Resuscitation (2008) **76**, 299—310

available at www.sciencedirect.com

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EXPERIMENTAL PAPER

Impact of resuscitation strategies on the acetylation status of cardiac histones in a swine model of hemorrhage☆

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Received 6 March 2007; received in revised form 16 July 2007; accepted 23 July 2007

KEYWORDS
Histone;
Acetylation;
Hemorrhagic shock;
Resuscitation;
Ringer's;
Ketone;
Gene transcription;
Post-translational modification;
Epigenetics;
Immediate-early response genes;
Histone deacetylase

Summary
Background: Chromatin remodeling through histone acetylation is a key control mechanism in gene transcription. We have shown previously that fluid resuscitation in rodents is coupled with highly structured post-translational modifications of cardiac histones. The current experiment was performed to validate this concept in a clinically relevant large animal model of hemorrhage and resuscitation, and to correlate the changes in histone acetylation with altered expression of immediate-early response genes.
Study design: Yorkshire swine ($n = 49$, 7/group, weight = 40—58 kg) were subjected to combined uncontrolled and controlled hemorrhage (40% of estimated blood volume) and randomly assigned to the following resuscitation groups: (1) 0.9% saline (NS), (2) racemic lactated Ringer's (DL-LR), (3) L-isomer lactated Ringer's (L-LR), (4) Ketone Ringer's (KR), (5) 6% hetastarch in saline (Hespan). KR contained an equimolar substitution of lactate with beta-hydroxybutyrate. No hemorrhage (NH) and no resuscitation (NR) groups were included as controls. Cardiac protein was used in Western blotting to analyze total protein acetylation and histone acetylation specifically. Lysine residue-specific acetylation of histone subunits H3 and H4 was further evaluated. In addition, Chromatin Immunoprecipitation (ChIP) technique was used to separate the DNA bound to acetylated histones (H3 and H4 subunits), followed by measurement of genes that

☆ A Spanish translated version of the summary of this article appears as Appendix in the final online version at 10.1016/j.resuscitation.2007.07.030.

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0300-9572/$ — see front matter
doi:10.1016/j.resuscitation.2007.07.030

are altered by hemorrhage/resuscitation, including immediate-early response genes (c-fos and c-myc), and heat shock protein (HSP) 70.
Results: The type of fluid used for resuscitation influenced the patterns of cardiac histone acetylation. Resuscitation with DL-LR and KR induced hyperacetylation on H3K9. KR resuscitation was also associated with increased acetylation on H3K14 and H4K5, and hypoacetylation on H3K18. The expression of genes was also fluid specific, with the largest number of changes following KR resuscitation (increased c-fos and c-myc, HSP 70 linked with H3; and increased c-myc linked with H4). Among the histone subunits studied, altered H3 acetylations were associated with the majority of changes in immediate-early gene expression.
Conclusions: Acetylation status of cardiac histones, affected by hemorrhage, is further modulated by resuscitation producing a fluid-specific code that is preserved in different species. Resuscitation with KR causes histone acetylation at the largest number of lysine sites (predominately H3 subunit), and has the most pronounced impact on the transcriptional regulation of selected (immediate-early response) genes.

Introduction

Tissue hypoperfusion during shock is treated conventionally with resuscitation fluids. Although resuscitation clearly improves hemodynamic profile it is now well recognized that these resuscitation fluids are not innocuous, and may actually exaggerate the cellular injury.[1] Among subjects resuscitated from hemorrhagic shock, a physiologic spectrum of responses is observed with overt multiple organ failure/dysfunction syndrome (MODS) manifested on one extreme and no apparent sequelae on the other. It is often assumed that the explanation for this variable response stems from the inherent variation in the human genome. When one considers however the remarkable homogeneity of that genome, there would appear to be an alternative, possibly epigenetic, mechanism to explain these profound individual differences.

Since the decoding of the human genome, it has become obvious that there are only 20,000—35,000 protein-coding genes responsible for millions of different phenotypes. Thus, it is not surprising that additional mechanisms that add genetic variability by altering the chromatin structure (and hence its function) have recently been identified.[2] Post-translational modifications of histones are among the most widely investigated mechanism in this evolving concept of epigenetics.[3] It is easier to understand this concept if we consider the fact that regulatory signals entering the cell nucleus interact not with DNA but chromatin, or the 1:1 complex of DNA and proteins. Chromatin, earlier thought to be merely a compacting/packaging protein, is now recognized as a regulatory protein, which critically influences the transcription of various genes.[4] Nucleosomes, the fundamental subunits of chromatin, contain an octomer of histones, two of each H2a, H2b, H3 and H4, surrounded by 146 base pairs of nucleotides.[5] Enzyme catalyzed chemical modifications such as acetylation, methylation, phosphorylation, ubiquitination and poly-ADP ribosylation, on the amino terminal tail of these histone proteins result in structural modification of the nucleosome, making the DNA available for transcription.[6] Among these modifications, acetylation is best known to correlate with transcriptional control of the genetic information.[3] Silencing of gene expression is associated with deacetylated histones, whereas activation of gene expression is associated with acetylated histones.[3]

There has been a recent surge in interest to identify precisely the factors that influence the acetylation status of chromatin,[7] and how the post-translational modification of histone control various nuclear functions including transcription of the genome. We now know that acetylation status of the histones is regulated precisely by interplay between two groups of enzymes; histone acetyl transferases (HATs) which increase acetylation, and histone deacetylases (HDACs) which exert the opposite effect. We have already tested the effects of hemorrhagic shock and resuscitation on histone acetylation in a rat model,[8] to demonstrate that shock leads to hypoacetylation of the histones, whereas resuscitation increases acetylation in a fluid specific fashion (''histone code''). However, in that study, changes in expression of the genes associated with hyperacetylated histones were not studied. With current technological advancement, particularly chromatin immunoprecipitation (CHIP) assay, it is now possible to link acetylated histones with their corresponding genes and thereby identify the genes whose transcription is altered. Another limitation of the previous study was that it used a small animal model of controlled hemorrhage. We therefore decided to reproduce these findings in a more clinically relevant large animal model of hemorrhage/resuscitation that has been established by our team.[9] We hypothesized that hemorrhage and resuscitation induced changes in histone acetylation in the swine model would be similar to those seen in rodents. Furthermore, we reasoned that alteration in histone acetylation would modulate the expression of associated acute phase genes.

Materials and methods

The Institutional Animal Care and Use Committee (IACUC) approved this study. All research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996 edition).

Animal preparation, monitoring and analytic sampling

Yorkshire swine (40—58 kg, Tom Morris Farms, Reisterstown, MD) were fed a standard diet and observed for at least a week to ensure a good state of health. Food was withheld the night before the experiment, but access to water was allowed. Anesthesia was induced with an intramuscular injection of ketamine (10 mg/kg) and inhaled isoflurane 4—5%. After placement of tracheal tubes, isoflurane concentration was reduced to approximately 1% for the rest of the experiment. Animals were allowed to breathe spontaneously using a mixture of 30% oxygen and air, delivered through a Narkomed M ventilator (North American Drager, Telford, PA).

Animals were placed in supine position on the operating table. The right external jugular vein and carotid artery were cannulated with an 8.5-Fr introducer sheath and 20-gauge angiocatheter, respectively, using a cut-down technique. A 7.5-Fr oximetric thermodilution pulmonary artery catheter (Baxter Health Care Corp., Irvine, CA) was positioned in the pulmonary artery through the introducer sheath. The catheters were attached to a hemodynamic monitoring system (Hewlett-Packard, Palo Alto, CA) for continuous monitoring of the pulmonary and carotid artery pressures. A Baxter system (Explorer, Baxter Edwards Critical Care, Irvine, CA) was used for continuous monitoring of mixed venous oxygen saturation and for recording the measured and derived pulmonary artery catheter variables.

