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Artesunate Protects Against the Organ Injury and Dysfunction Induced by Severe Haemorrhage and Resuscitation

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Running head: Artesunate reduces MOF in haemorrhagic shock

Mini-Abstract

The anti-malarial drug artesunate reduces the organ injury/dysfunction caused by haemorrhagic shock in rats. A similar dose of artesunate improves survival in patients with severe malaria with multiple organ injury/failure without causing any significant adverse effects. A phase II clinical trial evaluating the effects of artesunate in trauma/haemorrhage is planned.

Structured Abstract

Objective: To evaluate the effects of artesunate on organ injury and dysfunction associated with HS in the rat.

Summary Background Data: Haemorrhagic shock (HS) is still a common cause of death in severely injured patients and it is characterized by impairment of organ perfusion, systemic inflammatory response and multiple organ failure. There is no specific therapy that reduces organ injury/dysfunction. Artesunate exhibits pharmacological actions beyond its antimalarial activity, such as anti-cancer, anti-viral and anti-inflammatory effects.

Methods: Rats were submitted to HS. Mean arterial pressure was reduced to 30 mmHg for 90 min, followed by resuscitation. Rats were randomly treated with artesunate (2.4 or 4.8 mg/kg i.v.) or vehicle upon resuscitation. Four hours later, parameters of organ injury and dysfunction were assessed.

Results: Artesunate attenuated the multiple organ injury and dysfunction caused by HS. Pathway analysis of RNAseq provided good evidence to support an effect of artesunate on the Akt-survival pathway, leading to down-regulation of IRAK1. Using Western blot analysis, we confirmed that treatment of HS-rats with artesunate enhanced the phosphorylation (activation) of Akt and eNOS and the phosphorylation (inhibition) of glycogen synthase kinase-3β (GSK-3β). Moreover, artesunate attenuated the HS-induced activation of NF-κB and reduced the expression of pro-inflammatory proteins (iNOS, TNF-α and IL-6).

Conclusions: Artesunate attenuated the organ injury/dysfunction associated with HS by a mechanism that involves the activation of the Akt-eNOS survival pathway, and the inhibition of GSK-3β and NF-κB. A phase II clinical trial evaluating the effects of GMP-artesunate in patients with trauma and severe haemorrhage is planned.

Keywords: haemorrhagic shock, organ injury, artesunate, anti-malarial, Akt, NF-κB.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; DHA, dihydroartemisinin; eNOS, endothelial nitric oxide synthase; FOXO4, forkhead box O4; GSK-3β, glycogen synthase kinase-3β; HS, haemorrhagic shock; IL-6, interleukin 6; IRAK1, interleukin-1 receptor-associated kinase 1; MAP, mean arterial pressure; MOF, multiple organ failure; MPO, myeloperoxidase; NF-κB, nuclear factor kappa B; TNF-α, tumour necrosis factor-α; WHO, World Health Organization.

INTRODUCTION

Trauma is a leading cause of death with 5 million of victims yearly. About 40% of trauma deaths are due to haemorrhagic shock (HS), which causes hypoperfusion of organs leading to multiple organ failure (MOF).[1] The development of MOF occurs in approximately 30% of injured patients, and is the major cause of morbidity following trauma.[2] The development of MOF correlates with an increase in nosocomial infections and, if it persists, mortality.[3] The mechanisms underlying the MOF are not fully understood, although ischaemia-reperfusion injury and systemic inflammation are likely to be key players.[4] There are no specific treatments for the MOF associated with HS, thus, a therapeutic agent that reduces the incidence and severity of MOF is urgently needed and could have a major global impact on patient outcomes.

