Gene expression profiling of aortic smooth muscle cells treated with a novel H_2S-based compound

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ABSTRACT:
Despite improved healthcare and medical knowledge, cardiac failures after operation are still an important problem. Therefore, interest in alternative treatments like the use of gasotransmitters is high. Gasotransmitters are a novel class of pharmaceutically relevant components which can quickly induce complex biological responses in cells. H_2S, the newest member, exhibits anti-inflammatory and anti-apoptotic properties but is also toxic at higher concentrations. Thus, the need for a reliable H_2S donor is given. One candidate could be the compound SWS95.HCl, a H_2S/COS donor which exhibits additional vasodilatory effects on aortic smooth muscle cells (HCASMC). However, the underlying molecular mechanisms remain unclear so far. Hence, a whole genome approach was chosen to further elucidate the mode of action of SWS95.HCl on HCASMC cells. To study early and late responses alike, time-course experiments were performed and changes in the gene expression patterns were detected by microarray technology. To ensure the relevance of the subsequent analysis, evaluation of data prefiltering as well as comparison of two different statistical analysis methods was performed. Data analysis revealed the upregulation of a variety of genes in biological categories related to anti-apoptosis, smooth muscle contraction and angiogenesis. Furthermore, we showed an enhanced gene expression profile in pathways associated with ischemic preconditioning. SWS95.HCl is therefore a promising candidate for further drug development to treat cardiovascular diseases.

1 INTRODUCTION

Coronary artery disease is the leading cause of death worldwide[1] and characterized by the narrowing or blockage of coronary arteries. This is normally caused by atherosclerosis[2], a disease where cholesterol and fat deposits are built up and form plaques on the inner walls of blood vessels. Those plaques compromise the blood flow by influencing the arterial tone and function or clogging of the artery, which in turn can lead to an acute myocardial infarction - a life threatening condition, commonly known as heart attack.

The shortage of blood supply initially triggers an inflammation reaction. Injured myocardial cells release a cocktail of products that promote chemotaxis and attract leukocytes to the ischemic site. After migration through the endothelial layer of the blood vessels into the extracellular space, the leukocytes adhere onto cardiac myocytes, release their granular contents and produce reactive oxygen species (ROS). Cardiac muscle cells become necrotic in the process and leave collagen scars in the tissue. Those scars impair the function of the heart muscle and lead to the development of aneurysms, valve insufficiency and cardiac arrhythmia. If the duration of the ischemic event is long enough, the patient will die due to massive damage in the myocardium. The subsequent restoration of the blood flow worsens the situation and leads to reperfusion injury when oxygen is reintroduced into already stressed tissue. The resulting oxidative stress damages cellular proteins, DNA and plasma membranes and forces the cell into apoptotic

death.[3] This further increases the area of damaged tissue, which worsens the chances of the patient to survive the incident.

Acute myocardial infarction requires immediate medical treatment. Depending on the time until treatment and the medical equipment of the clinic or ambulance, two different reperfusion strategies are in place:[4]

- Percutaneous coronary intervention (PCI)

  A deflated balloon is feed through the femoral or radial artery into the blocked artery. There, the balloon is inflated and widens the artery, thus allowing the restoration of blood flow. Alternatively, a coronary artery bypass grafting (CABG) can be performed. Here, vessels from elsewhere in the body are used to bypass the blocked artery.

- Thrombolytic therapy

  Thrombolytic drugs are used to dissolve the blockage in the artery and allow the restoration of the blood flow. Examples for thrombolytic drugs are Heparin, Bivalirudin and Fondaparinux which can be combined with supporting drugs. In addition, rescue medications like the antiplatelet agent Aspirin or the glycosaminoglycan Heparin are in use to prevent further complications.