After instrumentation, samples for complete blood count, serum chemistry, and serum ELISA were drawn at baseline, end of hemorrhage (30 min), and end of experiment (210 min). Blood samples for ABG's were drawn at 15-min intervals. Arterial and mixed venous blood samples were analyzed on Nova Stat Profile Ultra (Nova Biomedical, Waltham, MA). Heart tissue was harvested at the end of the experiment and was stored at $-80\,^{\circ}C$.

Hemorrhage and resuscitation protocol

A lower midline abdominal incision was used to gain access into the peritoneal cavity. Two standardized longitudinal (medial and lateral) lacerations were made in the common iliac artery by passing a number 15-scalpel blade through the arterial walls. A venous injury was simultaneously created, by performing a 50% transection of a large branch of the internal iliac vein. This resulted in a brisk uncontrolled arterial and venous hemorrhage. The loops of bowel were allowed to fall back in place. After cessation of bleeding, the abdomen was temporarily closed with penetrating towel clamps.

Resuscitation was performed in different phases simulating various echelons of care in the battlefield:

- *Immediate post-injury period*: animals were kept in shock for 30 min (simulating response time by the medic in the field). To achieve similar blood loss in all animals, additional blood was withdrawn from the arterial line if needed during this period to adjust total blood loss to 40% of the estimated blood volume.
- *Limited volume resuscitation in the field*: after 30 min of shock, bled animals were either observed [(NR) no resuscitation] or resuscitated with either 25 ml/kg of crystalloid or 6 ml/kg of Hespan (HS) over 30 min. The groups were as follows ($n = 7$/group): (1) no hemorrhage control (NH); (2) hemorrhage with NR; (3) resuscitation with 0.9% saline (NSS); (4) resuscitation with racemic lactated Ringer's (DL-LR); (5) resuscitation with L-isomer lactated Ringer's (L-LR); (6) resuscitation with Ketone Ringer's (KR); (7) resuscitation with 6% hetastarch in 0.9% saline (HES). KR was identical to the lactated Ringer', except that instead of lactate it contained 28 mmoles of β-hydroxybutyrate.
- *Operative intervention and resuscitation*: vascular injuries were repaired and animals were resuscitated with 30 ml/kg of assigned crystalloid infused over 60 min. Animals that were resuscitated initially with 6% hetastarch (Hespan) solution were now switched to normal saline resuscitation.
- *Goal directed resuscitation*: postoperatively, additional infusions of 10 ml/kg every 30 min (up to a total resuscitation of 85 ml/kg) were given until the cardiac output (CO), lactate, and base deficit improved to baseline values (±10%).

The rationale behind the choice of these resuscitation strategies is described in more detail in the discussion section. The crystalloids used in this experiment were normal saline solution (NSS); commercially available lactated ringer's solution, containing both D- and L-isomers of lactate (DL-LR: B. Braun medical Inc., Irvine, CA); modified lactated Ringer's (L-LR; Baxter healthcare Corp., Deerfield, IL); and ketone Ringer's solution, which was manufactured by Hemonetics Corp (Braintree, MA), and generously provided as a gift by Dr. C. Robert Valeri (Boston, MA).

Subcellular protein fractionation

Heart tissue (50 mg wet weight) was sampled, immersed in extraction buffer and sonicated by an ultrasonic cell disruptor (Series 36810, Cole Parmer instrument company, Vernon Hill, IL). A subcellular proteome extraction kit (Calbiochem) was used according to instructions. Protease inhibitor cocktail was added to prevent protein degradation. The supernatants were obtained through different extraction procedures with sequential incubation and centrifugation as follows:

- Fraction I (F1): cytosolic protein extract.
- Fraction II (F2): membrane/organelle protein extract.
- Fraction III (F3): nucleic protein extract.
- Fraction IV (F4): cytoskeletal matrix protein extract.

Total protein concentration in each fraction was determined by BCA protein assay (Pierce, Rockford, IL).

Western blotting

The F3 proteins were electrophoresed by SDS-PAGE using 4—20% Tris—Glycine Gel (1.5 mm × 10 wells, Invitrogen, Carlsbad, CA), and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA). After being blocked with SuperBlock blocking buffer (Pierce, Rockford, IL), the immunoblots were probed with primary

antibodies. Initially F3 fractions of heart tissues were probed with anti-acetylated-lysine polyclonal antibody (1:1000; Cell Signaling Technology, Beverly, MA). The antibody detects all proteins containing acetylated lysine residues. It is therefore possible to determine whether the change in degree of protein acetylation was fraction-specific and treatment-specific with different resuscitation strategies after hemorrhagic shock. Finally, to determine specific pattern of core histone acetylation, the nuclear fractions were probed with multiple antibodies generated against acetylated histone on different lysine residues: acetyl histone H2A-lys9 (Upstate), acetyl histone H2B-lys20 (Cell Signaling Technology), acetyl histone H3-lys9 (Cell Signaling Technology), 14, 18, 23 (Upstate), and acetyl histone H4-lys5, 8, 12, 16 (Upstate). After incubation with primary antibodies, immunoblots were then incubated with corresponding secondary antibodies at room temperature for 45 min. The signals were visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Immunoblots were then stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) at 37 °C for 30 min and reblotted for total anti-histone H2A, H2B, H3, and H4 antibodies (Biovision, Exton, PA) to measure total core histone contents.

ChIP (chromatin immunoprecipitation) assay

Hundred and fifty milligram of pig heart frozen tissue was minced into small pieces with a razor blade. These pieces were then placed into a 15-ml conical tube containing 10 ml of cold phosphate buffered solution (PBS). After one stroke using the OMNI homogenizer, set at low-power, the protein-DNA was cross-linked by incubating in 1% formaldehyde solution which was achieved by adding 270 μl of 37% commercially formed solution PBS directly. Tubes were then incubated at room temperature for 15 min on a rotating platform. The pellet cells were obtained by centrifugation at 4 °C for 5 min, then washed twice in 4 ml of ice-cold PBS containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and scraped into 1.5 ml of centrifuge conical tube, re-suspended in 200 μl of SDS lysis buffer (Upstate, cat# 20—163, Lake Placid, NY) containing protease inhibitor cocktail and incubated for 10 min on ice. Lysates were sonicated with fifteen (15 s each) pulses at 30% of maximum power by Ultrasonic cell disruptor (Cole Parmer, cat# EW-368100-00,) in order to shear DNA to lengths between 100 and 500 bp. Shearing efficiency was verified by 2% agarose gel electrophoresis after reversing DNA-protein cross-link at 65 °C for 4 h. Debris was then removed from samples by centrifugation for 10 min at 13,000 rpm at 4 °C; 100 μl of supernatant was diluted 10-fold in ChIP dilution buffer (Upstate, cat# 20—153, Lake Placid, NY) adding protease inhibitor cocktail. Twenty microliter of the diluted solution was kept as input DNA. To reduce nonspecific background, the diluted supernatant was pre-cleared with 40 μl of 50% slurry of salmon sperm DNA/protein A agarose (Upstate, cat# 16—157C, Lake Placid, NY) for 30 min at 4 °C with agitation. Beads were pelleted by centrifugation at 1000 rpm for 1 min, and supernatants were transferred to fresh tubes and incubated with 5 μg of anti-acetylated H3 antibody (upstate, cat# 06—599, Lake Placid, NY), anti-acetylated H4 antibody (Upstate, cat# 06—866, Lake Placid, NY), or normal rabbit serum as negative control overnight at 4 °C with rotation.

