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Dopamine treatment attenuates acute kidney injury in a rat model of deep hypothermia and rewarming – The role of renal H_2S-producing enzymes

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ABSTRACT

Hypothermia and rewarming produces organ injury through the production of reactive oxygen species. We previously found that dopamine prevents hypothermia and rewarming-induced apoptosis in cultured cells through increased expression of the H_2S-producing enzyme cystathionine β-Synthase (CBS). Here, we investigate whether dopamine protects the kidney in deep body cooling and explore the role of H_2S-producing enzymes in an *in vivo* rat model of deep hypothermia and rewarming. In anesthetized Wistar rats, body temperature was decreased to 15 °C for 3 h, followed by rewarming for 1 h. Rats ($n \geq 5$ per group) were treated throughout the procedure with vehicle or dopamine infusion, and in the presence or absence of a non-specific inhibitor of H_2S-producing enzymes, amino-oxyacetic acid (AOAA). Kidney damage and renal expression of three H_2S-producing enzymes (CBS, CSE and 3-MST) was quantified and serum H_2S level measured. Hypothermia and rewarming induced renal damage, evidenced by increased serum creatinine, renal reactive oxygen species production, KIM-1 expression and influx of immune cells, which was accompanied by substantially lowered renal expression of CBS, CSE, and 3-MST and lowered serum H_2S levels. Infusion of dopamine fully attenuated renal damage and maintained expression of H_2S-producing enzymes, while normalizing serum H_2S. AOAA further decreased the expression of H_2S-producing enzymes and serum H_2S level, and aggravated renal damage. Hence, dopamine preserves renal integrity during deep hypothermia and rewarming likely by maintaining the expression of renal H_2S-producing enzymes and serum H_2S.

1. Introduction

Hypothermia represents a condition in which core body temperature drops below 35 °C (Jurkovich, 2007) interfering with normal metabolism and body function. The kidney is highly vulnerable to hypothermia and acute kidney injury has been reported in over 40% of accidental hypothermic cases (Megarbane et al., 2000), resulting in acute renal tubular necrosis caused by renal vasoconstriction and ischemia (Yoshitomi et al., 1998; Hottelart et al., 2004). Deep hypothermia, i.e. a temperature below 20 °C, is also employed intentionally as a therapeutic approach to preserve cells or organs, *e.g.* in transplantation, major cardiac surgery and neurological injuries (Polderman, 2008). However, hypothermia negatively affects oxygen and nutrient availability and the accumulation of metabolic waste, leading to organ injury, of which production of reactive oxygen species is an important meditator (Haugen and Nath, 1999). Further, restoration of blood supply upon rewarming results in excessive generation of reactive oxygen species (Carden and Granger, 2000).

Dopamine is a biogenic monoamine neurotransmitter, which is also synthesized in non-neuronal tissues including proximal renal tubular cells by decarboxylation of L-DOPA (Armando et al., 2015; Gottmann et al., 2006). Dopamine is known to reduce renal damage following hypothermic storage of donor kidney transplants in a rat model (Schnuelle et al., 2004) and in human transplantation (Yard et al., 2004; Schnuelle et al., 2009). We previously identified in cell culture the mechanism of action of dopamine to protect from cooling and rewarming injury, constituting of cellular dopamine uptake, in turn increasing the expression of the enzyme cystathionine β-synthase (CBS). Specificity of this route was demonstrated by the abrogation of dopamine effects when CBS upregulation was inhibited by specific siRNA treatment (Talaei et al., 2011). CBS is a cytosolic enzyme, which comprises the first and

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rate-limiting step in the transsulfuration pathway that leads to production of H_2S (Geng et al., 2004; Beard and Bearden, 2011). In addition to CBS, endogenous H_2S production can also be catalyzed by another cytosolic enzyme, cystathionine γ-lyase (CSE), and a mitochondrial enzyme, 3-mecaptopyruvate sulfurtransferase (3-MST). In turn, H_2S displays potent anti-oxidant actions and its therapeutic action to counteract reactive oxygen species associated damage upon exogenous administration has been demonstrated in various models and various organs (see for review (Wallace and Wang, 2015)). Nevertheless, the role of H_2S-producing enzymes and endogenous production of H_2S in such organ damage has received much less attention. Based on this we hypothesized that dopamine protects against acute kidney injury in whole body deep hypothermia by inducing or maintaining the expression of H_2S-producing enzymes. As a target body temperature we chose 15 °C, representing the hypothermia used in cardiac surgery with circulatory arrest and the lowest body temperature with completely recovery in humans (Gilbert et al., 2000). Thus, we examined dopamine actions in a rat model of deep hypothermia and rewarming (H/R) under general anesthesia by examining kidney damage, expression of H_2S-producing enzymes (CBS, CSE and 3-MST) and serum H_2S levels in the presence and absence of amino-oxyacetic acid (AOAA), a non-specific inhibitor of H_2S-producing enzymes (Asimakopoulou et al., 2013).