Despite improved medical treatment, the mortality rate from acute myocardial infarction remains significant. Hence, the need of developing new strategies for cardioprotection is clearly given. Ideally, such new therapeutic options could be applied in conjunction to the existing therapies, reduce myocardial infarct size and improve clinical outcome. One such strategy, discovered in the late 80's, is the protecting effect of "ischemia to protect the heart from ischemia".[5] In experiments, significantly delayed cell death during coronary artery ischemia occurred in dogs, which were subjected to ischemic preconditioning (IPC) by nonlethal coronary artery occlusion. Based on these results, a considerable amount of experiments was done to explain the effects of tissue damage prevention. The experiments revealed, that some of the key players involved in the pro-tective effect were phosphoinisitide-3-kinase (PI3K), protein kinase B (Akt) and the blockage of the mitochondrial permeability transition pore (MPT).[6] These findings are in line with a newer concept, which emerged nearly ten years later: the Reperfusion Injury Salvage Kinase pathway (RISK). It is based on the known fact that apoptotic cell death (due to MPT opening) contributes to lethal reperfusion injury. This can be countered by the activation of pro-survival anti-apoptotic kinases such as Akt, Erk1/2 and JNK which converge at the MPT by blocking its opening.[7] Hence, the blocking of the MPT is necessary in ischemic events to prevent further damage to the cells.[8]

In clinical applications, the use of IPC is limited to cases, where the ischemic event is known, such as coronary bypass surgery or heart transplantation. Therefore, another approach was tested: prevention of reperfusion injury through ischemia. It was shown, that intermitted reperfusion reduced the incidence of ventricularfibrillation in a cat model.[9] Based on these experiments, the cardioprotective effects of various substances were tested. Especially the relatively new group of gasotransmitters received a lot of attention because its prominent member nitric oxide was already known for its anti-neutrophilic properties[10] and commonly used for treatment of cardiac diseases. Gasotransmitters, diatomic gaseous molecules, present a novel form of signalling molecules. They have the ability to pass the cell membrane without relying on transport through receptors and therefore have significant advantages over conventional medications in terms of bioavailability and response time[11]. Additionally, they can have endocrine, paracrine or autocrine effects. Furthermore, gasotransmitter are produced endogenously by enzymes, have defined and regulated functions at physiological relevant concentrations, and have specific molecular targets for their cellular effects (which may be mediated by a second messenger).[12] Due to their nature as endogenously produced molecules, specific elimination pathways are already present in the body which makes responses more predictable. Examples for molecules that fulfil

these requirements are nitric oxide (NO), carbon monoxide (CO) and the newest member of the group, H_2S.[12] This gas, formerly known for its deadly image as “gas of rotten eggs”, is generated in many types of mammalian cells. Recent studies have shown anti-inflammatory and anti-apoptotic effects of H_2S on smooth muscle cells in the heart, as well as its potential to act as vasodilator.[13] Furthermore, it is suggested that H_2S could also serve as a potent agent for drug-induced ischemic preconditioning.[14] However, since H_2S is toxic at higher concentrations (>50 µM), the administration of the gaseous substance is greatly limited. Thus the need for a stable H2S donor arose, which releases the gas at controlled levels.

In our study, we investigated genome-wide transcriptional effects of the novel H_2S donor SWS95.HCl on human coronary artery smooth muscle cells (HCASMC) using Affymetrix GeneChip arrays. SWS95.HCl is a diester of the thiocarbonic acid SWS47.HCl (an O-thiocarbamate) and was chosen because of its excellent toxicological profile in preliminary studies on HCASMC cells (data not published).

2 MATERIAL AND METHODS

For microarray analysis, HCASMC cells were isolated from a patient undergoing heart transplan-tation as published earlier[15]. In short, an explant culture was prepared by rinsing the obtained tissue with phosphate buffered saline, cutting it into small pieces of 1-2 mm, placing it on a gelatine coated cell culture dish and adding a few drops of medium 199 (M199; Sigma, St. Luis, MO) containing 20% fetal calf serum (FCS), 100 U/ml penicillin, 100 U/ml streptomycin, 0,25 µg/ml fungizone and 2 mM L-Glutamine (Cambrex, East Rutherford, NJ). The tissue was incubated at 37°C and 5% CO_2 until smooth muscle cells migrated from the explant and formed a confluent layer on the cell culture dish. Subsequently, the cells were trypsinized, seeded in a new cell culture dish and incubated at 37°C and 5% CO_2 until they reached confluence again. Cell character-ization was done by analysis of the morphological appearance and immunostaining with a monoclonal antibody against alpha smooth muscle cell actin (Boehringer Mannheim, Mannheim).

