The role of endogenous H2S production during hibernation and forced hypothermia
Dugbartey, George Johnson

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Dopamine Treatment Attenuates Renal Injury via Production of H₂S in a Rat Model of Deep Hypothermia and Rewarming

George J. Dugbartery¹, Fatemeh Talaei², Martin C. Houwertjes², Maaike Goris¹, Anne H. Epema², Hjalmar R. Bouma¹ ³, Robert H. Henning¹

¹Department of Clinical Pharmacy and Pharmacology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

²Department of Anesthesiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

³Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Submitted
ABSTRACT

Background: Hypothermia and rewarming produces organ injury through the production of reactive oxygen species (ROS). We previously found that cellular uptake of dopamine prevents hypothermia and rewarming-induced apoptosis by H₂S formation through upregulation of the enzyme cystathionine β-Synthase (CBS) in cultured cells. Here, we investigate whether dopamine protects via H₂S pathway in an in vivo rat model of deep hypothermia and rewarming, examining the kidney as a highly vulnerable organ.

Method: In 4 groups of Wistar rats (n=5 each) anesthetized with ketamine, body temperature was decreased to 15°C at a rate of 1°C per 3 minutes, maintained at this temperature for 3 h followed by rewarming at 1°C per 2 minutes. Rats were treated throughout the procedure with vehicle or dopamine infusion, and in the presence or absence of a non-specific inhibitor of H₂S production, amino-oxyacetic acid (AOAA) and compared to non-cooled rats.

Results: Hypothermia and rewarming substantially lowered serum H₂S level, the renal expression of H₂S producing enzymes (CBS, CSE, 3-MST) and induced renal damage as demonstrated by pathological changes in glomeruli and tubuli, high KIM-1 expression and substantial influx of macrophages and neutrophils. AOAA further decreased serum H₂S levels and H₂S-producing enzymes, and aggravated renal damage and white blood cell influx. Infusion of dopamine increased serum H₂S and the expression of H₂S-producing enzymes, and fully protected kidneys from hypothermia and rewarming injury.

Conclusion: Dopamine infusion maintained H₂S production and fully preserved renal integrity during deep hypothermia and rewarming in the rat.
INTRODUCTION

Hypothermia represents a condition in which core body temperature drops below 35°C,\(^1\) interfering with normal metabolism and body function. Hypothermia can either be accidental or intentional, and can be further classified by severity. Deep hypothermia is clinically applied to preserve organs for transplantation and in major cardiac surgery to preserve organ integrity and function.\(^2,3,4\) Irrespective of its clinical usefulness, deep hypothermia is not a panacea. Hypothermia negatively affects oxygen and nutrient availability and the accumulation of metabolic waste, leading to organ injury, of which production of reactive oxygen species (ROS) is an important mediator.\(^5,6\) Further, restoration of blood supply upon rewarming results in excessive generation of ROS.\(^7\) Specifically in the kidney, hypothermia may result in acute tubular necrosis as a result of renal vasoconstriction and ischemia.\(^8\)

Dopamine is a central and peripheral nervous system biogenic monoamine neurotransmitter, which can be also synthesized in non-neuronal tissues including proximal renal tubular cells by decarboxylation of L-DOPA.\(^9,10\) Dopamine has been reported to reduce damage following hypothermic storage of donor kidney transplant in a rat model\(^11\) and in human transplantation.\(^12,13\) We previously identified in cell culture and whole tissue slices that the mechanism of action of dopamine constitutes of an increased synthesis of the endogenous production of hydrogen sulfide (H\(_2\)S) by cystathionine β-synthase (CBS).\(^14\) CBS is a cytosolic enzyme, which comprises the first and the rate-limiting step in the transsulfuration pathway that leads to production of H\(_2\)S.\(^15,16\) In addition to CBS, endogenous H\(_2\)S production may also be catalyzed by another cytosolic enzyme, cystathionine γ-lyase (CSE), and a mitochondrial enzyme, 3-mecaptopyruvate sulfurtransferase (3-MST).

To examine whether dopamine protects against kidney injury in the whole animal, we examined its actions in a rat model of deep hypothermia and rewarming under general anesthesia. Thus, we examined dopamine effects on kidney damage, blood H\(_2\)S levels and expression of H\(_2\)S producing enzymes in the absence and presence of amino-oxyacetic acid (AOAA), a non-specific inhibitor of endogenous H\(_2\)S production.\(^17\)

MATERIALS AND METHODS

Animals
The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC5920). Male Wister rats (Charles River, The Netherlands) with a weight of 400 ± 41g were used for this study. Rats were housed under standard conditions (standard rat chow; Hope Farms, Woerden, The Netherlands) and drinking water provided ad libitum throughout the study period.
Experimental Groups

Wistar rats were allocated to one of 5 experimental groups, being healthy non-cooled control (n=4, euthanized following brief isoflurane anesthesia), or one of the 4 experimental groups that underwent forced cooling and rewarming. Rats in the experimental groups (n=5 each) were treated with vehicle or dopamine, with and without administration of AOAA.