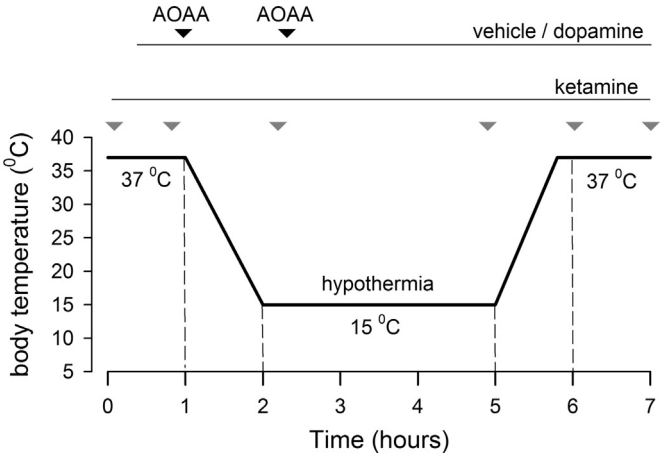

Fig. 1. The hypothermia and rewarming model in the rat. Body temperature of ketamine anesthetized rats was lowered from 37 °C using externally applied icepacks, at an average rate of ~1 °C per 3 min to reach a minimum body temperature of 15 °C, which was then maintained for 3 h. Rats were then rewarmed at a rate of 1 °C per 2 min until they reached a body temperature of 37 °C, which was maintained for 60 min until euthanization. Gray arrowheads indicate blood sampling at different time points; black arrowheads indicate administration of AOAA; infusions are indicated by the top horizontal lines.

2. Materials and methods

2.1. Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC #5920) and experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Male Wister rats (Charles River, The Netherlands) with a weight of 400 ± 41 g were used for this study. Rats were housed under standard conditions with food and drinking water provided *ad libitum*.

2.2. Experimental groups

Rats were randomly allocated to one of 5 experimental groups, being non-cooled control ($n=4$, euthanized following brief isoflurane anesthesia) and 4 experimental groups that underwent H/R ($n=5$ each), which were treated with vehicle or dopamine, with and without administration of aminooxyacetic acid (AOAA).

2.3. Experimental design

Fig. 1 shows a schematic representation of the experiment. Anesthesia was induced with 2.5% isoflurane in O_2/air (1:1) followed by intubation and mechanical ventilation (Infant Ventilator; Hoekloos, Amsterdam, Netherlands). Tidal volume was set to achieve normocapnia at a ventilation rate of 50 min^{-1} (0.5 s inspiration time). Body temperature was measured rectally. Carotid artery and jugular vein were cannulated to continuously monitor arterial blood pressure and heart rate, and take blood samples (Samarska et al., 2013). Subsequently, at $t=0$, anesthesia was switched to i.v. infusion of ketamine (5 μg/kg/min) combined with single bolus dose of pancuronium (1.5 mg/kg). Rats were maintained normothermic (37.0 ± 0.3 °C) for one h prior to cooling. At $t=30$ min, infusion of dopamine (5 μg/kg/min) was initiated and maintained throughout the cooling and rewarming phase. AOAA (20 mg/kg) was injected intravenously prior to cooling and repeated at 4 mg/kg when reaching 15 °C. Rats were maintained on a water-filled mattress, circulated with water of 4–10 °C or 37 °C. Hypothermia at a rate of 1 °C per 3 min was induced by additionally applying icepacks. At 25 °C, infusion rates of ketamine and dopamine were reduced by 50%. Three hours after reaching 15 °C, rats were rewarmed at 1 °C per 2 min until 37.0 ± 0.3 °C by the heating mattress and application of hot air. Subsequently, rats were maintained normothermic for 60 min followed by euthanization. As body temperature was entirely controlled by external cooling or heating, no differences exist in level and rate of change of body temperature between groups. Arterial blood samples were obtained at baseline 37 °C (without infusions), just prior to cooling at 37 °C (with infusions), upon reaching 15 °C and 1 and 3 h thereafter and 1 h after rewarming to 37 °C. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until further analysis or fixated followed by embedding in paraffin. Blood gas analysis was performed at the central laboratory of the University Medical Centre Groningen in 0.1 ml blood samples obtained from the carotid artery.

2.4. Histology

To evaluate glomerular and interstitial damage, 5 μm deparaffinized sections were oxidized in 0.5% periodic acid solution (PAS) and stained with Schiff reagent and counterstained with hematoxylin. Sections were examined blindly by two independent observers for glomerular damage at 400× in 100 glomeruli by a semiquantitative scoring from 0 to 4 (el Nahas et al., 1991). Tubulointerstitial damage was quantified by scoring tubular dilatation, atrophy of epithelial cells and widening of tubular lumen using the scoring system of Gross et al. (2006). Further details are provided in Supplementary data.

2.5. Immunohistochemistry

Sections were stained for kidney injury molecule (KIM-1) for tubular damage (goat polyclonal, diluted 1:50 v/v, Santa Cruz), HIS-48 for neutrophils (mouse monoclonal, generously provided by Prof. Kroese), ED-1 for macrophages (mouse monoclonal, diluted 1:500 v/v, Serotec Ltd, Oxford, UK) and cystathionine beta-synthase (CBS, diluted 1:100 v/v). Further details are provided in supplementary data.

2.6. Western blotting

Primary antibodies (1:1000 v/v dilution in 3% BSA/TBST) used were CBS (mouse monoclonal, Santa Cruz, Te Huissen, Netherlands), CSE (mouse monoclonal, Abnova, Walnut, CA, USA) and 3-MST (rabbit polyclonal, Santa Cruz). Protein bands were visualized and quantified using Gene Genome/Gene Tools (Westburg, Leusden, The Netherlands). β-actin was used as a housekeeping protein. Further details are provided in Supplementary data.