Artesunate (a semi-synthetic artemisinin derived from the plant Artemisia annua) is recommended by the World Health Organization (WHO) as the treatment of choice for severe malaria.[5] Artesunate is a safe, low-cost and well tolerated drug and has been used by thousands of patients with malaria without important adverse effects.[6] Although the mechanism behind the antimalarial activity of artesunate is not entirely clear, it is thought that the active metabolite, dihydroartemisinin (DHA) is responsible for the death of the parasite possibly through the inhibition of the parasitic calcium pump.[7]

Artemisinins, including artesunate, have many pharmacological effects beyond the eradication of Plasmodium species.[8] Artemisinins/artesunate have demonstrated cytotoxic effects against cancer cells,[9] viruses,[10] fungi,[11] but also powerful anti-inflammatory effects in animal models of asthma,[12] pancreatitis,[13] arthritis[14] and sepsis,[15] to name but a few. However, the effects of artesunate in HS, a condition associated with ischaemia and reperfusion injury, are unknown. Therefore, the aim of the present study was to investigate the effects of artesunate on the organ injury and dysfunction associated with HS. Having discovered that artesunate attenuates the organ injury and dysfunction associated with HS, we have then carried out an extensive evaluation of the potential mechanism of action of artesunate, including RNAseq

transcriptomics and pathway analysis. We report here for the first time that artesunate has pro-survival and anti-inflammatory properties, as it activates the Akt-eNOS cell survival pathway and inhibits a range of proinflammatory molecules, including IRAK1, FOXO4, GSK-3β and the transcription factor NF-κB.

METHODS

Animal welfare and ethical statements

The animal protocols used in this study were approved by the Animal Welfare Ethics Review Board (AWERB) of Queen Mary University of London (PPL: 70/7348) in accordance with Home Office guidance on Operation of Animals (Scientific Procedures Act 1986) and Guide for the Care and Use of Laboratory Animals of the National Research Council.

Haemorrhagic shock and quantification of organ injury and dysfunction

This study was carried out on 50 male Wistar rats (Charles River Ltd, Margate, UK) weighing 280 ± 20 g receiving a standard diet and water ad libitum. Hemorrhagic shock was performed as previously described.[16] Rats were anaesthetized using sodium thiopentone (120 mg/kg i.p. maintained using 10 mg/kg i.v.) and cannulation of the trachea, femoral and carotid arteries, jugular vein and bladder was performed. Blood was withdrawn in order to achieve a fall in mean arterial pressure (MAP) to 30 ± 2 mmHg, which was maintained for a period of 90 minutes. Resuscitation was performed with the remained shed blood. An infusion of Ringer Lactate (1.5 mL/kg/hour; i.v.) was maintained throughout the experiment for a total of 4 hours. The last 3 hours urine was obtained for the estimation of creatinine clearance. Blood samples were collected via the carotid artery for measurement of lactate (Accutrend Plus Meter, Roche Diagnostics, West Sussex, UK) and organ injury parameters [creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase, amylase and creatine kinase

(CK) (IDEXX Laboratories Ltd, West Yorkshire, UK)]. In addition, lung, kidney and liver samples were taken and stored at -80 ^{o}C for further analysis. Sham-operated rats were used as control and underwent identical surgical procedures but without haemorrhage or resuscitation.

Experimental design

Rats were randomly allocated into the following groups (n = 10 per group): sham + vehicle; sham + artesunate 4.8 mg/kg; HS + vehicle; HS + artesunate 2.4 mg/kg and HS + artesunate 4.8 mg/kg. Rats were administered vehicle (PBS; pH 8; 1 mL/kg) or artesunate (2.4 mg/mL/kg or 4.8 mg/mL/kg) i.v. upon resuscitation.

Determination of myeloperoxidase (MPO) activity

Samples were homogenized in phosphate buffer and centrifuged at 4°C. Pellets were re-suspended in a 0.5% hexadecyltrimethylammonium bromide 50 mM phosphate buffer solution, sonicated and put in ice for 20 minutes. After 15 minutes of centrifuge (13,000 g at 4°C), supernatant was allowed to react with a solution of o-Dianisidine (0.167 mg/mL) and H_2O_2 (0.0005%) in 50mM phosphate buffer. The rate of change in absorbance was measured spectrophotometrically at 460 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per minute and was expressed in milliunits per gram of wet tissue.