To collect the antibody/histone complex, 40 μl of 50% slurry of salmon sperm DNA/protein A agarose was added to samples, and incubated for 1 h at 4 °C with rotation. The protein A agarose/antibody/histone complexes were centrifuged and washed five times for 5 min each with 500 μl of different washing buffer from low salt immune complex wash buffer one wash at 4 °C, high salt immune complex wash buffer one wash at 4 °C, LiCL immune complex wash buffer one wash at 4 °C, and TE buffer two wash at room temperature. The complexes were eluted twice by 125 μl of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3) for 15 min at room temperature with rotation. Ten microliter of 5 M NaCl was added to the combined eluates, 1 μl of 5 M NaCl was added to input DNA samples, and reverse histone-DNA crosslink by heating at 65 °C for 4 h. Five microliter of 0.5 M EDTA, 10 μl of 1 M Tris—HCl, pH 6.5 and 1 μl of 10 mg/ml proteinase K were added to the combined eluates and incubated for 1 h at 45 °C. The DNA was purified by QIAquick PCR purification kit (QIAGEN, cat# 28106, Valencia, CA) following the manufacture protocol. The final immunoprecipitated DNA product was re-suspended in 50 μl of distilled water for PCR analysis. 2.5 μl from 50 μl of immunoprecipitated DNA with anti-acetylated H3 antibody, anti-acetylated H4 and normal rabbit serum as negative control and 50 μl of input DNA was used to perform 25 μl of PCR for c-fos, c-myc and HSP70 in a GeneAmp PCR System 9700 thermocycler.

The optimal reaction condition for PCR was determined for each primer pair. Parameters were as follows: denaturation at 94 °C for 45 s; annealing at 56 °C for 30 s for c-fos P1 and c-fos P2, 64 °C for 45 s for c-fos P3 and HSP70, 54 °C for 30 s for c-myc P1, 52 °C for 30 s for c-myc P2 and following by elongation at 72 °C for 45 s. PCR products were analyzed on 2% agarose gel at 150 V constant voltage for 40 min. The densities of bands were quantified using GEL DOC1000/2000 Quantity One program (Bio-Rad Laboratories, Hercules, CA) software. The ratio between immunoprecipitated DNA and input DNA was calculated for each animal and primer set. The percentage change of the experimental groups in which animals were resuscitated with different fluids after hemorrhagic shock was calculated by using control group- no hemorrhage (NH) 100% as reference.

The primer pairs used for c-fos ChIP analysis were: 5′-CTC CCT CCC GTT TAC ACA GG-3′ (FP1), 5′-ATT GCA GCT CTC GAG GGG TG-3′ (BP1), the product size was 265 bp; 5′-CTC CCT CCC GTT TAC ACA GG-3′ (FP2), 5′-TGC GGT TGG AGT ACT AGG CC-3′ (BP2), the product size was 340 bp; 5′-AAT GGG GGT GGA GGC GCA TT-3′(FP3), 5′-GGG AGG CGG GAA AGG CAG AG-3′ (BP3), the product size was 212 bp. The consensus sequences within the promoter, and a schematic representation of the porcine c-fos gene are displayed in Figure 1.

The primer pairs used for c-myc ChIP analysis were: 5′-TTT ATA GGC GAG GGT CTG CG-3′ (FP1), 5′-CTC TGC TCT TCC ACC CTG GA-3′ (BP1), the product size was 273 bp; 5′-CCC ATC TAC ACT TAC CCC AC-3′ (FP2), 5′-TGA CAA ACC GCA TAC TGG TC-3′ (BP2), the product size was 242 bp.

The primer set for HSP70-2 ChIP analysis was: 5′-AGG TGC CGT GCA AAC GCG AA-3′(FP), 5′-CCT GCC CCC TGG CTT TCT GG-3′(BP), the product size was 152 bp.

Data were analyzed using a commercially available statistical software package (GraphPad Prism, San Diego, CA) and expressed as mean ± standard error of the mean (S.E.M.).

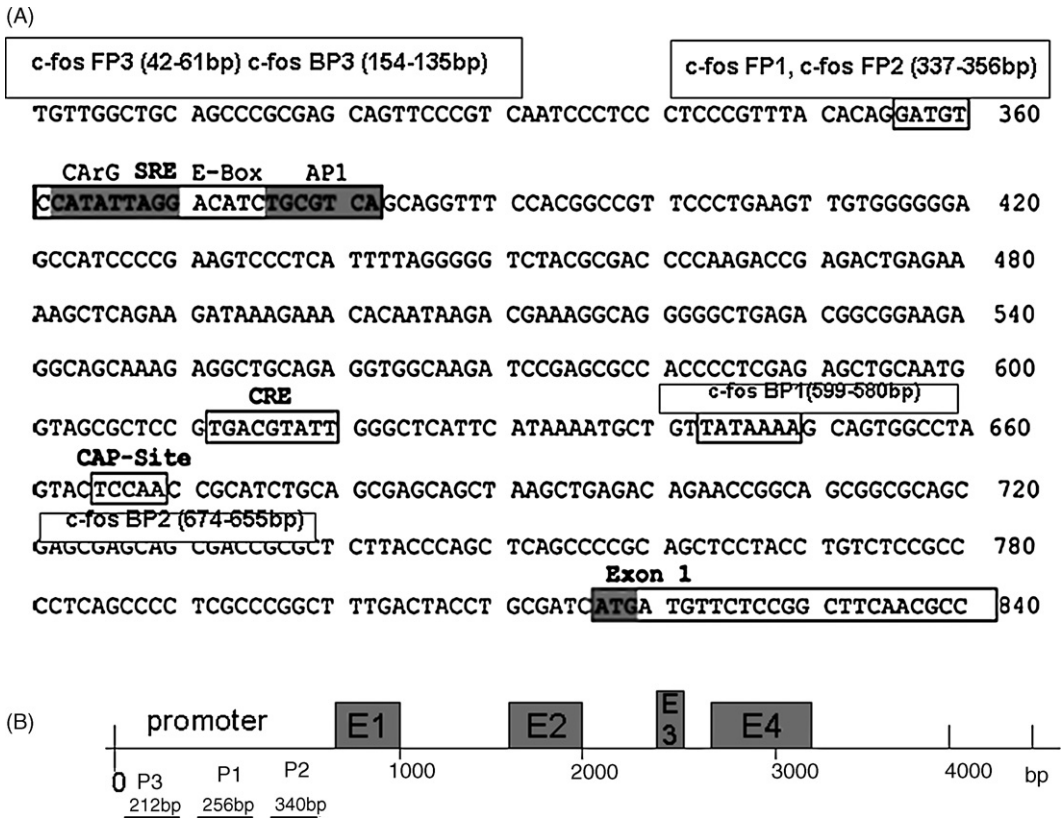

Figure 1 Porcine c-fos gene. (Panel A) Consensus sequences within the promoter of the porcine c-fos proto-oncogene. A TATA-box is situated at position −22, a CRE at position −53, an AP-1-box at position −289, an Ebox at position −294, and a SRE/CaRE at position −309. (Panel B) Schematic representation of the porcine c-fos gene in promoter region. Primer sets are indicated P3, P1 and P2.

Statistical significance of differences between groups was determined using one way analysis of variance (ANOVA) followed by Dennett's test for multiple comparisons. Significance was declared for $p < 0.05$.