Experimental Design

Figure 1 shows a schematic representation of the experiment. Anesthesia was induced with 2.5% isoflurane in O₂/air (1:1) followed by intubation and mechanical ventilation (Amsterdam Infant Ventilator; Hoekloos, Amsterdam, The Netherlands). Tidal volume was set to achieve normocapnia at a ventilation rate of 50 min⁻¹ (0.5 sec inspiration time). Body temperature was measured rectally. Next, the carotid artery and jugular vein were cannulated to continuously monitor arterial blood pressure and heart rate, and take blood samples. Following this initial preparative phase, isoflurane was stopped and anesthesia was maintained by i.v. infusion of ketamine (5 µg/kg/min) combined with single bolus dose of pancuronium (1.5 mg/kg). Animals were maintained normothermic at 37.0 ± 0.3°C. In this phase, infusion of dopamine (5 µg/kg/min) was initiated at a constant infusion rate and maintained throughout the cooling and rewarming phase in the corresponding experimental groups. Animals treated with AOAA were injected intravenously with 20 mg/kg prior to cooling. Following this normothermic phase, hypothermia was induced by applying icepacks externally, producing an average decrease of body temperature of 1°C per 3 min. Upon reaching a body temperature of 25°C, the infusion rate of ketamine and dopamine were reduced by 50%. When the body temperature reached 15°C, AOAA treated groups received another i.v. bolus injection of 4 mg/kg. Three hours after reaching a body temperature of 15°C, rats were rewarmed at a rate of 1°C per 2 min until they reached a body temperature of 37 ± 0.3°C. Subsequently, rats were maintained normothermic for 60 min followed by euthanization under anesthesia. Arterial blood samples were taken directly after the preparative phase ended at 37°C, just prior to cooling after one h of normothermia at 37°C, upon reaching 15°C, 1 h after reaching 15°C, just prior to rewarming, and 1 h after rewarming to 37°C (figure 1). Plasma samples were collected in heparinized tubes. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis or fixated followed by embedding in paraffin.

Histology

Kidney samples were fixated in zinc solution (0.1M Tris buffer, pH 7.4 with 0.5g calcium acetate, 5.0g zinc acetate and 5.0g zinc chloride) for 6 hours at room temperature followed by storage at 4°C for 24 hours. Next, samples were dehydrated in xylene and a decreasing series of ethanol from 100% to 70% for 12 hours and then embedded in paraffin. Five µm thick sections were deparaffinized in xylene and hydrated in decreasing series of ethanol
from 100% to 70% and distilled water. To evaluate glomerular and interstitial damage, the hydrated kidney samples were oxidized in 0.5% periodic acid solution (PAS) and stained with Schiff reagent and then counterstained with hematoxylin for 1 minute. Kidney sections were examined blindly by two independent observers for contraction of glomerular tuft, mesangial disorganization and shrinkage of glomerular content. The degree of glomerular damage was characterized at a magnification of 400x in 100 glomeruli by a semiquantitative scoring system with scores ranging from 0 to 4. Tubulointerstitial damage was examined and quantified on the basis of tubular dilatation, atrophy of epithelial cells and widening of tubular lumen using a scoring system described by Gross et al.9

**Immunohistochemistry**

Sections were stained for kidney injury molecule (KIM-1), a marker for renal tubular damage (goat polyclonal, diluted 1:50 v/v, Santa Cruz), HIS-48, a marker for neutrophils (mouse monoclonal, generously provided by Prof. F.G.M. Kroese, Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, Groningen, The Netherlands), ED-1, a marker for macrophages (mouse monoclonal, diluted 1:500 v/v, Serotec Ltd, Oxford, UK). In addition, samples were stained for cystathionine beta-synthase (CBS, diluted 1:100 v/v in 1% phosphate buffered saline/bovine serum albumen (PBS/BSA). Secondary and tertiary antibodies used are horseradish peroxidase (HRP)-linked polyclonal rabbit anti-mouse IgG (1:100 v/v, Thermo Scientific, Etten-Leur, Netherlands), HRP-linked polyclonal rabbit anti-goat IgG (1:100 v/v), and HRP-linked polyclonal goat anti-rabbit IgG (1:100 v/v, Thermo Scientific, Etten-Leur, Netherlands). Paraffin sections were dewaxed using a xylol and a decreasing series of ethanol from 100% to 70%, and then subjected to antigen retrieval in 0.1M Tris/HCl buffer, pH 9.0, by overnight incubation at 80°C. Next, sections were washed three times in PBS and blocked in 500 µl of 30% H$_2$O$_2$ and 50µl PBS for 30 minutes followed by incubation with the appropriate primary antibody for 60 minutes at room temperature. Following an additional three times washing step with PBS, samples were incubated for 30 minutes at room temperature with the appropriate secondary antibody after which they were washed three times in PBS and then with tertiary antibody at room temperature for 30 minutes. In the CBS staining however, samples were incubated with secondary and tertiary antibodies for 60 minutes. Finally, following a last washing step, samples were incubated with AEC high sensitivity substrate chromogen for 10-20 min and covered in DAKO Faramount (Dako, Heverlee, Belgium) aqueous mounting medium, and cover slip was applied over the mounting medium.