RNA sequencing analysis

Gene expression was evaluated by RNA sequencing (RNA-seq) which allows a dynamic range of detection and measurement of relatively limited differences in expression between samples. Whole blood RNA samples were extracted from 12 rats (3 samples per group). 1 µg of total RNA was used for library preparation, according to the Illumina TruSeq mRNA sample

preparation protocol (Illumina, San Diego, Calif). Libraries were sequenced using Illumina NextSeq platform and TruSeq protocol. Adaptor and poor quality sequences were removed using trim galore (v0.3.7) software. Trimmed sequences were aligned to the rat reference genome (rnor5) with TopHat2 (v2.0.13)/bowtie2 (v2.2.3). Read counts were calculated using the HTSeq-counts program and differential expression analyses were carried out with DESeq2, using the standard protocols. Pathway analysis was performed using Enrichr[17] and Ingenuity Pathway Analysis (http://www.ingenuity.com/).

Western blot

Samples were homogenized, centrifuged and the supernatants were removed and centrifuged at 16,000 g at 4°C for 40 minutes to obtain the cytosolic fraction. The pelleted nuclei were suspended in extraction buffer and centrifuged. The supernatants were removed, and protein content was determined on both nuclear and cytosolic extracts using bicinchoninic acid protein assay (Thermo Fisher Scientific Inc, Rockford, IL). Proteins were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinyldenedifluoride membrane, which was then incubated with a primary antibody [total Akt (1:1000); pAkt Ser^{473} (1:1000); total eNOS (1:200), peNOS Ser^{1177} (1:200); total GSK-3β (1:200); pGSK-3β Ser^{9} (1:200); NF-κB p65 (1:1000) and iNOS (1:200)]. Membranes were incubated with secondary antibody conjugated with horseradish peroxidase (1:2000) and developed using ECL detection system. The immunoreactive bands were visualised by autoradiography. The membranes were stripped and incubated with β-actin monoclonal antibody (1:5000) and subsequently with an anti-mouse antibody (1:10000) to assess gel-loading homogeneity. The densitometric analysis was performed using Gel Pro Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA) and is expressed as relative

optical density, corrected for the corresponding β-actin contents, and normalized using the related mean sham-operated band.

Cytokines

Serum cytokines TNF-α and IL-6 were determined using commercial immunoassay kits (R&D Systems, Minneapolis, MN) according to the manufacturer protocol.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). Ringer's Lactate was obtained from Baxter Healthcare Ltd.; sodium thiopentone (Thiovet©) from Link Pharmaceuticals, Horsham, U.K. The bicinchoninic acid protein assay kit and SuperBlock blocking buffer were from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Antibodies were from Cell Signalling Technology Inc. (Beverly, MA, USA).

Data analysis

All values described in the text and figures are expressed as mean ± SEM. Statistical analysis was carried out using GraphPad Prism 5.03 (GraphPad Software, San Diego, California, USA). Data were assessed by one or two-way ANOVA followed by Bonferroni's post hoc test. A p value of less than 0.05 was considered to be significant.

RESULTS

Artesunate attenuates the organ injury and dysfunction induced by HS

Rats subjected to HS exhibited a significant decrease in creatinine clearance (Fig. 1A), as well as a rise in serum creatinine (Fig. 1B) indicating the development of renal dysfunction. HS-rats also developed significant increases in AST (Fig. 1C), ALT (Fig. 1D), amylase (Fig. 1E), lipase (Fig. 1F), CK (Fig. 1G) and lactate (Fig. 1H), indicating the development of liver, pancreas and neuromuscular injury, and global ischaemia, respectively (Fig. 1).

The treatment of HS-rats with either 2.4 mg/kg or 4.8 mg/kg artesunate significantly attenuated the fall in creatinine clearance (Fig. 1A) and attenuated the rises in serum creatinine (Fig. 1B), AST (Fig. 1C) and CK (Fig. 1G) when compared to HS-rats treated with vehicle. However, when compared to vehicle HS-rats, only the treatment of HS-rats with 4.8 mg/kg artesunate attenuated the HS-induced rise in serum levels of ALT (Fig. 1D), amylase (Fig. 1E) and lactate (Fig. 1H). The higher dose of artesunate in sham-operated rats had no significant effect on any of the parameters evaluated.