To study early and late responses as well, timecourse experiments were performed. Confluent HCASMC cells were starved prior to the experiment for 24 hours in M199 containing 0,5% bovine serum albumin (BSA). On the next day, media was removed and cells were incubated at 37°C for 2, 4 or 24 hours in M199 containing 0,5% BSA and 200 µM of SWS95.HCl or mock control. All timecourse experiments were performed in duplicates. Preparation of total cellular RNA using High Pure RNA Isolation Kit (Roche, Basel), terminally labelled cDNA by using the GeneChip® 3’ IVT Express Kit and hybridization onto the GeneChip® Prime ViewTM Human Expression array and scanning of the arrays were carried out according to the manufacturer's protocols.

Preprocessing of the data as well as preliminary data filtering was done by using CARMAweb[16] software. First, preprocessing was performed using the RMA algorithm[17,18]. Afterwards, the preliminary data filtering step was evaluated by removal of 90%, 60 % and 0 % of the data set with the least variance, respectively. The filter cut-off value of 40% of the genes with the biggest variance across all samples yielded the highest number of regulated genes and thus was chosen for further analysis (data not shown). After a comparison of two statistical approaches (t-test limma moderated[19] and fold-change), the preprocessed and statistical evaluated data (with t-test, p= 0,05) were further analysed with Gene Set Enrichment software (GSEA).[20,21] For the first analysis, c2 and c5 filters were used, which identify functional sets (pathways) or GO terms (biological categories). To perform a more detailed analysis, seven gene sets were chosen specifically. They represent important aspects of the effect of H2S on the cardiac system, namely “Anti-Apoptosis”, “Inflammatory and Stress Response”, “Smooth Muscle Contraction” and “Angiogenesis”.

3 RESULTS AND DISCUSSION

The genome-wide transcriptome approach yielded an overview of different regulated genes and helped to shed light on the mechanisms of action of SWS95.HCl in the simultaneous activation of various pathways. Importantly, we demonstrate for the first time genome-wide effects of a H_2S releasing donor drug on HCASMC cells over a predefined timecourse. For the Microarray ana-lysis, two statistical approaches were evaluated: t-test and fold-change analysis. T-tests offer an insight into the relation between gene expression data and the underlying noise, the fold change approach analyses the absolute expression value change between two states and highlights huge differences.[22] The direct comparison of both methods revealed a striking difference. While fold change analysis suggested that there was only a handful of relevant gene expression changes, the t-test analysis identified approximately ten times as many relevant genes. An evaluation of the top 50 up- and downregulated genes for each timepoint revealed the underlying cause. The treatment of cells with SWS95.HCl led to a large amount of gene expression changes, however the mean fold change of most genes was not greater than Log +1 or -1. This indicates that the main effect of SWS95.HCl doesn't originate from a strong up- or downregulation of a few selected genes; it rather affects gene expression modestly. Additionally, both methods showed only mod-erate gene expression changes at early timepoints after treatment, while after 24 hours of treatment most gene changes occur (see Table 1). These findings suggest that the effect of SWS95.HCl takes place at late timepoints, probably due to the uptake of the substance into the cell or its stability within the cell before H_2S is released. However, at this late stage of the experi-ment, secondary effects on cells can be seen, too. Thus, gene changes at this level don't neces-sarily reflect the true mode of action of SWS95.HCl but rather could be side effects of the exper-imental setup or indirectly caused by the substance.

Table 1. Comparison of the two statistical methods t-test and fold-change.

Hours	t-test (P <0,05, BH corr.)	Fold-change
2	262	47
4	598	66
24	11138	1448

The above results didn't allow a verified assumption that our substance releases H_2S at all. To ascertain the release assumption, we took a look at cystathione gamma lyase (CSE) expression levels. CSE is the main producer of H_2S in vascular tissue and acts cytoprotective during reperfusion injury through elevated expression levels and hence extended H_2S production in the infarct area[23]. Upregulation of CSE has been reported to occur in response to stimulation with H_2S donors[14] and can be thought as a surrogate marker for actual H_2S release from compounds.