Results

Physiological variables

Mean arterial pressure (MAP) and cardiac output (CO) remained stable during the pre-hemorrhage equilibration (40 min), and markedly decreased during the 30 min of hemorrhage. The degree of hypotension and decrease in CO were similar in all the groups that were subjected to hemorrhage (Figure 2). Although MAP readings in hemorrhaged animals were lower than the NH group during the entire experiment, after 30 min of resuscitation, the differences between the various treatment groups were statistically insignificant. As the normalization of blood pressure and CO were the endpoints for resuscitation, by the end of final resuscitation phase these variables were back to the baseline values. Physiological changes in this model have been reported in detail separately.[9] Selected data are presented in Table 1.

The efficiency of protein fractionation

Before proceeding with detailed analysis of histones, the efficiency of protein fractionation was verified to ensure that only fraction III (nucleic proteins) was selected for further testing. Most of heat shock protein 70 (HSP70) exists in the cytosolic compartment. In heart, the strongest signal was detected in fraction I, the cytosolic fraction, though there were also weaker signals detected in the

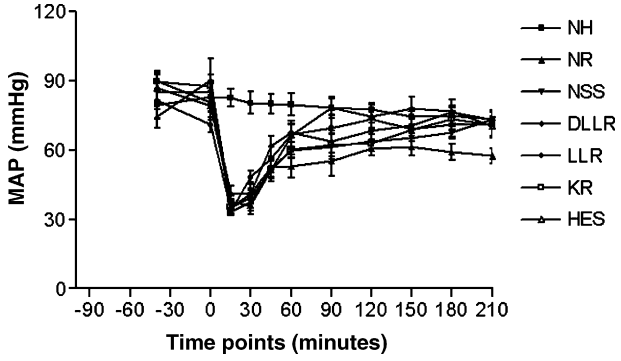

Figure 2 Graph showing mean arterial pressure (MAP) over time. Data presented as group means ± S.E.M. ($n = 7$/group). MAP measured in mmHg and time in minutes. NH = no hemorrhage control, NR = hemorrhage with no resuscitation, NSS = resuscitation with 0.9% saline, DL-LR = resuscitation with racemic lactated Ringer's, L-LR = resuscitation with L-isomer lactated Ringer's, KR = resuscitation with Ketone Ringer's, and HES = resuscitation with 6% hetastarch in 0.9% saline.

Table 1 Physiological values during experiment

	Baseline	Post-Hem	Initial Resuscitation	Final resuscitation
Hg (g/dl)				
NH	9.1 ± 0.3	9.2 ± 0.3	9.6 ± 0.4	9.7 ± 0.3
NR	8.8 ± 0.5	9.5 ± 0.5	9.3 ± 0.5	9.3 ± 0.5
NSS	8.2 ± 0.3	8.4 ± 0.3	6.7 ± 0.4*	6.4 ± 0.3*
DL-LR	10.0 ± 0.3	9.1 ± 0.3	6.7 ± 0.3*	6.3 ± 0.2*
L-LR	9.9 ± 0.6	9.4 ± 0.4	7.6 ± 0.6*	6.2 ± 0.6*
KR	9.4 ± 0.3	8.9 ± 0.4	6.5 ± 0.4*	5.8 ± 0.2*
HES	9.3 ± 0.6	9.1 ± 0.4	7.6 ± 0.5*	6.4 ± 0.4*
Cardiac output (l/min)				
NH	4.2 ± 0.3	4.9 ± 0.3	4.8 ± 0.5	4.9 ± 0.4
NR	4.2 ± 0.5	1.9 ± 0.2*	2.3 ± 0.3*	2.8 ± 0.4*
NSS	4.3 ± 0.3	2.5 ± 0.2*	4.2 ± 0.3	4.3 ± 0.5
DL-LR	4.4 ± 0.5	2.2 ± 0.4*	3.5 ± 0.4	4.8 ± 0.4
L-LR	3.8 ± 0.3	2.2 ± 0.4*	3.7 ± 0.5	3.8 ± 0.4
KR	4.7 ± 0.3	2.0 ± 0.1*	3.7 ± 0.4	4.1 ± 0.4
HES	4.2 ± 0.3	2.0 ± 0.2*	3.2 ± 0.4*	5.0 ± 0.2
Base excess (mmol/l)				
NH	5.0 ± 1.5	5.8 ± 2.0	9.3 ± 1.2	9.8 ± 0.8
NR	7.8 ± 1.2	4.1 ± 1.8	4.4 ± 1.6	6.1 ± 1.7
NSS	7.6 ± 0.6	3.5 ± 1.6	2.1 ± 1.3*	3.5 ± 1.2*
DL-LR	8.1 ± 0.8	7.0 ± 1.1	2.6 ± 1.6*	7.3 ± 0.9
L-LR	7.1 ± 0.5	3.5 ± 0.6	2.1 ± 1.3*	2.7 ± 1.5*
KR	9.0 ± 0.5*	5.2 ± 0.9	3.4 ± 1.1*	5.1 ± 1.7
HES	7.3 ± 1.0	4.0 ± 1.3	2.7 ± 2.0*	4.4 ± 1.4*
Lactate (mmol/l)				
NH	0.6 ± 0.1	1.0 ± 0.5	1.0 ± 0.3	0.6 ± 0.1
NR	1.4 ± 0.7	2.1 ± 0.6	3.8 ± 1.4	2.3 ± 1.0
NSS	0.8 ± 0.2	2.4 ± 0.7	2.1 ± 0.6	0.6 ± 0.2
DL-LR	1.7 ± 0.6	5.0 ± 2.7	7.4 ± 3.3*	3.2 ± 1.0
L-LR	0.6 ± 0.2	2.0 ± 0.4	4.3 ± 1.2	2.3 ± 0.9
KR	1.1 ± 0.2	1.6 ± 0.3	2.5 ± 0.4	2.1 ± 1.2
HES	0.9 ± 0.3	2.2 ± 0.9	2.7 ± 0.5	0.7 ± 0.3

Data is presented as group means ± S.E.M. *Represents $p < 0.05$ using ANOVA and Dunnett's test for multiple comparisons against no hemorrhage group. NH = no hemorrhage, NR = no resuscitation, NSS = normal saline solution, DL-LR = DL-lactated Ringer's solution, L-LR = L-isomer lactated Ringer's solution, KR = ketone Ringer's solution, HTS = 7.5% hypertonic saline, HES = Hespan. Post-Hem = at the end of 30 min of hemorrhage, Initial resuscitation = at the end of limited volume resuscitation (60 min post-injury), Final resuscitation = at 180 min post-injury.

three other fractions. Anti-histone H3 antibody was used to probe histone H3 whose signal was only detected in fraction III—the nucleic fraction. Therefore, the protein extraction method effectively separated the proteins into four different fractions.

The effect of resuscitation strategies on core histone (F3) Acetylation after hemorrhagic shock

The amount of total (non-acetylated) core histones—H2A, H2B, H3 and H4 was confirmed not to be different among groups except the DL-LR group where the amount of H4 was statistically different. We then measured the core histone acetylation levels in all group subjected to hemorrhage. These are described in detail below (as percent change compared to the sham animals).

The impact of hemorrhagic shock on core histone acetylation: in heart tissue of the animals subjected to shock but not resuscitated, there was no significant increase in the acetylation on lysine residues of histones.

The effect of resuscitation strategies on core histone acetylation (Table 2): resuscitation altered histone acetylation. These changes at different lysine residues are listed below for the different histone sub-units. Changes were considered significant when p value was <0.05 on inter group comparisons.