2.7. Measurement of reactive oxygen species

Kidney samples were homogenized in 100 μl PBS containing butylatedhydroxytoluene (Cell Biolabs, Netherlands) and centrifuged at 10.000 g for 5 min at 4 °C. SDS-Lysis solution (50 μl, Cell Biolabs) was added to 50 μl of supernatant and malondialdehyde (MDA) standards, incubated at room temperature (5 min), addition of thiobarbituric acid (125 μl, TBA) Reagent (Cell Biolabs) and incubated at 95 °C for 60 min, followed by cooling to room temperature in 5 min and centrifugation at 1000 g for 15 min. 2-Butanol (150 μl, Merck, Darmstadt, Germany) was added to supernatants, vortexed for 2 min and centrifuged for 5 min at 10,000 g. Lipid peroxidation was calculated by measuring optical density at 532 nm in 200 μl of the butanol fraction and expressed as μM MDA per mg of tissue.

2.8. Serum H_2S measurement

Sulfide antioxidant buffer was prepared from 25 g of sodium salicylate, 6.5 g of ascorbic acid and 8.5 g of NaOH in 100 mL water and pH adjusted to ≥ 13. Sulfide antioxidant buffer (100 μl) was added to 100 μl serum. A sulfide sensitive electrode (Lazar Research Laboratories, CA, USA) was immersed into the mixture and the stabilized electrode potential was recorded. The sulfide ion concentration of the serum was calculated using a standard curve prepared from 10 mL of the sulfide antioxidant buffer and 24 mg of $Na_2S \cdot 9H_2O$, according to the manufacturer's instructions.

2.9. Statistical analysis and data presentation

Variables are expressed as mean ± standard error of the mean (S.E.M.). Differences between groups were tested 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).

3. Results

3.1. Dopamine marginally affects hemodynamic changes in hypothermia and rewarming

Cardiovascular effects of hypothermia and rewarming (H/R) were 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 ($P < 0.01$; Fig. 2). In addition, dopamine increased heart rate by about 15% during the normothermic phase ($P < 0.05$),

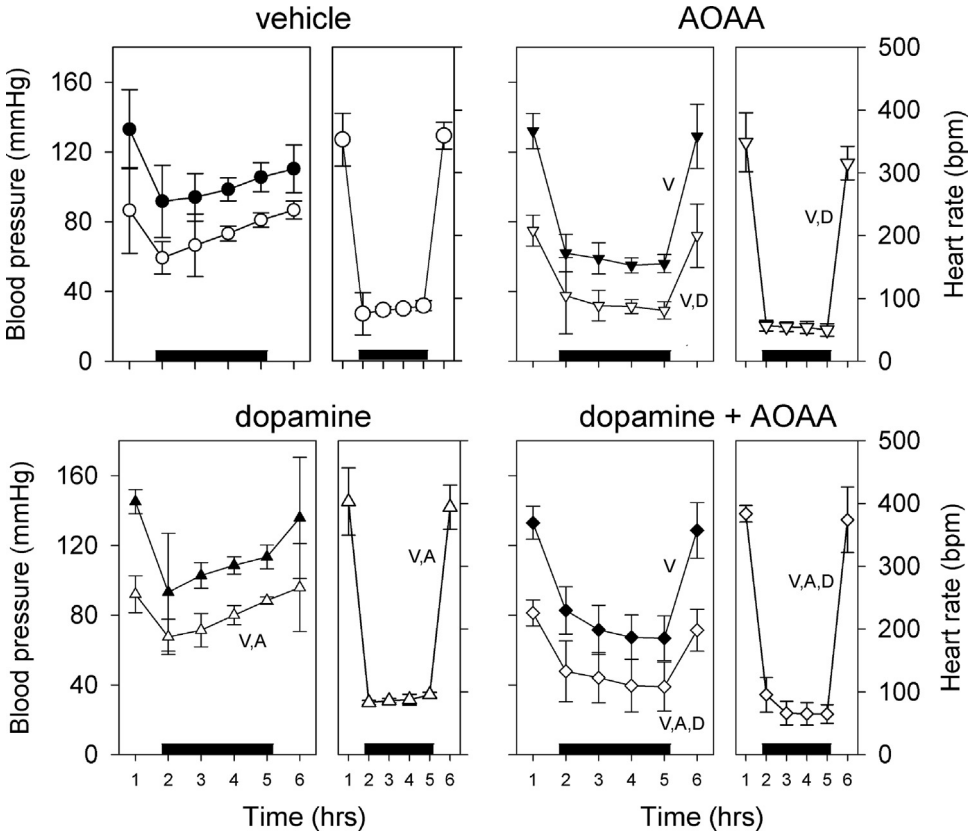

Fig. 2. Hypothermia induced reduction in heart rate and blood pressure. Induction of hypothermia to 15 °C resulted in a significant drop in systolic blood pressure (left panels, closed symbols) and diastolic blood pressure (left panels, open symbols) and heart rate (right panels) in all groups compared to baseline values. Rewarming after 3 h of hypothermia (time = 6 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 (time = 1 h) and after the rewarming phase compared to other groups ($P < 0.05$). Timepoints $t = 2$–5 h represent the hypothermic period. Data are presented as mean ± S.E.M., $n = 5$ per group. 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.