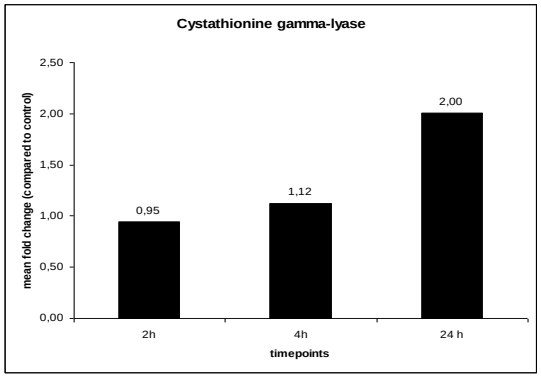

Figure 1. Regulation of Cystathione gamma lyase over time. On the y-axis, mean fold change is shown.

Importantly, an examination of our single gene data revealed a steady upregulation of (CSE) throughout the time course (0,95-, 1,12-, 2-fold after 2, 4 and 24 hrs, respectively; see Figure 1). As we have not directly measured H_2S release in our cells following stimulation with SWS95.HCl, we conclude that this compound does in fact release H_2S, as measured by our microarray screen.

In the last analysis step, the preprocessed and statistical evaluated data were further analysed with Gene Set Enrichment software (GSEA). This software applies predefined filters onto the data and identifies regulated pathways or transcription factors by bringing them into biological context.[21] For the first analysis, c2 and c5 filters were used, which identify pathways or biological categories. The results revealed an even more striking difference in terms of regulated gene sets over time. After 2 hours and 4 hours of treatment with SWS95.HCl, only a few gene sets were statistically significant regulated. In contrast, after 24 hours, 96 gene sets were upregulated, indicating a variety of different pathways affected by SWS95.HCl. To perform a more detailed analysis, seven gene sets were chosen specifically. They represent important aspects of the effect of H_2S on the cardiac system and belong to four major groups, namely “Anti-Apoptosis”, “Inflammatory and Stress Response”, “Smooth Muscle Contraction” and “Angiogenesis”.

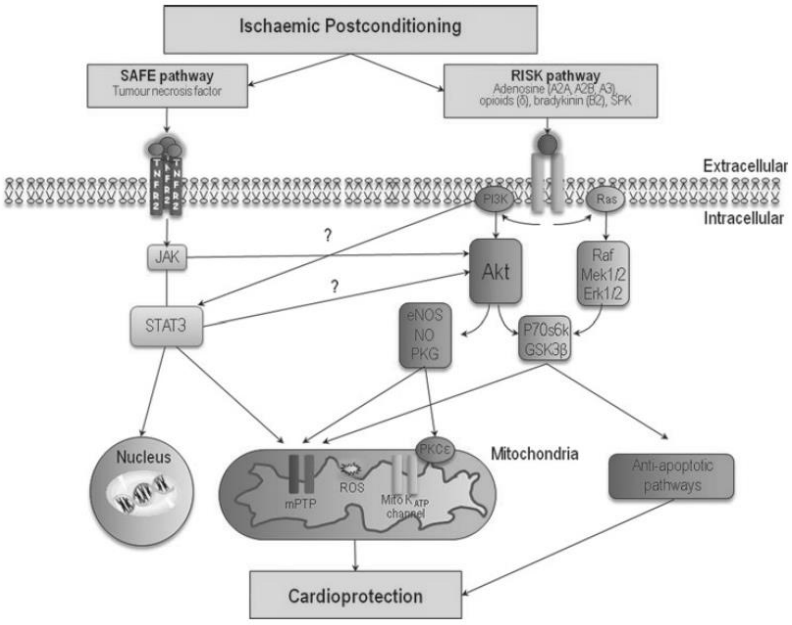

Figure 2. Known mechanisms of ischaemic Postconditioning (IP). The inhibition of the MPT pore is accomplished via two pathways. The SAFE pathway is activated by TNFα and leads to activation of STAT3. The RISK pathway is activated by a various signalling molecules and leads to activation of PI3K and Ras. Image courtesy of D. Hausenloy, 2011[7]