(1) H2A

Lysine 9: there was no significant increase in any group compared to sham.

(2) H2B

Lysine 20: there was a significant increase in the HES group compared to sham, NR, DL-LR, and L-LR groups.

Table 2 Acetylation pattern in cardiac histone subunits

	H2A H2B		H3				H4			
	Lys9	Lys20	Lys9	Lys14	Lys18	Lys 23	Lys 5	Lys 8	Lys 12	Lys 16
NR										
NS										↑175
DL-LR			↑260							
L-LR										
KR			↑238	↑245	↓60		↑164			
HES		↑136								

Data presented as %change compared to sham hemorrhage animals. Only statistically significant changes shown in table. NR = hemorrhage with no resuscitation, NS = resuscitation with 0.9% saline, DL-LR = resuscitation with racemic lactated Ringer's, L-LR = resuscitation with L-isomer lactated Ringer's, KR = resuscitation with Ketone Ringer's, and HES = resuscitation with 6% hetastarch in 0.9% saline. Arrows point direction of change. Detailed description of different histone subunits is included in the text.

(3) H3

Lysine 9: there was a significant increase in DL-LR and KR groups compared to sham, NR, and HTS groups.

Lysine 14: there was a significant increase in KR group compared to sham, DL-LR, L-LR, HTS, and NS groups.

Lysine 18: there was a significant decrease in KR compared to sham.

(4) H4

Lysine 5: KR group had a significantly higher level compared to all other groups.

Lysine 8: there was no significant difference in all experimental groups compared to sham.

Lysine 12: there was no significant difference in all experimental groups compared to sham.

Lysine 16: NS group had significantly higher level compared to all other groups.

ChIP statistical analysis

Although Chromatin immunoprecipitation (ChIP) is an important assay to study protein and gene interactions within the context of the cell, the reaction conditions of the procedure must be optimized to use this assay successfully. Initially, we varied the time of fixation in 1% formaldehyde of the frozen heart tissues, as well as used a variety of sonication conditions in order to break DNA to 100—500 bp fragments. The ChIP assay was performed to obtain immunoprecipitated DNA by using anti-acetylated histone H3 and anti-acetylated histone H4 antibodies. In addition, we detected the early expression of genes (c-fos and c-myc) and heat shock protein (HSP) 70 gene by PCR analysis. The primer sets for c-fos were selected from the different promoter regions of the gene: c-fos P1 located in between distal and proximal sites from transcriptional start site, c-fos P2 in the proximal site including TATA-box and AP-1 box, and c-fos P3 in distal site.

KR resuscitation after hemorrhagic shock increased the expression of H3 c-fos P1 and H3 c-fos P2. Hemorrhagic shock without resuscitation also induced an increase expression of H3 c-fos P2. There was no change in the expression of c-fos P3 among all groups (Figure 3). DL-LR resuscitation up-regulated the expression of H4 c-fos P2.

To examine whether this effect was selective for another gene, we also detected the levels of histone H3 and H4 associated with the c-myc and HSP70 genes. The primer sets for c-myc PCR analysis were chosen from the two promoter regions of the gene, which are c-myc P1 in proximal site and c-myc P2 in distal site. The expression of c-myc P1 was increased by DL-LR resuscitation (associated with acetylated H3), the expression of c-myc P2 was increased by KR resuscitation (associated with acetylated H3 and H4), and hemorrhagic shock without resuscitation (NR) (H4 antibody) (Figure 4). As shown in Figure 4, DL-LR, KR, HES and NR groups all increased the expression of HSP70 associated with acetylated H3 antibody (Figure 5). There was no alteration for the expression of HSP70 associated with acetylated H4. Taken all together, these data suggest that hemorrhagic shock and resuscitation strategies could induce changes in the expression of specific genes that correlate with histone acetylation.

Discussion

Resuscitation fluids are given routinely after hemorrhagic shock in an effort to restore intra-vascular and interstitial volume deficits. Traditionally, effectiveness of fluid resuscitation was measured simply in terms of improvement in physiological variables, but now we know that these strategies can have an enormous impact on sub-cellular regulatory mechanisms.[1] Recent studies from our laboratory and others suggest that long-term adaptations at the level of gene expression are influenced by shock and further modulated by resuscitation.[10,11] The goal of the present

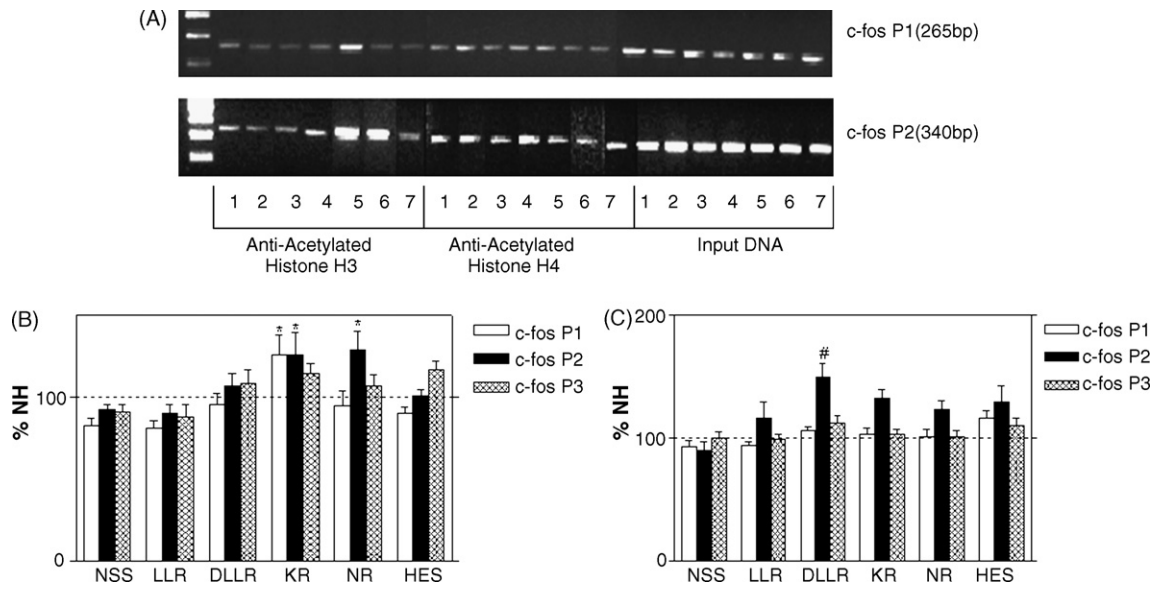

Figure 3 Expression of cardiac c-fos in different resuscitation groups. Panel A displays the group specific PCR products for c-fos gene (primer sets 1 and 2). Numbers 1—7 as follows: 1 = NH, 2 = NSS, 3 = LLR, 4 = DLLR, 5 = KR, 6 = NR, 7 = HES. The ratio between immunoprecipitated DNA and input DNA was calculated for each animal and primer set. The bottom panels displays the findings as group means ± S.E.M., for anti-acetylated H3 (Panel B) and anti-acetylated H4 (Panel C). Data presented as percentage change in immunoprecipitated DNA of the different fluid experimental groups as compared to no hemorrhage (NH) taken as 100% ($n = 7$/group). *$p < 0.05$ and #$p < 0.01$ vs. NH group. KR resuscitation after hemorrhagic shock increased the expression of H3 c-fos P1 and H3 c-fos P2. Hemorrhagic shock without resuscitation also induced an increase expression of H3 c-fos P2. NH = no hemorrhage control, NR = hemorrhage with no resuscitation, NSS = resuscitation with 0.9% saline, DL-LR = resuscitation with racemic lactated Ringer's, L-LR = resuscitation with L-isomer lactated Ringer's, KR = resuscitation with Ketone Ringer's, and HES = resuscitation with 6% hetastarch in 0.9% saline.