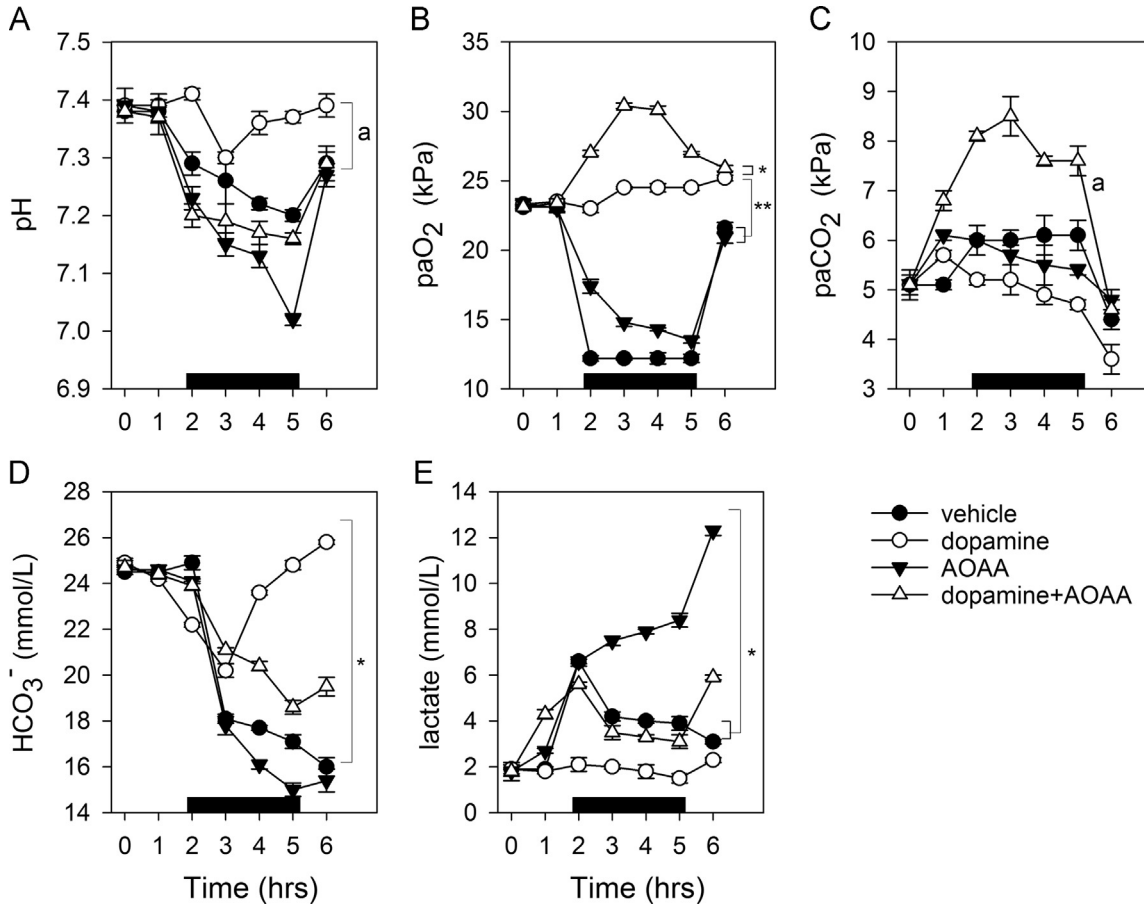

Fig. 3. Dopamine infusion prevents hypothermia and rewarming induced metabolic changes. (A) Dopamine treatment maintained blood pH during hypothermia and rewarming compared to other groups, which show a significant decrease compared to their baseline values ($P<0.05$). (B) Dopamine maintains baseline PaO_2 throughout H/R, whereas vehicle and AOAA treated groups show a significant reduction throughout hypothermia ($P<0.01$). AOAA further increase PaO_2 in dopamine treated animals ($P<0.05$). (C) Dopamine infusion in AOAA treated rats significantly increases $PaCO_2$ compared to other groups ($P>0.05$). (D) HCO_3^- levels normalize in dopamine treated animals during H/R as opposed to other groups ($P<0.05$). (E) AOAA strongly increases plasma lactate levels compared to other groups ($P<0.05$). */** represents $P<0.05$/0.01; 'a' represents significant differences from all other groups ($P<0.05$); RM ANOVA. Data are presented as mean ± S.E.M., $n=5$ per group.

but showed a similar decrease during hypothermia compared to other groups (Fig. 2). Heart rate was not affected by AOAA (Fig. 2).

Hypothermia resulted in a significant drop in baseline systolic blood pressure ($P<0.001$), while rewarming restored systolic blood pressure to baseline values in all groups (Fig. 2). In addition, dopamine induced a small increase in systolic blood pressure amounting 9.1 ± 5.8 and 25.5 ± 11.0% in normothermia, respectively before and after hypothermia ($P<0.05$; Fig. 2B). AOAA did not affect systolic blood pressure. Diastolic blood pressure in all groups followed a similar profile as systolic blood pressure (Fig. 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.

3.2. Dopamine prevents metabolic effects of hypothermia and rewarming

Hypothermia induced a significant drop in blood pH as compared to baseline values ($P<0.01$; Fig. 3A). Whereas pH increased after rewarming, it did not fully restore to baseline level ($P<0.05$). Remarkably, dopamine fully prevented the change in pH induced by H/R (Fig. 3A). Infusion of AOAA attenuated the normalization of blood pH by dopamine during H/R periods ($P<0.05$; Fig. 3A). Taken together, both hypothermia and AOAA induce acidosis, whereas dopamine infusion precludes mainly the acidosis induced by hypothermia.