**Western Blotting**

Frozen kidney tissue samples (~500mg) were homogenized in 0.4 ml of RIPA buffer containing 100 mM protease cocktail inhibitor, 100 mM sodium orthovanadate and 10 mM
mercaptopethanol. After 30 minutes of incubation on ice, the homogenized samples were centrifuged at 14000 rpm at 4°C for 20 minutes. The supernatants were collected and soluble protein concentrations were determined using a Bradford protein assay. Samples were boiled for 5 min, cooled and then frozen at -20°C until use. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was run using 40 µg of protein per slot at 100V for 60 minutes. Proteins were then wet blotted onto nitrocellulose membranes with a pore size of 0.45 µm (Bio-Rad Laboratories, Inc, Germany) using a transfer buffer solution that consisted of 0.25mM Tris (pH 8.5), 192mM glycine and 10% v/v methanol at 4°C for 60 min at 0.3mA. Following blotting, the nitrocellulose membranes were blocked for 30 minutes in TBS + Tween 20 (50mM Tris-HCl, pH 6.8, 150mM NaCl, 0.5% v/v Tween 20, Sigma-Aldrich, USA) with 5% w/v powdered skim milk (Sigma-Aldrich, Switzerland). After decantation of the blocking buffer, membranes were incubated overnight at 4°C with the following primary antibodies at a 1:1000 v/v dilution in 3% BSA/TBST: CBS (mouse monoclonal, Santa Cruz, Te Huissen, Netherlands), CSE (mouse monoclonal, Abnova, Walnut, CA, USA) and 3-MST (rabbit polyclonal, Santa Cruz, Te Huissen, Netherlands). Subsequently, membranes were incubated with HRP-linked polyclonal rabbit anti-goat IgG secondary antibody (1:1000 v/v dilution) in 3% BSA/TBST for 60 minutes and then blots were developed using the SuperSignal West Dura Extended Duration Substrate (IL, USA) according to the manufacturer’s protocol. Protein bands were visualized using Gene Genome (Westburg B.V., Leusden, The Netherlands) and band intensities were quantified using the associated Gene Tools software (Westburg B.V., Leusden, The Netherlands). β-actin was used as a house-keeping protein to normalize protein concentrations.

Measurement of Reactive Oxygen Species
To determine the levels of reactive oxygen species (ROS) in renal tissue, samples from the left kidney were homogenized with 100 ml PBS containing butylatedhydroxytoluene (BHT, Cell Biolabs, Inc, Netherlands) and centrifuged at 10.000g for 5 minutes at 4°C. 50µl of SDS-Lysis solution (Cell Biolabs, Inc, Netherlands) was added to 50 µl of the supernatant and malondialdehyde (MDA) standards in separate microcentrifuge tubes followed by 5 minutes incubation at room temperature. Next, 125 µl of thiobarbituric acid (TBA) Reagent (Cell Biolabs, Inc, Netherlands) was added to each supernatant and standard and incubated at 95°C for 60 minutes. Following this incubation, the samples were cooled to room temperature in an ice bath for 5 minutes and then centrifuged at 1000 g for 15 minutes. The supernatants were transferred to another tube and 150 µl of 2-butanol (Merck, Darmstadt, Germany) was added, vortexed vigorously for 2 minutes and centrifuged for 5 minutes at 10,000g. The level of lipid peroxidation in the tissue sample was estimated by measuring the optical density at 532 nm on a spectrophotometer in 200 µl of the butanol fraction. Using the standard curve, readings were transformed into µM MDA per milligram of tissue.
Serum H$_2$S Measurement
Sulfide antioxidant buffer was prepared from 25 g of sodium salicylate, 6.5 g of ascorbic acid and 8.5 g of sodium hydroxide in 100 mL of distilled water and pH adjusted to ≥ 13. One hundred µl of the sulfide antioxidant buffer was added to 100 µl serum samples. A sulfide sensitive electrode (Lazar Research Laboratories Inc., CA, USA) was immersed into the mixture and the electrode potential was monitored and the stabilized mV reading was recorded. The sulfide ion concentration of the serum was calculated using a standard curve prepared from a stock of 10 mL of the sulfide antioxidant buffer and 24 mg of Na$_2$S.9H$_2$O, according to the manufacturer’s instructions.

Statistical Analysis and Data Presentation
All variables are expressed as mean ± standard error of the mean (SEM). Differences between groups were tested for significance using a One-Way ANOVA (Bonferroni Post-Hoc testing) and Repeated Measures ANOVA (Tukey HSD Post-Hoc testing). P-values < 0.05 were considered statistically significant (SPSS version 22).

RESULTS

Figure 1. The hypothermia and rewarming model in the rat. Body temperature of ketamine anesthetized rats was lowered from 37°C using externally applied icepacks, at an average rate of ~1 °C per 3 minutes to reach a minimum body temperature of 15°C, which was then maintained for 3 h. Rats were then rewarmed at a rate of 1°C per 2 minutes until they reached a body temperature of 37 °C, which was maintained for 60 minutes until euthanization. Arrows indicate blood sampling at different time points.

Dopamine is largely without effect on hemodynamic changes in hypothermia and rewarming
The cardiovascular effect of hypothermia and rewarming was assessed by continuous measurement of heart rate, blood pressure and arterial blood gas analyses. In all groups,
heart rate was reduced to about 75% of normothermic baseline values upon reaching 15°C \( (p < 0.01; \text{figure } 2) \). In addition, animals treated with dopamine experienced a significant increase in heart rate of about 15% during the normothermic phase \( (p < 0.05; \text{figure } 2) \), while dopamine-treated animals showed a similar drop in heart rate during hypothermia as compared to the other groups (figure 2). Heart rate was not affected by AOAA at any of the time points as compared to the other groups (figure 2).