study was to determine if some of these transcriptional effects are mediated at the level of chromatin remodeling. We have previously shown that hemorrhagic shock in a small animal model (rodents) is associated with post-translational modification of histones, and that application of various resuscitative strategies leads to the development of highly distinctive acetylation/deacetylation profiles in cardiac histones.[8] In the same study, we also discovered that intravenous infusion of specific HDAC inhibitors (HDACI) during resuscitation can upregulate histone acetylation. This increase in acetylation appears to be protective, as HDACI before hemorrhagic shock has been shown to prolong survival (in the absence of any fluid resuscitation) in a lethal model of hemorrhagic shock.[12] Even more impressively, our ongoing experiments (unpublished) show that HDACI treatment can improve survival even when given after 1 h of severe hemorrhage (60% blood loss). However, all of these studies were conducted in small animal models and it remains unknown whether these histone modifications could be replicated in a higher-level animal subjected to a more realistic hemorrhage/resuscitation protocol.

The animal model for the current study consisted of uncontrolled hemorrhage from combined arterial and venous injuries, and used strategies (low volume initial resuscitation, use of hetastarch for prehospital resuscitation, realistic delays in start of resuscitation) that reflect the recommendations of the recent consensus conferences on combat resuscitation.[13,14] The delay from time of injury to surgical repair in our experiment was modeled after the experience of the US Navy Forward Resuscitative Surgery Systems (FRSS)[15] and the Army Forward Surgical Teams (FST).[16,17] during the current war. This model also had some obvious limitations such as lack of blast injury, no major non-vascular trauma, withdrawal of additional blood in addition to the uncontrolled hemorrhage (to minimize animal to animal variability). This was essentially a ''proof of concept'' study with short observation period that was not designed to provide direct evidence to link the sub-cellular changes to organ function and long-term outcomes. The choice of the heart as a target organ was made for several reasons. Myocardial dysfunction after hemorrhage is well described and demonstrated to persist hours after fluid resuscitation.[18] Histone/DNA ratios in the heart cluster closely around a value of 1 g/g, making efficient isolation possible. In contrast to classical biochemical manifestations of global ischemia-reperfusion, the content of myocardial acetyl coenzyme A, the sole source of acetyl groups for acetylation,[19] does not decrease in hemorrhagic shock.[20] Finally, the obtained data allowed for cross-species correlation with our previously published data on cardiac acetylation patterns in rats to validate our theory.

We investigated the effect of hemorrhagic shock followed by different resuscitative treatments on common post-translational modifications of cardiac histones H2A, H2B, H3 and H4. In addition to attempting to reproduce our rat study findings in a clinically relevant large animal study, we also opted to deepen the study by analyzing the state of histones H3 and H4 at three hemorrhage-regulated gene pro-

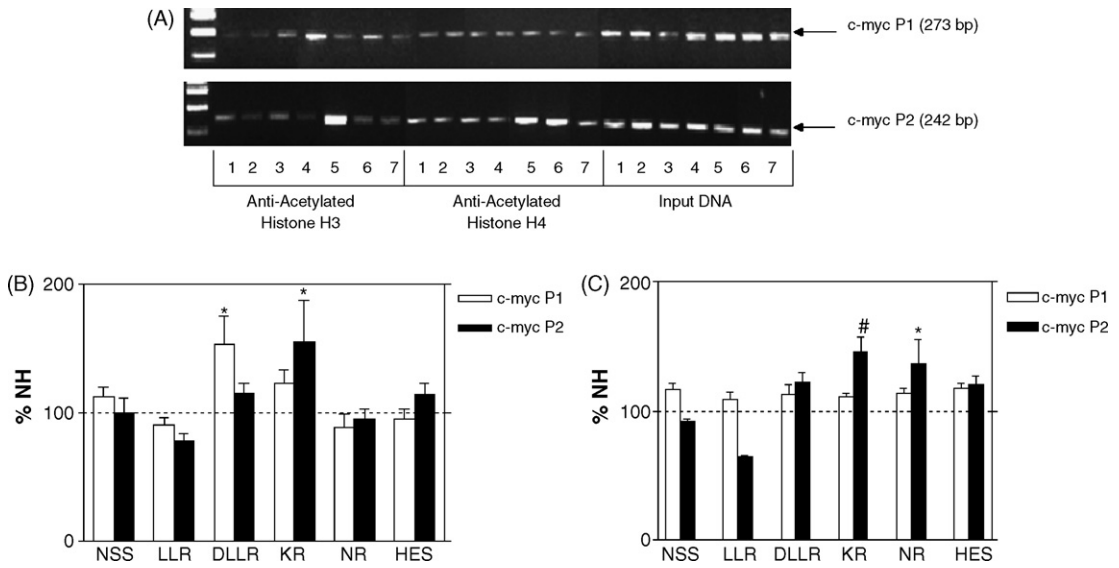

Figure 4 Expression of cardiac c-myc in different resuscitation groups. Panel A displays the group specific PCR products for c-myc gene (primer sets 1 and 2). Numbers 1—7 as follows: 1 = NH, 2 = NSS, 3 = LLR, 4 = DLLR, 5 = KR, 6 = NR, 7 = HES. The ratio between immunoprecipitated DNA and input DNA was calculated for each animal and primer set. The bottom panels displays the findings as group means ± S.E.M., for anti-acetylated H3 (Panel B) and anti-acetylated H4 (Panel C). Data presented as percentage change in immunoprecipitated DNA of the different fluid experimental groups as compared to no hemorrhage (NH) taken as 100% ($n = 7$/group). *$p < 0.05$ and #$p < 0.01$ vs. NH group. The expression of c-myc P1 was increased by DL-LR resuscitation (associated with acetylated H3), the expression of c-myc P2 was increased by KR resuscitation (associated with acetylated H3 and H4), and hemorrhagic shock without resuscitation (NR) (H4 antibody). NH = no hemorrhage control, NR = hemorrhage with no resuscitation, NSS = resuscitation with 0.9% saline, DL-LR = resuscitation with racemic lactated Ringer's, L-LR = resuscitation with L-isomer lactated Ringer's, KR = resuscitation with Ketone Ringer's, and HES = resuscitation with 6% hetastarch in 0.9% saline.

moters: the immediate-early response genes c-*fos*, c-myc, and HSP70. We found that acetylation patterns in cardiac histones are altered by hemorrhage and further modified by resuscitation in a fluid specific fashion. Resuscitation with ketone based fluids caused the largest number of transformations. As expected, changes in histone acetylation resulted in altered transcription of the associated genes. Finally, by comparing these findings to our previously published data (rat model) we discovered that these responses were well conserved across different species.[8,12]