Hypothermia led to a slight increase in partial arterial carbon dioxide pressure ($PaCO_2$), a substantial increase in serum lactate and a decrease in bicarbonate level (HCO_3^-) ($P<0.05$; Fig. 3C–E). Rewarming partially restored lactate levels, although still significantly higher than baseline values ($P<0.05$; Fig. 3E). Infusion of dopamine prevented these changes (Fig. 3C–E). Addition of AOAA in animals infused with dopamine partially precluded the beneficial effect of dopamine on HCO_3^- and lactate, while sole administration of AOAA amplified the effects of H/R (Fig. 3D). Increased $PaCO_2$ in the group treated with dopamine and AOAA, which shows similar lactate patterns as dopamine, seems of respiratory origin possibly caused by excessive ventilation-perfusion mismatch (*e.g.* shunting). 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 (PaO_2), which was restored upon rewarming, while dopamine treatment precluded changes in PaO_2 throughout the experiment ($P<0.01$; Fig. 3B). Thus, infusion with dopamine prevented the decrease in PaO_2 level upon hypothermia.

3.3. Dopamine prevents hypothermia and rewarming-induced kidney dysfunction and damage

As a marker of kidney function, serum creatinine levels were measured throughout the procedure. Serum creatinine level

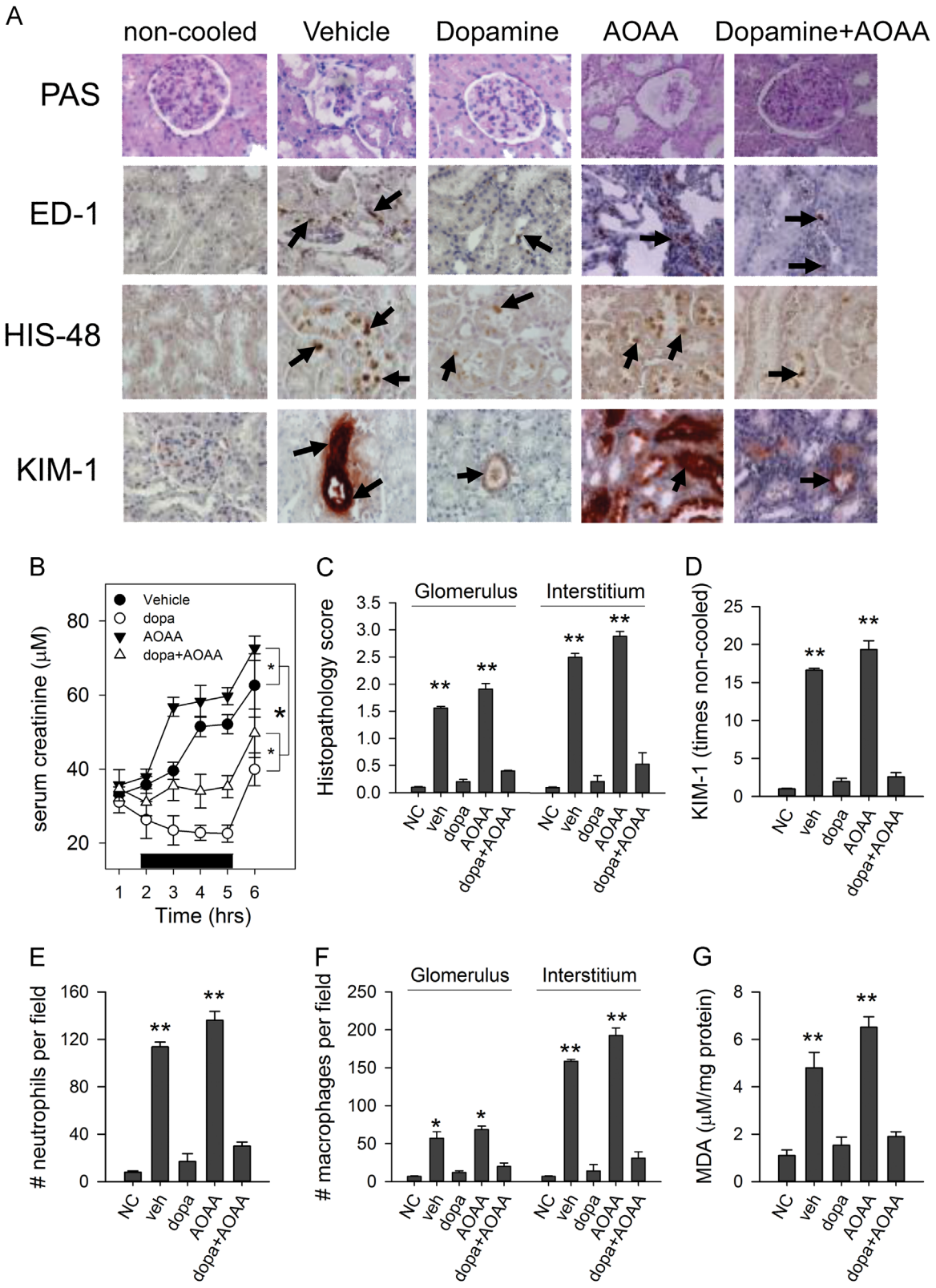

Fig. 4. Dopamine infusion prevents hypothermia and rewarming induced kidney injury. (A) Representative sections of the kidney (magnification × 400) from all groups showing PAS, KIM-1, ED-1 and HIS-48 stainings. Arrows point to positively stained areas (brown). (B) H/R induced increase in serum creatinine is counteracted by treatment with dopamine, both in the absence and presence of AOAA. (C–G) quantification of immuno(histochemical) stainings of (C) PAS, (D) KIM-1, (E) HIS-48, (F) ED-1. (G) Dopamine infusion attenuated the increase in renal MDA found in vehicle, irrespective of AOAA co-administration ($P > 0.05$). PAS = Periodic Acid Schiff, KIM-1 = Kidney Injury Molecule, HIS-48 = Neutrophil marker, ED-1 = Macrophage marker, MDA = malondialdehyde. Data are presented as mean ± S.E.M., n = 5 per group. */** $P < 0.05/0.01$ compared to non-cooled.