Forced hypothermia resulted in a significant drop in basal systolic blood pressure \( (p < 0.001; \text{figure } 2) \), while rewarming restored systolic blood pressure to baseline values in all groups (figure 2). In addition, dopamine treatment was associated with a small increase in systolic blood pressure amounting about 9.1 ± 5.8 and 25.5 ± 11.0 % in normothermia, respectively before induction of hypothermia and after rewarming \( (p < 0.05; \text{figure } 2) \). AOAA did not affect

---

**Figure 2. Hypothermia induced reduction in heart rate and blood pressure.** Induction of hypothermia to 15 °C resulted in a significant drop in (A) heart rate and (B) systolic blood pressure (closed symbols) and diastolic blood pressure (open symbols) in all groups compared to baseline values. Rewarming after 3 h of hypothermia \( (\text{time } = 6 \text{ h}) \) restored heart rate and blood pressure to baseline values. Heart rate and blood pressure in dopamine-treated animals was significantly higher during the normothermic period prior to the induction of hypothermia \( (\text{time } = 1 \text{ h}) \) and after the rewarming phase compared to other groups \( (p < 0.05) \). Timepoints \( t = 2 - 5 \text{ h} \) represent the hypothermic period. Data are presented as mean ± SEM. V.D.A: \( P < 0.05 \); curve differs significantly from vehicle treated (V), dopamine treated (D) or AOAA treated (A), RM ANOVA with Kruksal-Tukey correction.
systolic blood pressure. Diastolic blood pressure in all groups followed a similar profile as systolic blood pressure (figure 2). Taken together, dopamine slightly increased heart rate and blood pressure under normothermia, but was without effect on the marked reduction in these parameters during hypothermia.

**Dopamine prevents metabolic effects of hypothermia and rewarming**

Arterial blood gas analyses revealed a significant drop in pH upon hypothermia as compared to baseline values at the start of the experiment \((p < 0.01;\) Table 1). Whereas pH increased upon subsequent rewarming, it did not fully restore to baseline level \((p < 0.05;\) Remarkably, dopamine treatment fully prevented the change in pH induced by hypothermia and rewarming (Table 1). Infusion of AOAA attenuated the normalization of blood pH by dopamine during hypothermia and rewarming periods \((p < 0.05;\) Table 1). Thus, dopamine infusion precluded the acidosis induced by forced hypothermia.

Forced hypothermia led to a slight increase in partial arterial carbon dioxide pressure \((\text{PaCO}_2)\), substantial increase in serum lactate and a decrease in bicarbonate level \((\text{HCO}_3^-)\) \((p < 0.05;\) Table 1). Rewarming partially restored lactate levels, although still significantly higher than baseline values \((p < 0.05;\) Table 1). Infusion of dopamine prevented these changes (Table 1). Addition of AOAA in animals infused with dopamine partially precluded the beneficial effect of dopamine on \(\text{HCO}_3^-\) and lactate values, while sole administration of AOAA amplified the effects of hypothermia and rewarming (Table 1). These data show that hypothermia is associated with metabolic lactate acidosis, which was fully attenuated by dopamine treatment.

The lactate acidosis upon hypothermia is likely due to hypoperfusion or poor oxygenation. Hypothermia substantially reduced the partial arterial pressure of oxygen \((\text{PaO}_2)\), which was restored upon rewarming, while dopamine treatment precluded changes in PaO\textsubscript{2} throughout the experiment \((p < 0.01;\) Table 1). Thus, infusion with dopamine prevented the decrease in PaO\textsubscript{2} level upon hypothermia.