When compared to sham-operated rats, HS-rats developed a significant increase in lung MPO activity (Fig. 2) indicating the infiltration of inflammatory cells. Treatment of HS-rats with both doses of artesunate significantly attenuated the rise in MPO activity in the lung (Fig. 2). The administration of the higher dose of artesunate in sham-operated rats had no significant effect on MPO activity.

Artesunate does not prevent the development of the circulatory failure induced by HS

When compared to sham-operated rats (open circles), HS-rats treated with vehicle demonstrated a significant reduction in MAP after resuscitation (closed squares; Fig. 3). The administration of artesunate (2.4 or 4.8 mg/kg; closed triangles) on resuscitation failed to attenuate the reduction in MAP caused by HS (Fig. 3). The administration of artesunate (4.8 mg/kg) in sham-operated rats had no significant effect on the hemodynamic function (closed diamonds).

Artesunate acts predominantly on the AKT-survival pathway

RNAseq transcriptome sequencing was performed on white blood cells. Differential expression analysis identified a large number of differentially expressed transcripts between treatment groups (Supplementary Table 1). Enrichr pathway analysis of genes showing differential expression in both the sham and HS-rats, identified AKT1 protein interaction as the strongest enrichment on a genome wide basis (10/119 genes; adjusted p value = 0.00116; Fig. 4A; Supplementary Table 1). The AKT1 interactor genes identified in the analysis were PPM1A, IRAK1, AKT2, ITGB3, LMNA, NCOA4, BPGM, FOXO4, BCL2L1 and CDC25B. To investigate the possible mechanistic basis of AKT1 interaction further, we constructed a mechanistic model of differentially expressed direct AKT1 interactors using the molecule activity predictor algorithm in Ingenuity Pathway analysis (IPA).[18] The algorithm predicted AKT activation (Fig. 4B), on the basis of down regulation of a number of genes in the AKT core network that are known to be down-regulated by AKT, including IL-1 receptor-associated kinase 1 (IRAK1) and forkhead box O4 (FOXO4).

Artesunate attenuates the activation of NF-κB pathway induced by HS

As we had some evidence to support the action of artesunate on the Akt-survival pathway (Fig. 4), and as the most striking beneficial effects of artesunate in organ protection were observed in liver and kidney (Fig. 1), we subsequently investigated the possible mechanisms by which artesunate (4.8 mg/kg) attenuated the organ injury and dysfunction in liver and kidney biopsies. When compared to sham-operated rats, HS-rats treated with vehicle exhibited a trend but do not show significantly differences in the phosphorylation of Akt on Ser^{473} (Figs 5A, 6A), eNOS on Ser^{1177} (Figs 5B, 6B) or GSK-3β on Ser^{9} (Figs 5C, 6C) in liver or kidney, respectively. On the other hand, HS-rats exhibited an increase in the nuclear translocation of the p65 NF-κB subunit

(Figs 5D, 6D). When compared to HS-rats treated with vehicle, HS-rats treated with artesunate significantly increased the phosphorylation of Akt (Figs 5A, 6A), eNOS (Figs 5B, 6B), GSK-3β (Figs 5C, 6C) and attenuated the subsequent translocation of the p65 NF-κB subunit to the nucleus (Figs 5D, 6D). The treatment of sham-rats with artesunate had no significant effect (Figs 5, 6).

Effects of IKK16 treatment on iNOS expression and cytokines formation induced by HS

As we have found a decrease in NF-κB pathway, and iNOS is regulated by this transcription factor, we investigated the expression of iNOS in HS-rats. When compared with sham-operated rats, livers and kidneys from rats submitted to HS (treated with vehicle) exhibited a significant increase in iNOS expression (Fig. 7A, B, respectively). The treatment of HS-rats with artesunate at the onset of resuscitation significantly attenuated iNOS expression in both tissues (Fig. 7A, B).

When compared to sham-operated rats, HS-rats treated with vehicle developed significant serum increases in the pro-inflammatory cytokines TNF-α (Fig. 7C) and IL-6 (Fig. 7D). The treatment of HS-rats with artesunate significantly reduced the rises in serum TNF-α and IL-6 (Fig. 7C, D). The treatment of sham-rats with artesunate had no significant effect.