increased during hypothermia, while rewarming induced a further rise ($P<0.05$; Fig. 4B) as compared to pre-procedure values. Dopamine infusion prevented the increase in serum creatinine levels during both H/R ($P<0.01$; Fig. 4B). AOAA treatment alone significantly increased serum creatinine compared to vehicle-treated animals, which was substantially reduced, albeit not normalized, by dopamine ($P<0.05$; Fig. 4B). Thus, dopamine prevented the hypothermia-induced increase in serum creatinine irrespective of the presence of AOAA, while AOAA increased serum creatinine on top of H/R.

Next, we examined histopathological changes in kidney obtained one hour after rewarming. H/R induced substantial glomerular and tubular injury as assessed by PAS- and KIM-1 stains ($P<0.01$; Fig. 4A,C,D). Dopamine infusion fully prevented these morphological changes, while AOAA induced excess renal injury as compared to vehicle-treated animals ($P<0.05$; Fig. 4A,C,D). Furthermore, the protection from kidney damage offered by dopamine was unaffected by AOAA ($P<0.05$; Fig. 4A,C,D).

H/R induced a substantial influx of macrophages and neutrophils in the kidney as compared to non-cooled animals

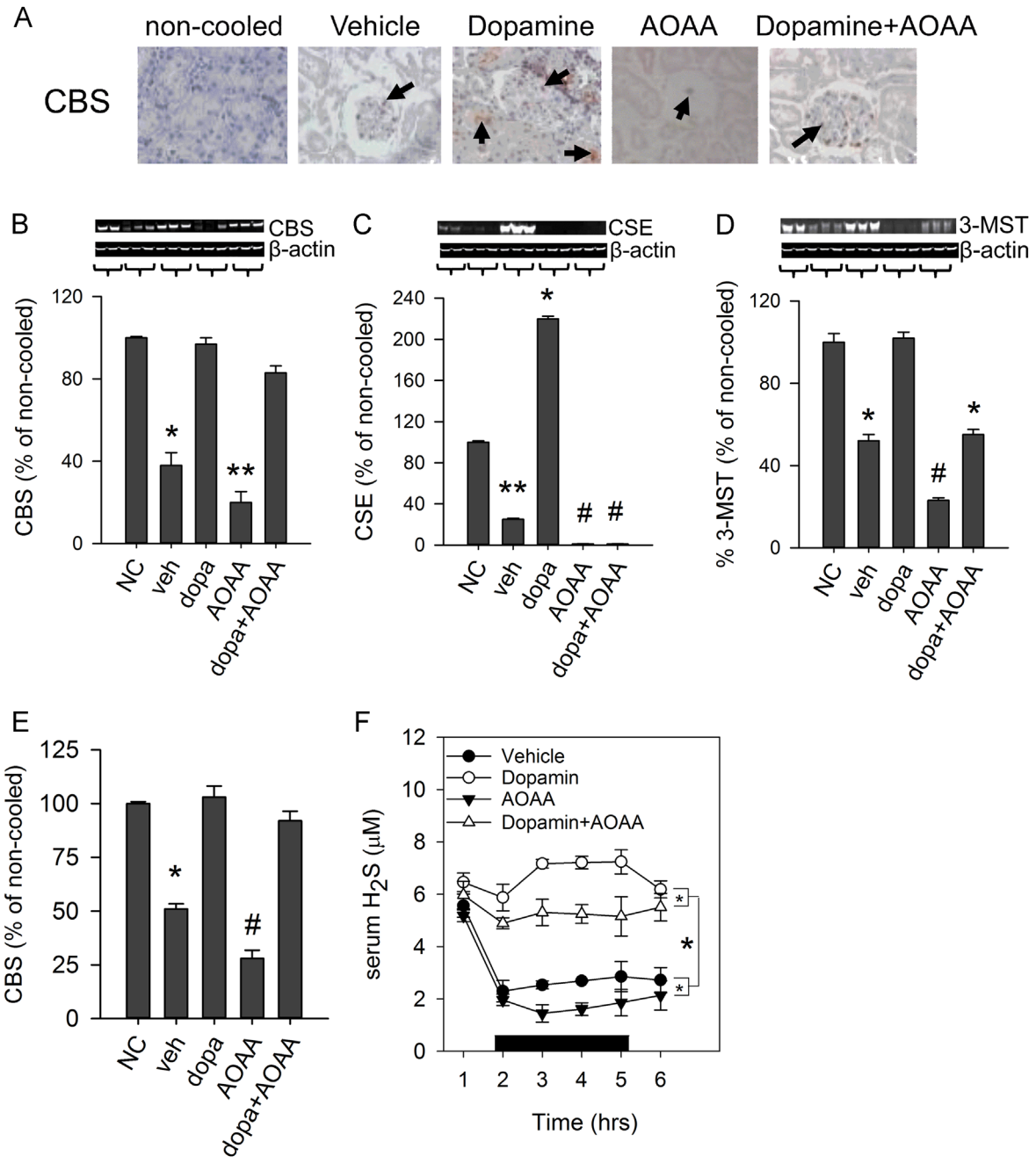

Fig. 5. Dopamine prevents metabolic effects of hypothermia and rewarming and maintains the expression of H_2S-producing enzymes. (A) Representative sections of the kidney (magnification × 400) from all groups showing CBS staining. Arrows point to positively stained areas (brown). (B) Dopamine maintained renal CBS expression both in the absence and presence of AOAA treatment ($P<0.01$). (C) Dopamine strongly upregulates renal CSE expression, which was fully annihilated by AOAA ($P<0.01$). (D) Dopamine maintains renal 3-MST expression, which was blocked by co-treatment with AOAA. Insets: cropped pictures of Western Blots; full blot is depicted in Supplemental Fig. 1. (E) Quantification of immunohistochemical staining of CBS. (F) Dopamine infusion maintains serum H_2S 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_2S, which is not restored after rewarming ($P<0.01$). */** represents $P<0.05/0.01$; # represents difference from all other groups ($P<0.05$); RM ANOVA (B) or ANOVA (C–F). Data are presented as mean ± S.E.M., $n=5$ per group.

($P<0.01$; Fig. 4A,E,F). Similarly to damage markers, administration of dopamine fully prevented the increase in influx of these cells, which was unaffected by co-administration of AOAA ($P>0.05$; Fig. 4A,E,F). Treatment with AOAA on the other hand, resulted in an excess influx of macrophages and neutrophils as compared to vehicle-treated animals ($P<0.05$; Fig. 4A,E,F).

Finally, reactive oxygen species production in kidney tissue was assessed by measurement of MDA levels. H/R induced a 5-fold increase in MDA ($P<0.01$; Fig. 4G). Treatment with dopamine fully prevented the increase in MDA ($P>0.05$), which was not affected by AOAA administration in these animals ($P>0.05$; Fig. 4G). Also, MDA levels were higher in animals treated with AOAA compared to vehicle treated animals ($P<0.05$; Fig. 4G). Collectively, these data demonstrate that dopamine attenuates kidney injury following hypothermia and rewarming.