Histone modifications represent a prominent form of chromatin remodeling. According to the ''histone code theory,'' different modifications of histones at a particular promoter region, alone or in combination, define a specific epigenetic state that encodes gene activation versus gene silencing.[21] Acetylation of histones allows the transcriptional machinery access to the promoters of particular genes. Hyper-acetylation at promoters indicates an increase in gene activity, whereas hypoacetylation marks a decrease in activity.[22] Furthermore, gene activity is marked by post-translational alterations on specific, but not random, lysine residues (K).[23] Acetylation at K5, K8, K12, K16 residues on the N-terminal tail of histone H4 are common modifications associated with genes active in transcription.[24,25] The deacetylation of K16 on H4 has been implicated in gene silencing. In addition, acetylation of K9 and K14, but not K18 or K23 on histone H3 is thought to be directly related to the regulation of gene transcription.[26] In our study, analysis of acetylation profiles in cardiac tissues revealed that resuscitation with KR induced the most changes, leading to hyperacetylation on most residues of H3 and K5 of H4 histones (Table 2). Remarkably, KR treatment induced the largest number of histone modifications in the rat model as well.[8] Hyperacetylation on H4 K5 and H3 K9 was the most pronounced in both the rat and swine models. Also of note is the fact that resuscitation with DL-LR was associated with hyperacetylation on only 1 residue out of 10 tested—K9 of H3. That was the only residue hyperacetylated after this treatment in rat model as well. The current study corroborates the original observation that resuscitative treatments indeed posses the ability to induce specific histone modification patterns, or in other words, they have special ''histone codes''. As the rat and swine models used very different hemorrhage and resuscitation protocols (type and rate of blood loss, volume and rate of fluid administration) the histone code seems to be dependent mostly upon the composition of resuscitation fluids. More importantly, it appears to be a highly conserved response, which is reproducible in different animal species.

Since hyperacetylation on specific lysine residues of particular histones implies variability in affected gene activity, we attempted to identify the gene-candidates. Using chromatin immunoprecipitation (ChIP) assays, we measured levels of H4 acetylation and H3 acetylation at the c-*fos*, c-myc, and HSP70 promoters in the cardiac tissues after hemorrhage and resuscitation. External stimuli (e.g. stress, cytokines, growth factors, pharmacological agents, fluids, etc.) exert their influence on the cells through activation

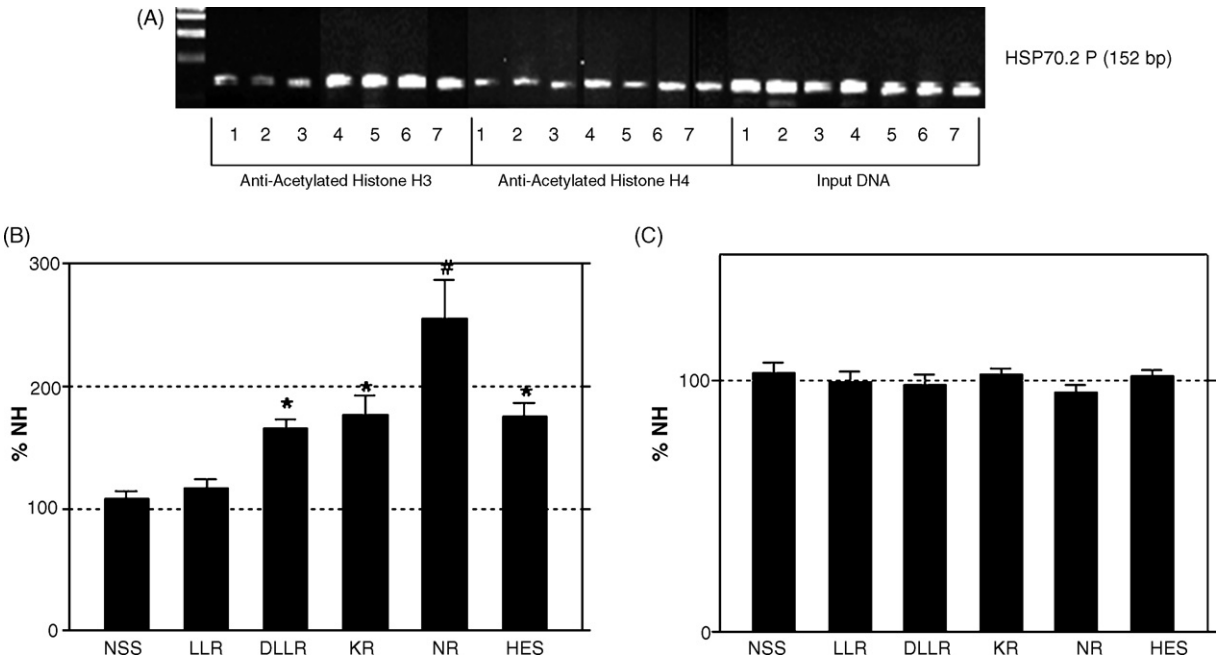

Figure 5 Impact of hemorrhage and different fluid resuscitation strategies on the expression of cardiac heat shock protein (HSP) 70. Panel A displays the group specific PCR products for HSP-70 gene. Numbers 1—7 as follows: 1 = NH, 2 = NSS, 3 = LLR, 4 = DLLR, 5 = KR, 6 = NR, 7 = HES. The ratio between immunoprecipitated DNA and input DNA was calculated for each animal and primer set. The bottom panels displays the findings as group means ± S.E.M., for anti-acetylated H3 (Panel B) and anti-acetylated H4 (Panel C). Data presented as percentage change in immunoprecipitated DNA of the different fluid experimental groups as compared to no hemorrhage (NH) taken as 100% ($n = 7$/group). *$p < 0.05$ and #$p < 0.01$ vs. NH group. DL-LR, KR, HES and NR groups all increased the expression of HSP70 associated with acetylated H3 antibody. There was no alteration for the expression of HSP70 associated with acetylated H4. NH = no hemorrhage control, NR = hemorrhage with no resuscitation, NSS = resuscitation with 0.9% saline, DL-LR = resuscitation with racemic lactated Ringer's, L-LR = resuscitation with L-isomer lactated Ringer's, KR = resuscitation with Ketone Ringer's, and HES = resuscitation with 6% hetastarch in 0.9% saline.

of signaling pathways that rapidly alter patterns of gene expression. Among the first genes that are induced by the intracellular signaling pathways, through histone modulation, are the immediate-early genes (IEGs). It has already been demonstrated that hemorrhage shock/resuscitation very rapidly stimulate the expression of two immediate early genes; c-fos and c-myc.[27] IEGs are rapidly activated in the absence of de novo protein synthesis and encode for secreted proteins, chemo-attractants, ligand-dependent and inducible transcription factors.[28] Recently, c-fos rapid induction and transcriptional regulation was shown to be under the control of chromatin remodeling enzymes and histone modifications, in particular, histone acetylation, which allows for access of the promoter regions of the IEGs.[29] Levels of c-*fos* mRNA peak 0—2 h after reperfusion, and it has been suggested that alterations in the pattern of IEG expression might represent an indication of the degree of organ damage or the repair processes subsequent to hypotension/reperfusion.[28]

To understand the molecular actions of shock/reperfusion better, we studied the histone architecture at the promoter regions of the IEGs. Heart tissue was processed to cross-link proteins bound to DNA *in vivo*. The cross-linked chromatin was then sheared to fragments of 500 bp in length. We then performed chromatin immunoprecipitation assays with antibodies against polyacetylated H4 (acH4) and acetylated H3 (acH3) and quantified the amount of DNA associated with the modified histones using PCR. Several controls were performed to confirm the specificity and validity of our assays. To control for the specificity of antibody binding, we immuno-precipitated chromatin samples with nonimmune IgG, which precipitated negligible levels of the various genes studied. To test the degree to which H3 and H4 acetylation correlates with the transcriptional activity of the c-*fos* gene, we measured c-*fos* mRNA levels. The *c-fos* mRNA levels immunoprecipitated with anti-acetylated H3 were increased in KR group, providing additional evidence that, for the cardiac c-*fos* gene, levels of H3 acetylation correlate with levels of transcription, whereas no such general correlation was apparent between c-*fos* transcription and levels of H4 acetylation. It is important to emphasize, of course, that these are correlations only and that causal relationships between histone modification and gene expression *in vivo* will require additional investigation.