DISCUSSION

The main finding of the present report is that the safe antimalarial drug artesunate attenuates the organ injury and dysfunction associated with HS. We have discovered that administration of artesunate upon resuscitation reduces the MOF by enhancing the resistance of organs against injury/dysfunction by activating a well-known cell survival pathway and reducing excessive inflammation. The lower dose of artesunate shown here to be effective in HS (e.g. 2.4 mg/kg)

is identical to the dose used as an i.v. bolus injection in patients with malaria, many of which also have multiple organ dysfunction. Indeed, in these patients artesunate is superior to quinine in improving survival.[6]

In order to gain a better insight into the potential mechanism of action of artesunate in HS, we carried out a controlled RNAseq transcriptome analysis of white blood cells. Pathway analysis of genes showing differential expression in both sham and HS-rats, were strongly enriched for AKT1 interactor genes. Although AKT1 itself was not differentially expressed, this does not reflect protein activity and a mechanistic model of the AKT1 core network predicted AKT activation, on the basis of down regulation of several pro-inflammatory genes in the AKT core network that are known to be down-regulated by AKT. These include IRAK1 and FOXO4. IRAK1 is a signalling molecule that induces nuclear translocation of NF-κB and activator protein (AP)-1, resulting in the production of inflammatory cytokines such as TNF-α and IL-6.[19] Previous studies have reported that down regulation of IRAK1 is protective in ischemia/reperfusion injury.[20] Similarly, FOXO4 specifically activates MMP9 expression in response to TNF-α, acting as a convergence point of cytokine and growth factor signalling pathways, leading to the proposal that inhibition (or down-regulation as seen in artesunate treatment) of FOXO4 could provide a therapeutic target for inflammatory arterial diseases.[21]

In our gene expression studies we also observed a decrease in gene expression of MEMO1 in sham-operated animals and in HS-rats (data not shown). This finding was somewhat contradictory to the hypothesis of AKT activation, as the protein has been shown to activate the Akt pathway.[22] Subsequently, we have evaluated MEMO1 by Western Blot analysis, and found a consistent increase in MEMO1 protein, confirming that artesunate does indeed enhance the expression of MEMO1 protein in liver/kidney of sham and HS-rats (data not shown). The observed reduction in MEMO1 transcript expression may represent a feedback control mechanism.

We discovered that treatment of HS-rats with artesunate activates the kinase Akt, which is involved on inflammatory responses and cell survival.[23] Activation of Akt has a number of beneficial effects including reduction/prevention of i) ventilation-induced lung injury,[24] ii) sepsis-induced cardiac dysfunction,[25] iii) sepsis-induced acute kidney injury,[26] iv) myocardial ischaemia-reperfusion[27] and v) HS-induced organ dysfunction.[28] Activation of Akt results in phosphorylation and activation of eNOS at Ser1177[29] which enhances the formation of small amounts of NO, which is pivotal for the preservation of microvascular perfusion[30], reducing organ injury.[25,31] Altogether, these data support our hypothesis that activation of Akt and consequently activation of eNOS contribute to the attenuation of organ injury and dysfunction caused by HS.

Activation of Akt was also associated with an increase in the phosphorylation of GSK-3β. Phosphorylation of the constitutively-active, serine-threonine kinase GSK-3β by Akt turns off the catalytic activity of GSK-3β[23]. Inhibition of GSK-3β reduces inflammation[32] and organ injury in myocardial ischaemia,[25] colitis,[33] endotoxemia[34,35] and haemorrhagic shock.[36] Thus, the inactivation of GSK-3β may also contribute to the beneficial effects of artesunate in HS.