3.4. Dopamine maintains renal expression of H_2S-producing enzymes and serum levels of endogenous H_2S in hypothermia and rewarming

To explore the potential mechanism by which dopamine protects from H/R damage in kidney, we determined the renal expression of the three different H_2S-producing enzymes, i.e. CBS, CSE and 3-MST. H/R resulted in a significant decrease in the expression of CBS, CSE and 3-MST as compared to non-cooled animals ($P<0.05$; Fig. 5A–E). Notably, dopamine infusion maintained renal CBS expression at the level of non-cooled animals both in immunostaining and western blot ($P>0.05$; Fig. 5A,B,E). Addition of AOAA did not affect the maintenance of CBS expression in dopamine-treated animals ($P>0.05$; Fig. 5B). Similar to CBS, renal CSE expression was substantially downregulated following H/R as compared to non-cooled animals ($P<0.01$; Fig. 5C). However, dopamine treatment not only restored, but markedly upregulated CSE expression by 210% compared to non-cooled animals ($P<0.01$; Fig. 5C). Administration of AOAA, both in vehicle and dopamine treated animals, strongly downregulated CSE expression level below the detection limit (Fig. 5C). The expression of renal 3-MST was markedly reduced in hypothermic and rewarmed rats compared to non-cooled animals ($P<0.05$), which was prevented by dopamine ($P>0.05$; Fig. 5D). Addition of AOAA lowered the expression levels of 3-MST both in vehicle and dopamine-treated animals (Fig. 5D). Thus, H/R substantially downregulated the expression of all H_2S-producing enzymes in the kidney, which was fully attenuated by dopamine infusion. Finally, we investigated whether changes in the expression of H_2S-producing enzymes in the kidney is paralleled by changes in serum levels of H_2S (Fig. 5F). Hypothermia induced a strong reduction in serum H_2S levels that was maintained throughout hypothermia compared to baseline value ($P<0.01$). Remarkably, subsequent rewarming did not restore serum H_2S levels (Fig. 5F). Dopamine infusion slightly increased baseline serum H_2S level ($P<0.05$) and completely attenuated its decrease during the subsequent H/R phases (Fig. 5F). Administration of AOAA to animals infused with dopamine partly prevented the effect of dopamine on the serum H_2S level upon hypothermia ($P<0.05$), while administration of AOAA alone further reduced the H_2S level ($P<0.05$). Thus, dopamine prevented the hypothermia induced decrease in serum H_2S.

4. Discussion

In this study, we demonstrate dopamine infusion to protect against renal injury caused by whole body deep hypothermia and rewarming in the rat. It is evident that our *in vivo* H/R protocol provokes substantial renal injury, as *e.g.* demonstrated by the increase in serum creatinine and renal reactive oxygen species production, expression of KIM-1 and influx of immune cells in the renal interstitium. Further, our model closely matches the clinical situation of whole body deep hypothermia in which there is unavoidable (compensated) metabolic acidosis, increased serum creatinine, and a drastic impairment of cardiovascular performance (Shida, 1974). Consequently, the hypothermic and rewarmed rat represents an adequate model to study therapeutic interventions that limit the associated renal injury.