**Dopamine prevents hypothermia and rewarming-induced kidney dysfunction and damage**

As a marker of kidney function, we measured serum creatinine levels in all experimental groups throughout the procedure. Serum creatinine level increased in the vehicle-treated group during hypothermia. In addition, rewarming induced a further rise of the serum creatinine level \((p < 0.05;\) figure 4B) as compared to the pre-procedure value. Infusion of dopamine prevented the increase in serum creatinine levels during both hypothermia and rewarming \((p < 0.01;\) figure 4B). Dopamine maintenance of serum creatinine levels was unchanged by administration of AOAA \((p > 0.05;\) figure 4B). However, AOAA treatment alone increased the level of serum creatinine to a level significantly higher than the vehicle-treated
Table 1. Arterial blood gas analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Body temp (°C)</th>
<th>Time from start (h)</th>
<th>Vehicle</th>
<th>Dopamine</th>
<th>AOAA</th>
<th>Dopamine + AOAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>37</td>
<td>0</td>
<td>7.38 ± 0.01</td>
<td>7.39 ± 0.01</td>
<td>7.39 ± 0.03*</td>
<td>7.38 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1</td>
<td>7.38 ± 0.01</td>
<td>7.39 ± 0.02</td>
<td>7.38 ± 0.02</td>
<td>7.37 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>7.29 ± 0.02</td>
<td>7.41 ± 0.01</td>
<td>7.23 ± 0.02*</td>
<td>7.20 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>7.26 ± 0.04</td>
<td>7.30 ± 0.01</td>
<td>7.15 ± 0.02</td>
<td>7.19 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>7.22 ± 0.01</td>
<td>7.36 ± 0.02</td>
<td>7.13 ± 0.02</td>
<td>7.17 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>7.20 ± 0.01</td>
<td>7.37 ± 0.01</td>
<td>7.02 ± 0.01*</td>
<td>7.16 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6</td>
<td>7.29 ± 0.03</td>
<td>7.39 ± 0.02</td>
<td>7.27 ± 0.02*</td>
<td>7.29 ± 0.02*</td>
</tr>
<tr>
<td>PaO₂</td>
<td>37</td>
<td>0</td>
<td>23.1 ± 0.1</td>
<td>23.3 ± 0.3</td>
<td>23.4 ± 0.2</td>
<td>23.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1</td>
<td>23.1 ± 0.4</td>
<td>23.5 ± 0.2</td>
<td>23.0 ± 0.2</td>
<td>23.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>12.2 ± 0.2^2</td>
<td>23.0 ± 0.3</td>
<td>17.4 ± 0.5^*</td>
<td>27.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>12.2 ± 0.1</td>
<td>24.5 ± 0.1</td>
<td>14.8 ± 0.3</td>
<td>30.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>12.2 ± 0.4</td>
<td>24.5 ± 0.3</td>
<td>14.3 ± 0.1</td>
<td>30.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>12.2 ± 0.3</td>
<td>24.5 ± 0.2</td>
<td>13.5 ± 0.2</td>
<td>27.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6</td>
<td>21.6 ± 0.4^*</td>
<td>25.2 ± 0.2</td>
<td>20.9 ± 0.4^*</td>
<td>25.9 ± 0.2</td>
</tr>
<tr>
<td>PaCO₂</td>
<td>37</td>
<td>0</td>
<td>5.1 ± 0.2</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.1</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1</td>
<td>5.1 ± 0.1</td>
<td>5.7 ± 0.1</td>
<td>6.1 ± 0.1</td>
<td>6.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>6.0 ± 0.3</td>
<td>5.2 ± 0.1^*</td>
<td>6.0 ± 0.3^*</td>
<td>8.1 ± 0.1^*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>6.0 ± 0.2</td>
<td>5.2 ± 0.3</td>
<td>5.7 ± 0.2</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>6.1 ± 0.4</td>
<td>4.9 ± 0.2</td>
<td>5.5 ± 0.4</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>6.1 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6</td>
<td>4.4 ± 0.2^*</td>
<td>3.6 ± 0.3^*</td>
<td>4.8 ± 0.2^*</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>37</td>
<td>0</td>
<td>24.5 ± 0.1</td>
<td>24.9 ± 0.2</td>
<td>24.6 ± 0.2</td>
<td>24.7 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1</td>
<td>24.5 ± 0.1</td>
<td>24.2 ± 0.1</td>
<td>24.6 ± 0.2</td>
<td>24.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>24.9 ± 0.3^*</td>
<td>22.2 ± 0.1</td>
<td>24.1 ± 0.1^*</td>
<td>23.9 ± 0.2^*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>18.1 ± 0.2</td>
<td>20.2 ± 0.3</td>
<td>17.8 ± 0.4</td>
<td>21.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>17.7 ± 0.1</td>
<td>23.6 ± 0.1</td>
<td>16.1 ± 0.2</td>
<td>20.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>17.1 ± 0.3</td>
<td>24.8 ± 0.2</td>
<td>15.0 ± 0.3</td>
<td>18.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6</td>
<td>16.0 ± 0.4^*</td>
<td>25.8 ± 0.1</td>
<td>15.4 ± 0.5^*</td>
<td>19.5 ± 0.4^*</td>
</tr>
<tr>
<td>Glu</td>
<td>37</td>
<td>0</td>
<td>12.5 ± 0.1</td>
<td>12.7 ± 0.3</td>
<td>12.6 ± 0.1</td>
<td>12.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1</td>
<td>12.4 ± 0.2</td>
<td>10.8 ± 0.2</td>
<td>13.3 ± 0.1</td>
<td>12.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>15.6 ± 0.3^*</td>
<td>12.8 ± 0.3</td>
<td>23.5 ± 0.1^*</td>
<td>16.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>19.5 ± 0.2</td>
<td>15.2 ± 0.3</td>
<td>24.0 ± 0.3</td>
<td>16.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>19.7 ± 0.3</td>
<td>15.7 ± 0.2</td>
<td>24.4 ± 0.2</td>
<td>17.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>19.7 ± 0.1</td>
<td>16.8 ± 0.1</td>
<td>23.0 ± 0.1</td>
<td>17.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6</td>
<td>20.8 ± 0.2^*</td>
<td>11.7 ± 0.2</td>
<td>19.0 ± 0.3^*</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>Lac</td>
<td>37</td>
<td>0</td>
<td>1.9 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.4</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1</td>
<td>1.9 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>2.7 ± 0.1</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2</td>
<td>6.6 ± 0.2^*</td>
<td>2.1 ± 0.3</td>
<td>6.6 ± 0.1^*</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>4.2 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>7.5 ± 0.2</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4</td>
<td>4.0 ± 0.1</td>
<td>1.8 ± 0.3</td>
<td>7.9 ± 0.1</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>5</td>
<td>3.9 ± 0.3</td>
<td>1.5 ± 0.2</td>
<td>8.4 ± 0.3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>6</td>
<td>3.1 ± 0.1^*</td>
<td>2.3 ± 0.1</td>
<td>12.3 ± 0.2^*</td>
<td>5.9 ± 0.1</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. Interventions are compared to vehicle using Repeated Measures ANOVA

