit of thiol-based cardioprotection against I/R injury, does not necessarily mean that we should give up on thiol- and H_2S-related cardioprotection, nor other therapies reducing I/R injury, in general. Lessons learned from the GIPS-IV trial, may help future clinical trialists within the I/R injury- and H_2S-related research field.

Although we used a dosage of STS with proven efficacy in other diseases, we could not exclude that for cardioprotection higher dosages were required. To overcome such uncertainty, future trials that aim to target I/R injury should consider evaluation of efficacy on surrogate markers (e.g. enzymatic infarct size or effects on underlying mechanisms involved in I/R injury) before undertaking a proof-of-principle clinical trial. In the near future, planned sub-studies using stored samples from the GIPS-IV trial may obtain more insights into the specific actions of STS, e.g. effects on oxidative stress and inflammation. If detected, it could be of interest to test STS in a population at higher risk for large myocardial infarction, since in this population, more benefit of this therapy on the reduction of I/R injury may be expected.[44] In this case, recruitment of patients with anterior myocardial infarction, higher Killip Class, and/or only TIMI 0/1 flow may be considered. The latter, however, implies the study medication will be started only after coronary angiography is completed. However, since the most detrimental effects of reperfusion injury occur in the first minutes to hours after reperfusion, this approach may either delay the appropriate therapy to re-open the vessel or actually lead to late and thus ineffective administration of the protective compound and should therefore be carefully considered. On the other hand, some pathophysiological mechanisms of reperfusion injury last longer than the first hours after reperfusion, including the inflammatory response and metabolic replenishment of ATP.[38,45] The infarct healing and remodeling phase subsequently takes at least another 6-8 weeks.[46] Since H_2S has also effects on angiogenesis and fibrosis, future trials with H_2S-related compounds may also consider a combination of treatments: first administration of an intravenous or intracoronary compound that targets the acute reperfusion injury, followed by oral (H_2S) treatments that affect important mechanisms involved in cardiac remodeling, including fibrosis, angiogenesis, microvascular function and metabolism.

Although the scope of this thesis is mainly focused on administration of STS to reduce I/R injury in the setting of myocardial infarction, the pleiotropic effects of H_2S and the results of **Chapter 4** also suggest that thiol-based cardioprotection may hold promise for other cardiovascular conditions, such as heart failure, but also for protection of donor hearts preceding heart transplantation. As for heart failure, associations between more profound H_2S depletion and more severe cardiac dysfunction have already been established in pre-clinical models of shock.[47] This thesis adds proof that free thiol depletion is associated with worse outcomes in patients with heart failure. Moreover, in **Chapter 3** we observed that the association between free thiols and LVEF was stronger than the association with infarct size. Therefore, we speculate that there might be a larger window of opportunity for H_2S/thiol-based therapies in the remodeling phase and after the development of heart failure post myocardial infarction. Interestingly, favorable effects of H_2S have also been established in experimental models of hypertensive and diabetic heart disease.[48,49] Since these entities often coincide with heart failure with preserved ejection fraction (HFpEF), H_2S treatment might also be of interest in this heart failure subtype. In **Chapter 4** we observed similar associations between free thiols and clinical outcomes for both heart failure subtypes, substantiating that treatment might be beneficial in both HFrEF and HFpEF. However, as already mentioned, before undertaking clinical trials with H_2S-donating and/or thiol-modulating compounds in heart failure, we should gain more mechanistic insights, e.g. whether free thiols are markers or important mediators, and which patients to treat with which dosage. Namely, it has been suggested that interfering in oxidative stress may potentially disturb physiological redox signaling.

Nowadays the pool of donor hearts is not sufficient to meet the rising demand, due to an increasing incidence of end-stage heart failure.[50] Enlargement of the donor pool with non-heart beating donors (donation after circulatory death; DCD) is gaining still more interest.[51] However, an important hurdle to overcome in these (heart) organ transplantations is the accompanying I/R injury. To protect the graft against I/R injury, a hypothermic diastolic arrest is induced using cold crystalloid cardioplegia followed by cold storage during transportation.[52] Because the heart is a metabolically demanding organ and the primary organ leading to a circulatory arrest, the acceptable period for cold storage of the heart is much shorter than for kidney- or liver transplantation.[53] Currently, the DCD procedure for hearts is only implemented in a few countries worldwide. Hence, protecting a donor heart with a H_2S-donor might be an appealing option to provide additional cardioprotection, which, when effective, can be easily and broadly applied. In comparison with H_2S application in myocardial infarction, advantages in the setting of heart transplantation include that H_2S-donating compounds can be administered shortly after the onset of ischemia and timely before reperfusion and can also be delivered more specifically to the heart when added to the cold storage. H_2S induces a reversible hypometabolic state, caused by a reversible cytochrome oxidase c inhibition-related blockade of the mitochondrial respiratory chain,[54] which is especially warranted during the DCD procedure, since reductions in metabolism may preserve the heart for the longer

time and yield more suitable donor hearts. Currently there is early pre-clinical evidence that supports H_2S-donating compounds in heart transplantation, and the use of STS (amongst others) in a 'cold' kidney transplantation model.[55–57]

Last but not least, we would suggest that future I/R trials also aim to target metabolism during the first 24 hours after myocardial infarction. In **Chapter 2** we investigated circulating KBs, and demonstrated that their concentrations at 24 hours were associated with functional outcomes after STEMI. The replenishment of ATP in the mitochondriae takes at least up to 24 hours after the restoration of blood flow and thus supplementation of nutrients during this period may lead to additional myocardial salvage.[58] Although targeting metabolism post STEMI has been investigated for decades now, for example with glucose, insulin and potassium (GIK; aim: improved glycemic control and reduction in free fatty acid metabolism), most trials administered GIK for ≤12 hours, while we hypothesize there might be benefit from longer metabolic modulation. To date, no trials investigated therapeutic effects of exogenous KBs after STEMI. However, KBs, as a more efficient source of energy, when compared with free fatty acids, or glucose, may prove to be beneficial in this setting.[45] Furthermore, other pleiotropic effects of KBs might protect against myocardial I/R.[12] For heart failure and cardiogenic shock, clinical trials with exogenous KBs are rapidly accumulating and several clinical trials are underway (*www.clinicaltrials.gov*). In myocardial I/R injury only pre-clinical studies are ongoing,[59] and one clinical trial with SGLT2-inhibitors, which are also considered to increase circulating KBs, was recently published. The Emmy clinical trial investigated empaglifozine vs. placebo in patients with large myocardial infarction. This trial demonstrated a significant increase in KBs and greater reductions in NT-proBNP in the empaglifozine arm at 26 weeks.[60] Participants were, however, randomized within 72 hours after admission for STEMI and also the future EMPACT study on clinical outcomes initiates empaglifozine within 14 days after STEMI.[61] Therefore, there remains an unmet need for studies investigating exogenous KBs during the acute phase of STEMI.

In conclusion, targeting I/R injury remains challenging, and although many studies in an ideal and academic setting have been neutral, it is premature to give up on cardioprotection. First, because in less developed countries, infarct sizes are still predominantly large and therefore better cardioprotective strategies are of utmost importance. In addition, lessons learned from previous studies, together with advancing (digital) technologies enable us to better understand I/R injury and may further aid us in the development of new pharmacologic strategies against I/R injury.

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