Up to date, there are two major pathways known that are key players in cardiac protection through ischemic postconditioning (see Fig. 2). Both pathways lead mainly to an inhibition of the mito-chondrial permeability transition pore (MPT pore). It has been shown, that the MPT pore is closed during ischemia, but opens once reperfusion starts, leading to mitochondrial swelling and subse-quently cell death. Hence, an inhibition of its opening is favourable during acute myocardial in-farction[7]. The SAFE pathway induces this inhibition by activation of the JAK/STAT pathway via phosphorylation of TNFα[24]. A subsequent member in the signalling cascade is Interleukin 6 (IL-6). In our experiment, a significant upregulation of IL-6 gene expression was seen after 24 hours of treatment with SWS95.HCl. Moreover, we saw an upregulation of sphingosin kinase

1 and 2 as well as phosphatidylinositide-3-kinase (PI3K). Those genes are both associated with activation of STAT3[25] through formation of sphingosine-1-phosphate (S1P) or crosstalk between SAFE and RISK pathway in case of PI3K. Hence, we speculate that treatment with SWS95.HCl may lead to an activation of the SAFE pathway through several targets.

The main players of second inhibitory pathway (called RISK) are PI3K-Akt as well as the Ras-MEK1-2-Erk1/2 signalling cascades. As already mentioned, we saw an upregulation of PI3K after 24 hours of treatment with SWS95.HCl. This further strengthens our hypothesis, that SWS95.HCl acts like a H_2S releasing agent since in vivo and ex vivo studies have proven the activating effect of H_2S donors on the PI3K/Akt pathway.[26] Additional, IGF-1 binding protein 1 was upregulated. It serves as stabilizer for IGF-1, which is known to be a potent activator of the Akt signalling path-way.[27] In contrast to published data[28], we found no increase in gene expression of eNOS after treatment of cells with SWS95.HCl. However, eNOS exists in the endothelial cells as preformed protein and is activated through phosphorylation by Akt in ischemic events[7]. Hence, SWS95.HCl could enhance eNOS activity by the upregulation of IGF-1 binding protein which subsequently leads to Akt activation. Interestingly, we saw no enhanced gene expression of members of the Ras-MEK1-2Erk1/2 signalling cascades. Instead, we observed the upregulation of various other MAP Kinases (p38, GLK, HGK and MAPKAPK2). The effect of H_2S donors on the upregulation or downregulation of p38 in the event of ischemic preconditioning is discussed controversially[26]. However, it was speculated that the various active isoforms of p38MAPK and their apoptotoic as well as anti-apoptotic functions in cardiomyocytes may be responsible for the contradictory re-sults.[29] In summary, based on our gene expression data, the effects of SWS95.HCl on the RISK pathway remain unclear.

Last and in line with recent literature about the cardioprotective effects of H2S donors, we demonstrated an upregulation of VEGF genexpression in cells after treatment with SWS95.HCl. VEGF is strongly associated with angiogenic stimuli and most likely plays a role in left ventricular remodelling after myocardial infarction.[30]

In summary, treatment of HCASMC cells with SWS95.HCl led to a significant upregulation of genes in categories related to anti-apoptosis, smooth muscle contraction and angiogenesis. These findings are in line with recent literature about the cardioprotective effects of H_2S donors. Furthermore, we showed an enhanced genexpression profile in the survival factor enhancement (SAFE) pathway, one of the key players in cardioprotection through ischemic postconditioning.[24]. Looking at the overall picture of regulated genes, one could speculate that the substance causes some sort of hypoxic stress onto HCASMC cells, therefore mimicking ischemic preconditioning. Based on our analysis we further verified our assumption that SWS95.HCl acts as a H_2S gasotransmitter. It steadily upregulates cystathione gamma lyase (CSE) over time – an enzyme that is the main producer of endogenous H_2S in vascular tissue. SWS95.HCl is therefore a promising candidate for further drug development to treat cardiovascular diseases. To further elucidate the mode of action(s) of SWS95.HCl, subsequent gain of function/loss of function experiments should be performed to confirm the data obtained from the microarray experiment. One definitely interesting target for additional experiments is IL-6. It is a prominent member of the survival activation factor enhancement (SAFE) pathway and loss of function experiments should allow additional insight into the cardioprotective actions of H_2S.

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