The inhibition of GSK-3β directly inhibits NF-κB dependent gene transcription[37], and NF-κB plays a pivotal role in inflammation[38] and organ injury[28,31,39]. HS increased the translocation of NF-κB subunit p65 to the nucleus, thus, indicating its activation. The treatment of HS-rats with artesunate caused inactivation of GSK-3β (by phosphorylation), which was associated with reduced translocation of p65 subunit to the nucleus. Indeed, inhibition of NF-κB (by inhibiting activation of the IKK complex) reduces the organ injury/inflammation in HS.[28] Thus, inhibition of GSK-3β and NF-κB may both importantly contribute to the observed anti-inflammatory effects (e.g. neutrophil recruitment into end organs such as the lung, reduction in the formation of pro-inflammatory cytokines; see below, prevention of the expression of iNOS, see below) of artesunate in HS.

As NF-κB regulates iNOS expression and iNOS plays an important role in the pathophysiology of HS,[40,41] we have investigated the effects of artesunate treatment on iNOS expression in HS-rats. HS increased the expression of iNOS in liver and kidney, and artesunate attenuated iNOS expression in both organs. As an excessive formation of NO by iNOS contributes to the organ injury in HS[16,40] and sepsis,[42,43,44] and also causes cellular damage and tissue injury through peroxynitrite formation,[41] the reduction in iNOS expression may contribute to the organ protection afforded by artesunate.

An increase circulating pro-inflammatory cytokines such as TNF-α and IL-6 is associated with organ injury/dysfunction and lethality following HS and sepsis.[43,45,46,47] Elevated serum IL-6 concentrations predict the development of organ failure in patients with trauma-haemorrhage.[48] We report here that HS results in the excessive formation of TNF-α and IL-6, and that treatment of HS-rats with artesunate reduced the production of these cytokines. As patients who either produce excessive amounts of cytokines or cannot clear them from the circulation have an increased risk of MOF and death,[45] the reduction of circulating TNF-α and IL-6 may have importantly contributed to the attenuation of organ injury and dysfunction observed in HS-rats treated with artesunate.

Taken together, our results show that the protective effect of artesunate in HS is associated with an increased activation of Akt and eNOS phosphorylation and consequently inhibition of GSK-3β, which in turn, inhibited the translocation of NF-κB to the nucleus. This mechanism is supported on several levels. At a transcriptome level using pathway analysis, we find strong support for AKT activation on the basis of genome-wide enrichment of differential expression among AKT interactors. Expression of pro-inflammatory, inhibition targets of AKT, such as IRAK1 and FOXO4 are reduced. At the protein level, artesunate also attenuated iNOS expression and reduced the formation of pro-inflammatory cytokines, all of which may have contributed for the attenuation of organ injury and dysfunction associated with HS. Thus, the

use of the anti-malarial drug artesunate, which is a safe, low-cost and already-developed drug, could represent a therapeutic approach for the treatment and/or prevention of the organ injury and dysfunction associated with HS.

Currently, here is no specific treatment for organ failure and dysfunction associated with HS, which is associated with a high morbidity and mortality. Thus, a safe and low cost therapeutic intervention, which reduces the incidence and severity of MOF may have a great impact on the patient quality of life and the costs of healthcare. The doses of artesunate used here (2.4 or 4.8 mg/kg i.v. bolus injection) are very similar to those used in patients with malaria. Artesunate has a very good safety profile with no or minimal adverse effects in both healthy volunteers (up to 8 mg/kg i.v.) and patients with severe malaria (2.4 mg/kg i.v. followed by 2.4 mg/kg i.v. every 12 h until the patient has regained consciousness). The excellent safety profile of artesunate is (at least in part) due to its very short half-life in man: the intravenous injection of artesunate results in high, transient peak concentrations, which decline rapidly with an estimated half-life of 15 minutes. The concentration of the active metabolite DHA peaks within 25 minutes of injection of artesunate, and the half-life is estimated 30 to 60 minutes. The metabolism of artesunate is not affected by the presence of either renal or liver failure and the dose of artesunate does not need to be adjusted in patients with multiple organ failure.[49] Thus, we are planning a phase II placebo-controlled randomized clinical trial designed to evaluate the effects of GMP-artesunate (2.4 or 4.8 mg/kg i.v.) in 105 patients with trauma and severe haemorrhage. This single-centre trial will commence at the Royal London Hospital in autumn of 2015.