*P < 0.01: time point 1 vs 5; *P < 0.05: time point 2 vs 6.
Figure 3. Dopamine infusion prevents hypothermia and rewarming induced kidney injury. (A) Representative sections of the kidney (magnification x400) from all groups showing PAS, KIM-1, ED-1, HIS-48 and CBS stainings. Arrows point to positively stained areas (in brown). (B-F) panels show the quantification of immunohistochemical stainings of (B) PAS, (C) ED-1, (D) HIS-48, (E) KIM-1 and (F) CBS. PAS = Periodic Acid Schiff, ED-1 = Macrophage marker, HIS-48 = Neutrophil marker, KIM-1 = Kidney Injury Molecule, CBS = Cystathionine β-synthase. NC = Non-cooled; veh = Vehicle; dopa = Dopamine; A0AA = A0AA; dopa+AOAA = Dopamine+AOAA. Data are presented as mean ± SEM. */** P < 0.05/0.01 compared to non-cooled.
group ($p < 0.05$; figure 4B). Thus, dopamine prevented the hypothermia-induced increase in
serum creatinine, which was further increased by blockade of H$_2$S-producing enzymes with
AOAA in the absence of dopamine.

To further evaluate the effect of hypothermia on the kidney, we examined histopathological
changes in kidney tissues obtained at one hour after rewarming. Hypothermia is associated
with substantial glomerular and tubular injury as assessed by PAS- and KIM-1 stains
compared to the untreated group ($p < 0.01$; figure 3A, 3B, 3E). Dopamine infusion fully
prevented these morphological changes (figure 3A, 3B, 3E), while AOAA induced excess
renal injury as compared to vehicle-treated group ($p < 0.05$; figure 3A, 3B, 3E). Furthermore,
the protection from kidney damage offered by dopamine infusion was unaffected by AOAA
($p < 0.05$; figure 3A, 3B, 3E). Thus, dopamine treatment reduces morphological changes
and the expression of markers of tubular damage induced by hypothermia and rewarming.

Further, hypothermia and rewarming induced a substantial influx of macrophages and
neutrophils in the kidney in hypothermia and rewarmed animals as compared to non-cooled
control animals ($p < 0.01$; figure 3A, 3C, 3D). Similarly to damage markers, administration
of dopamine fully prevented the increase in influx of these cells, which was unaffected by
coadministration of AOAA ($p > 0.05$; figure 3A, 3C, 3D). Treatment with AOAA on the other
hand, resulted in an excess influx of macrophages and neutrophils as compared to vehicle-
treated animals ($p < 0.05$; figure 3A, 3C, 3D).

Finally, ROS production in kidney tissue was assessed by measurement of MDA levels.
Hypothermia and rewarming induced a 5-fold increase in MDA ($p < 0.01$; figure 4C).
Treatment with dopamine fully prevented the increase in MDA ($p > 0.05$; figure 4C), which
was not affected by AOAA administration in these animals (figure 4C, $p > 0.05$). Also, MDA
levels were higher in animals treated with AOAA compared to vehicle treated animals ($p <
0.05$; figure 4C). Thus, dopamine reduces renal ROS production associated with hypothermia
and rewarming in parallel to changes observed in serum H$_2$S levels. Collectively, these data
demonstrate that dopamine attenuates kidney injury following hypothermia and rewarming.

**Dopamine maintains renal expression of H$_2$S-producing enzymes and serum levels of
endogenous H$_2$S in hypothermia and rewarming**

To explore the potential mechanism by which dopamine protects from hypothermia and
rewarming damage in kidney, we determined the renal expression of the three different
H$_2$S-producing enzymes (CBS, CSE and 3-MST). Hypothermia and rewarming resulted
in significant decrease in the expression of CBS, CSE and 3-MST as compared to non-
cooled animals ($p < 0.05$; figure 4D). Notably, dopamine infusion maintained renal CBS
expression at the level of non-cooled animals both in immunostaining and western blot ($p$
Addition of AOAA did not affect the maintenance of CBS expression in dopamine-treated animals ($p > 0.05$; figure 4D). Similar to CBS, renal CSE expression was substantially downregulated following hypothermia and rewarming as compared to non-cooled animals ($p < 0.01$; figure 4E). However, dopamine treatment not only restored, but markedly upregulated CSE expression by 210% compared to non-cooled animals ($p < 0.01$; figure 3E). Administration of AOAA, both in vehicle or dopamine treated animals, strongly downregulated CSE expression level below the detection limit (figure 4E). The expression of renal 3-MST was markedly reduced in hypothermic and rewarmed rats compared to non-cooled animals ($p < 0.05$; figure 4F), which was prevented by dopamine ($p > 0.05$; figure 4F). Addition of AOAA in either vehicle-treated or dopamine-treated animals, did not affect

Figure 4. Dopamine prevents metabolic effects of hypothermia and rewarming and maintains the expression of H$_2$S-producing enzymes. (A) Dopamine treatment maintained blood pH during hypothermia and rewarming, while other groups show a significant decrease compared to their baseline values ($p < 0.05$). (B) Dopamine infusion maintains serum H$_2$S level during hypothermia near levels found in non-cooled animals, irrespective of AOAA co-administration. Vehicle- and AOAA-treated groups show substantial decrease in serum H$_2$S, which is not restored after rewarming ($p < 0.01$). (C) Dopamine infusion attenuated the increase in renal MDA found in vehicle, irrespective of AOAA co-administration ($p > 0.05$). (D) Dopamine maintained renal CBS expression both in the absence and presence of AOAA treatment ($p < 0.01$). (E) Dopamine strongly upregulates renal CSE expression, which was fully annihilated by AOAA ($p < 0.01$). (F) Dopamine maintains renal 3-MST expression, which was blocked by co-treatment with AOAA. */** represents $p < 0.05/0.01$; RM ANOVA (A,B) or ANOVA (C-F). Data are presented as mean ± SEM.
the expression levels of 3-MST (figure 4F). Thus, hypothermia and rewarming substantially
downregulated the expressions of all H$_2$S-producing enzymes in the kidney, which was fully
attenuated by dopamine infusion.