As its main novel finding, our study documents dopamine infusion throughout the *in vivo* H/R procedure, at a dosage that hardly influences cardiovascular performance, to prevent both renal deterioration and systemic metabolic effects. Previously, dopamine protection from cooling and rewarming injury in cells has been demonstrated to involve maintenance of SH equivalents (Brinkkoetter et al., 2008) and to be conveyed through maintenance of CBS expression (Talaei et al., 2011). Therefore, we here studied in depth dopamine effects on renal expression of H_2S-producing enzymes. Similar to decreased CBS expression in cells and organ slices subjected to cooling and rewarming (Talaei et al., 2011), *in vivo* H/R substantially decreases the renal expression of all three H_2S-producing enzymes. Importantly, dopamine fully preserved the expression of renal CBS and 3-MST and even increased renal CSE expression following H/R. However, in the presence of AOAA, dopamine reduced renal reactive oxygen species production and expression of damage markers, while fully preserving the renal expression of CBS. In contrast, dopamine failed to maintain the expression of 3-MST and particularly of CSE in AOAA treated animals, suggesting that dopamine's preservation of kidney integrity is mostly 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 H/R in cell culture (Talaei et al., 2011). Also, Sen et al. observed sodiumhydrosulfide (an H_2S donor) to prevent chronic kidney injury in uninephrectomized CBS-knockout mice (Sen et al., 2009). However, also in CSE-knockout mice, increased vulnerability to kidney injury following ischemia-reperfusion injury was reported (Tripatara et al., 2008; Bos et al., 2013).

Our data on the involvement of H_2S-producing enzymes in dopamine protection from H/R is further substantiated by observations on serum H_2S. The decrease of serum H_2S following H/R, its rescue by dopamine and the small but significant decrease by AOAA all match the observed changes in the expression of H_2S-producing enzymes. H_2S levels provide insight into the time course of changes, as they were assessed during the whole H/R procedure, showing that hypothermia induces an immediate decrease of H_2S and absence of increase after rewarming. Moreover, dopamine maintains normal serum H_2S straight from the onset of hypothermia, indicating that dopamine need to be present throughout the full procedure. The source of serum H_2S is currently unknown, although main candidates are synthesis by circulating enzymes (Krijt et al., 2011) and rapid diffusion from tissues following local production (Jennings, 2013; Ramos-Alvarez et al., 2013). The concomitant reduction in renal expression of H_2S-producing enzymes and the decrease in serum H_2S level in H/R groups treated with vehicle or AOAA is compatible with the last option.

The modest effects of AOAA on top of H/R across the board may question the role of H_2S in protection from H/R injury. *E.g.* AOAA administration did not increase kidney injury resulting from H/R. Nevertheless, AOAA did moderately lower serum H_2S levels, increased serum creatinine and renal reactive oxygen species production, and further reduced renal 3-MST in H/R animals. The modest effect of AOAA may thus likely be explained by a relative low dosing.

Taken together, our data imply a substantial effect of dopamine

on expression of H_2S-producing enzymes and serum H_2S levels. Because of the anti-apoptotic and anti-inflammatory properties of H_2S conveying renoprotection in several animal models of ischemia/reperfusion injury (Bos et al., 2013, 2009; Lobb et al., 2014), the protective effects of dopamine during H/R may well result from maintenance of the expression levels of H_2S-producing enzymes and H_2S. However, additional effects of dopamine and/or H_2S may also explain dopamine's beneficial action. Dopamine has been reported to maintain renal blood flow (RBF) and glomerular filtration rate (GFR) in both animal and human studies (Fink et al., 1985; Abay et al., 2007), attributed to the activation of specific dopamine receptors (Mathur et al., 1999; Narkar et al., 2004; Fontana et al., 2005; Li et al., 2011). In addition, central nervous system effects of dopamine may influence kidney indirectly through modulation of the sympathetic nervous system (Pyner, 2009). Alternatively, maintenance of the expression levels of renal H_2S-producing enzymes as well as H_2S levels by dopamine, either systemically or locally, may induce H_2S mediated vasodilation (Hosoki et al., 1997), resulting in an increased RBF and GFR, as found in L-cysteine infused rat (Xia et al., 2009). Our results further demonstrate dopamine to convey a potent antioxidant action in kidney tissue, which may be explained by 2 mechanisms. First, dopamine may lower reactive oxygen species levels through providing adequate H_2S levels, as found in cells (Talaei et al., 2011) and following exogenous administration of H_2S (Kimura and Kimura, 2004; Liu et al., 2009; Calvert et al., 2009; Yang et al., 2013). Second, dopamine itself also possesses reactive oxygen species-scavenging properties, thought to prevent cold-preservation and ischemia-reperfusion injury in cells (Gottmann et al., 2006; Yard et al., 2004; Li et al., 2011). However, as AOAA failed to lower serum H_2S in dopamine-infused animals, we cannot discriminate between the two options.

Finally, effects on kidney observed in our *in vitro* H/R protocol transplantation are remarkably similar to those observed in cooled and rewarmed renal transplants, including *e.g.* upregulation of KIM-1, reactive oxygen species production and interstitial influx of immune cells (Shoskes and Cecka, 1998; Salahudeen, 2004). Their resemblance may be instrumental in further dissecting the mechanisms of action of dopamine to prevent hypothermia injury to the kidney.

In conclusion, we demonstrate for the first time that dopamine preserves kidney function and integrity in whole body hypothermia and rewarming in the rat. Dopamine-induced maintenance of expression of H_2S-producing enzymes in rat kidney seems of crucial importance. Therefore, implementing dopamine treatment in clinical conditions requiring hypothermia and rewarming may have potential to ameliorate renal injury.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2015.11.022.

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