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FIGURE LEGENDS

Fig. 1. Artesunate attenuates the organ injury and dysfunction induced by HS. (A) creatinine clearance, (B) serum creatinine, (C) serum aspartate aminotransferase (AST), (D) serum alanine aminotransferase (ALT), (E) serum amylase, (F) serum lipase, (G) serum creatine kinase (CK) and (H) lactate of HS-rats treated with vehicle or artesunate (2.4 or 4.8 mg/kg) on resuscitation are shown. Sham rats were treated with vehicle or artesunate 4.8 mg/kg. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$ versus sham + vehicle and #$p < 0.05$ versus HS + vehicle.

Fig. 2. Artesunate attenuates MPO activity in lungs induced by HS. MPO activity in lungs of sham and HS-rats treated with vehicle or artesunate (2.4 or 4.8 mg/kg) on resuscitation are shown. Sham rats were treated with vehicle or artesunate 4.8 mg/kg. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$ versus sham + vehicle and #$p < 0.05$ versus HS + vehicle.

Fig. 3. Artesunate does not prevent the development of the circulatory failure induced by HS. HS-rats were treated with vehicle or artesunate (2.4 or 4.8 mg/kg) on resuscitation. Sham animals were used as control and received vehicle or artesunate (4.8 mg/kg). The mean arterial

pressure (MAP) was recorded during the whole experiment. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$ versus sham + vehicle and #$p < 0.05$ versus HS + vehicle.

Figure 4. Enrichr Protein Protein Interaction hub (PPI hub) analysis and core network of AKT1 interactor proteins constructed using Ingenuity pathway analsyis. (A) The top ten PPI hubs represented in the HS v HS + ART comparison are shown with combined scores in the HS v HS + ART and the SH v SH + ART comparisons. Combined score is based on log fisher exact p-value multiplied by the Z-score of the deviation from expected rank. (B) The network was constructed using AKT1 as a seed and all genes showing differential expression in SH v SH + ART and HS v HS + ART analyses. Ingenuity Molecule Activity Predictor analysis predicts activation of AKT1 on the basis of observed down regulation in several interactors that are known to be inhibited by AKT1.

Fig. 5. Artesunate attenuates the activation of NF-κB pathway induced by HS in the liver. The phosphorylation of (A) Ser^{473} on Akt, (B) Ser^{1177} on eNOS, (C) Ser^{9} on GSK-3β and (E) the nuclear translocation of p65 subunit of NF-κB in the liver of sham and HS rats treated with vehicle or artesunate (4.8 mg/kg) on resuscitation were determined by western blotting. Protein expression was measured as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related sham band. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$ versus sham + vehicle and #$p < 0.05$ versus HS + vehicle.

Fig. 6. Artesunate attenuates the activation of NF-κB pathway induced by HS in the kidney. The phosphorylation of (A) Ser^{473} on Akt, (B) Ser^{1177} on eNOS, (C) Ser^{9} on GSK-3β and (E) the nuclear translocation of p65 subunit of NF-κB in the kidney of sham and HS rats treated with vehicle or artesunate (4.8 mg/kg) on resuscitation were determined by western blotting. Protein expression was measured as relative optical density (O.D.), corrected for the

corresponding β-actin contents and normalized using the related sham band. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$ versus sham + vehicle and #$p < 0.05$ versus HS + vehicle.

Fig. 7. Artesunate attenuates iNOS expression in liver and kidney and serum TNF-α and IL-6 induced by HS. The iNOS expression in the liver (A) and kidney (B) of sham and HS rats treated with vehicle or artesunate (4.8 mg/kg) on resuscitation were determined by western blotting. Protein expression was measured as relative optical density (O.D.), corrected for the corresponding β-actin contents and normalized using the related sham band. The serum concentrations of (C) TNF-α and (D) IL-6 were determined by ELISA in sham and HS-rats treated with vehicle or artesunate (4.8 mg/kg) on resuscitation. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *$p < 0.05$ versus sham + vehicle and #$p < 0.05$ versus HS + vehicle.