Next, we investigated whether changes in the expression of H$_2$S producing enzymes in
the kidney is paralleled by changes in serum levels of H$_2$S. Hypothermia induced a strong
reduction in serum H$_2$S levels that was maintained throughout hypothermia compared to
baseline value ($p < 0.01$; figure 4A). Remarkably, subsequent rewarming did not restore serum
H$_2$S levels (figure 4A). Dopamine infusion slightly increased baseline serum H$_2$S level ($p <
0.05$; figure 4A) and completely attenuated its decrease during the subsequent hypothermia
and rewarming phases (figure 4A). Administration of AOAA to animals infused with dopamine
partly prevented the effect of dopamine on the serum H$_2$S level upon hypothermia ($p < 0.05$;
figure 4A), while administration of AOAA alone further reduced the H$_2$S level ($p < 0.05$; figure
4A). Thus, dopamine prevented the hypothermia induced decrease in serum H$_2$S.

DISCUSSION

In the present study, we demonstrate the protective effect of dopamine infusion against renal
injury caused by whole body hypothermia and rewarming in the rat. Our data implicate the
maintenance of production of H$_2$S as a major mechanism of action of dopamine, as dopamine
prevented the drop in serum H$_2$S levels upon hypothermia. Further, dopamine preserved the
expression of the H$_2$S producing enzymes CBS and 3-MST and even increased expression of
CSE in the kidney following hypothermia and rewarming. Further evidence for the importance
of H$_2$S production in hypothermia and rewarming-induced renal damage is derived from
the group infused with AOAA, a non-specific blocker of H$_2$S production, in which renal
damage was substantially aggravated. Remarkably, the detrimental effects of AOAA on
kidney damage and ROS production in hypothermia and rewarming were fully overcome by
dopamine co-infusion. However, dopamine only maintained a normal expression of CBS in
the kidney of AOAA treated animals, but did not rescue the AOAA invoked downregulation
of CSE and 3-MST. Thus, our data extend our findings in cell culture and organ slices, by
showing that the beneficial effect of dopamine maintains endogenous H$_2$S production during
hypothermia and rewarming in the whole animal.

In vivo hypothermic and rewarmed rat as a model of hypothermia

To the best of our knowledge, this study is the first demonstrating the beneficial action of
dopamine in hypothermia and rewarming injury in vivo, thus extending previous in vitro
observations in cells and isolated organs. Although the in vitro hypothermia and
rewarming protocol we used is less extensive in magnitude and duration than usually applied
in the transplantation setting, its effects are remarkably similar. Particularly, the increase in
renal ROS production, expression of KIM-1 and influx of immune cells in the renal interstitium we found in our model are also common findings in 4 °C cold-preserved kidney transplants.\textsuperscript{18,19} In addition, the downregulation of CBS following hypothermia and rewarming was previously observed in cultured cells and organ slices.\textsuperscript{14} Thus, our model bears great resemblance to transplant cooling, and may explain the beneficial effect of dopamine pretreatment of donors on kidney outcome following transplantation.\textsuperscript{20} Moreover, our model seems to closely match the clinical situation in deep hypothermia in which there is unavoidable (compensated) metabolic acidosis,\textsuperscript{21} elevated level of serum creatinine, and drastic fall in cardiovascular parameters such as heart rate and blood pressure.\textsuperscript{21} Thus, the hypothermic and rewarmed rat represents an adequate model for examining hypothermia and rewarming-induced renal injury in the \textit{in vivo} situation.

\textit{Influence of hypothermia and rewarming on H}_2\textit{S biology}

In developing our model, we identified a profound impact of hypothermia and rewarming on H\textsubscript{2}S biology. Hypothermia induced a substantial decrease in serum H\textsubscript{2}S level, which did not restore during the rewarming. Further, hypothermia and rewarming substantially reduced the renal expression of all three H\textsubscript{2}S-producing enzymes, extending previous observations in cells and tissue slices in which hypothermia down regulated CBS expression.\textsuperscript{14} While the source of plasma H\textsubscript{2}S is currently unknown, there are speculations that it is derived from circulating enzymes\textsuperscript{22} or rapidly diffuses from tissues following local production.\textsuperscript{23,24} The concomitant decrease in serum H\textsubscript{2}S and the reduction in renal expression of H\textsubscript{2}S-producing enzymes in hypothermic and rewarmed animals treated with vehicle or AOAA seems compatible with the last option. To further substantiate the involvement of H\textsubscript{2}S, animals were treated with AOAA, a non-specific inhibitor of H\textsubscript{2}S-producing enzymes.\textsuperscript{17} Administration of AOAA reduced serum H\textsubscript{2}S levels both at baseline and throughout the hypothermic and rewarming periods, and resulted in a substantial increase in ROS production and renal damage. Moreover, the decrease in renal expression of all three H\textsubscript{2}S-forming enzymes by hypothermia and rewarming was amplified by AOAA treatment. Therefore, the decrease in H\textsubscript{2}S and/or H\textsubscript{2}S-producing enzymes during hypothermia may represent the mechanism inducing the observed renal damage following hypothermia and rewarming. As H\textsubscript{2}S has been reported to be renoprotective in several animal models of ischemia/reperfusion injury via its anti-apoptotic and anti-inflammatory properties,\textsuperscript{25,26,27} such properties may have conferred a similar renoprotection under hypothermia and rewarming.

\textit{Dopamine increases the endogenous production of H\textsubscript{2}S}

Dopamine treatment increased baseline serum H\textsubscript{2}S level and maintained this level during the entire hypothermia and rewarming procedure, giving support to findings in cells where dopamine increased H\textsubscript{2}S production.\textsuperscript{14} Dopamine infusion maintained serum H\textsubscript{2}S even
during the hypothermic phase, most likely reflecting increased H$_2$S production, as reported previously *in vitro* during hypothermia at 3°C. Moreover, dopamine preserved (CBS, 3-MST) or even increased (CSE) expression of H$_2$S-producing enzymes in the kidney following hypothermia and rewarming. Our data show that much of the beneficial effect of dopamine was maintained under co-infusion of AOAA, including the maintenance of serum H$_2$S levels, albeit at a slightly lower level than observed in dopamine-treated animals. Further, in the presence of AOAA, dopamine reduced renal ROS production and expression of damage markers, and fully preserved the expression of CBS. However, dopamine infusion failed to maintain the renal expression of 3-MST and particularly of CSE in hypothermic and rewarmed animals treated with AOAA. Our data show that much of the beneficial effect of dopamine was maintained under co-infusion of AOAA, including the maintenance of serum H$_2$S levels, albeit at a slightly lower level than observed in dopamine-treated animals. Further, in the presence of AOAA, dopamine reduced renal ROS production and expression of damage markers, and fully preserved the expression of CBS. However, dopamine infusion failed to maintain the renal expression of 3-MST and particularly of CSE in hypothermic and rewarmed animals treated with AOAA. This observation suggests that preservation of kidney integrity is most dependent on the maintenance of CBS expression, rather than CSE or 3-MST. Such notion is in accord with previous data showing that selective down-regulation of CBS by siRNA abrogates the beneficial action of dopamine during hypothermia and rewarming. Also, Sen *et al.* observed sodium hydrosulfide (an H$_2$S donor) to prevent chronic kidney injury in uninephrectomized CBS-knockout mice. However, also in CSE-knockout mice, high vulnerability to kidney injury following ischemia-reperfusion injury (IRI) was reported. Taken together, our data imply a substantial effect of dopamine on serum H$_2$S levels and expression of H$_2$S-producing enzymes, which is highly consistent with its action observed in cells and organs. Therefore, the protective effects of dopamine during hypothermia and rewarming may well result from maintenance of H$_2$S level.

*Additional Dopamine effects in preservation of kidney function and integrity*

Dopamine has been reported to maintain renal blood flow (RBF) and glomerular filtration rate (GFR) in both animal and human studies, which has been attributed to the activation of specific dopamine receptors. Alternatively, preservation of H$_2$S levels by dopamine, either systemically or locally, may induce H$_2$S mediated vasodilation, resulting in an increase RBF and GFR, as found in L-cysteine infused rat. Our results further demonstrate dopamine to convey a potent antioxidant action in kidney tissue, which likely protects the organ in hypothermia and rewarming. This action of dopamine may be explained by 2 mechanisms. First, dopamine may protect cells from the damaging effect of ROS through increased formation of H$_2$S. Protection from ROS through enhanced H$_2$S availability is supported by several *in vivo* and *in vitro* studies demonstrating that exogenous administration of H$_2$S increases the production and activity of antioxidant enzymes and protects against oxidative stress. Second, dopamine itself also possesses ROS-scavenging properties, which is thought to prevent injury by cold-preservation and ischemia-reperfusion in cells. In accord, dopamine-infused animals co-treated with AOAA still show a reduced ROS formation following hypothermia and rewarming compared to vehicle-treated animals. However, dopamine infusion also produces high serum H$_2$S level and maintains expression of CBS in
AOAA co-treated animals. Thus, dopamine attenuation of ROS production and subsequent kidney damage may originate from antioxidant effects either through the maintenance of $H_2S$ production or its scavenging properties. Our data are, however, consistent with the view that maintenance of $H_2S$ by dopamine limits ROS production.

Conclusion
In summary, we have shown for the first time that dopamine preserves kidney function and integrity in whole body cooling and rewarming in the rat. A major mechanism involved seems to consist of the dopamine-induced increase in the endogenous $H_2S$ production, possibly through its maintenance of the expression of $H_2S$-producing enzymes in rat kidney. Therefore, implementing dopamine treatment in clinical conditions, which require hypothermia and rewarming, may have the potential to ameliorate renal injury.

ACKNOWLEDGEMENTS
This study was supported by grants from Groningen University Institute for Drug Exploration (GUIDE). The University of Groningen together with University Medical Centre Groningen (UMCG, Netherlands) provided the laboratory and all materials and equipment for this study. We thank Prof. Frans Kroese and Prof. Harry van Goor (UMCG, Netherlands) for kindly providing the HIS-48 and CSE antibody, respectively.

DISCLOSURES
None
REFERENCES