Similar to c-fos, H3-precepitated c-myc expression was increased after resuscitation with KR, but also DL-LR, which correlated with increased degree of basal acetylation in these groups established previously. Again, no general correlation was apparent between HSP70 transcription and levels of H4 acetylation. Therefore, we found that, with few exceptions, levels of H3 acetylation correlate best with the expression of c-*fos*, c-myc, and HSP70 mRNA levels after hemorrhage and reperfusion. This important observa-

tion supports the hypothesis that histone hyperacetylation is associated with increased levels of gene activity in the reperfused heart and suggests that measurement of H3 acetylation at a promoter might serve as a novel marker for the dynamic, *in vivo* state of the activity of a gene during resuscitation phase. There were important exceptions to this observed correlation, however, that should be highlighted. Unresuscitated hemorrhagic shock led to significant up-regulation of HSP70, c-myc and c-fos mRNA although in contrast to the rat study, we did not observe any reliable changes in levels of H3 or H4 acetylation in this study. One possible explanation for this discrepancy is that other forms of post-translational modifications at this promoter (e.g. phosphorylation, methylation, etc.) may participate in the response, mimicking the effect of histone acetylation. Although phosphorylation appears to occur prior to and may be involved in promoting acetylation,[29] in case of IEGs specifically, blocking H3-Ser10 phosphorylation does not affect H3 acetylation or the induction of c-fos.[30] Since (1) H3 acetylation affects downstream induction of IEGs to a much greater extent, compared to other modifications and (2) we assume that by administering fluids we attempt to diminish the effects of shock, we propose that H3 acetylation in heart tissue and subsequent IEG induction could be the mechanism of therapeutic response to fluid therapy. In case of unresuscitated shock, when histone hyperacetylation is not present, dynamic recruitment of different factors and histone modifications, such as methylation of H3 on lysine 4, followed by the assembly of pre-initiation c-jun/c-fos complex,[31] could be important for IEGs activation.

Chromatin remodeling is a dynamic process in which several histones can be modified within close temporal and spatial proximity. As suggested by the histone code hypothesis, a combination of several histone modifications may ultimately determine the outcome of gene expression. Our data indicate complex changes in histone modifications at these promoters, which provide new insight into the mechanisms governing shock/reperfusion-induced regulation of gene expression in the heart. Is it possible that one fluid is more ''protective'' than others? Based on the number of hyperacetylated sites and associated gene activity, KR resuscitation stands out. Previously, we have shown that resuscitation with KR attenuates expression of adhesion molecules and apoptosis in affected tissues.[32,33] Shires et al. reproduced these findings in a recent study.[34] Employing a pressure-controlled model of hemorrhage in rats, they were able to detect increased level of pulmonary apoptosis upon resuscitation with DL-LR, and significant attenuation of apoptosis after KR resuscitation. It is entirely possible that these beneficial properties were due to an upregulation of pro-survival pathways controlled by acetylated histones. Regardless of the underlying mechanisms, these studies lend credence to new concept that resuscitation fluids should be treated as sophisticated pharmacological agents rather than simple volume expanders.

Ketone Ringer's-induced changes in histone acetylation could be regulated via recruitment of specific histone deacetylases (HDACs; enzymes that decrease histone acetylation), histone acetyltransferases (HATs; enzymes that increase histone acetylation), or proteins that regulate these enzymes. In this particular study, due to logistical reasons, we did not test fluids supplemented with known pharmacological inhibitors of HDAC. However, in our previous study, done in rats, the pattern of cardiac histone acetylation in group resuscitated with KR was remarkably similar to the one induced by pharmacological inhibition of HDAC.[8] Since in the current study the sites of histone hyperacetylation after KR resuscitation have shown high reproducibility, it is reasonable to assume that in KR exerted its action through the same mechanism (as seen in rats), namely, by inhibiting HDAC and promoting hyperacetylation on selective lysine residues. Interestingly, a short-chain fatty acid, sodium butyrate, is a known inhibitor of HDAC class I.[3] It is found normally in GI tract and, in addition to its potent anti-tumor properties, is currently being recognized for being responsible for the protective effects of high-fiber diets. However, its poor bioavailability, short half-life and difficulties in achieving active millimolar plasma concentrations have limited its clinical use. There are, obviously, two functional differences between beta hydroxybutyrate (BHB) that was used in KR and the salts of butyric acid: one is the substitution of sodium for the acidic hydrogen of the carboxylate and the second is the presence of the alcohol group in the BHB. The similarity of the action of these compounds depends on the chemical species responsible for HDAC inhibiting activities of the sodium butyrate. If it is the carboxylic acid, the action would be expected to be similar, if not identical. Therefore, KR appears to be an inexpensive and potent HDAC inhibitor that is suitable for clinical use.

In summary, in a large animal model of uncontrolled hemorrhage and clinically relevant resuscitation strategies, we have shown that resuscitation affects the acetylation patterns of nucleosomal histones in a fluid specific fashion. This ''histone code'' is highly conserved and reproducible across different animal species. Furthermore, changes in histone acetylation (specifically cardiac histone H3) influences transcriptional regulation of associated immediate-early response genes.

Conflict of interest statement

None to declare.

Acknowledgements

Funded by Office of Naval Research grant number N0014-05-MP-2-0006 (to HBA). Some of these data were presented at the 91st Clinical Congress of the American College of Surgeons (Surgical Forum), San Francisco, CA (October, 2005).

References

1. Committee on Fluid Resuscitation for Combat Casualties. Fluid resuscitation: State of the science for treating combat casualties and civilian injuries. Report of the Institute of Medicine. Washington, DC: National Academy Press; 1999.
2. Jenuwein T, Allis CD. Translating the histone code. Science 2001;293:1074—80.
3. Thiagalingam S, Cheng K, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. Histone deacetylases: unique players in shaping the epigenetic histone code. Ann NY Acad Sci 2003;983:84—100.

4. West AG, van Attikum H. Chromatin at the crossroads. Meeting on signaling to chromatin epigenetics. EMBO Rep 2006;7:1206—10.
5. Spotwood HT, Turner BM. An increasingly complex code. J Clin Invest 2002;110:577—82.
6. Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000;403(6765):41—5.
7. Roh TY, Cuddapah S, Zhao K, et al. Active chromatin domains are defined by acetylation islands revealed by genome-wide mapping. Genes Dev 2005;19:542—52.
8. Lin T, Koustova E, Chen H, Kirkpatrick J, Alam HB. Cardiac histones are substrates of histone deacetylase activity in hemorrhagic shock and resuscitation. Surgery 2006;139:365—76.
9. Ayuste EC, Chen H, Koustova E, et al. Hepatic and pulmonary apoptosis after hemorrhagic shock in swine can be reduced through modifications of conventional Ringer's solutions. J Trauma 2006;60:52—63.
10. Chen H, Alam HB, Querol RI, Rhee P, Li Y, Koustova E. Identification of expression patterns associated with hemorrhage and resuscitation: integrated approach to data analysis. J Trauma 2006;60:701—23.
11. Alam HB. An update on fluid resuscitation. Scand J Surg 2006;95:136—45.
12. Gonzales E, Chen H, Munuve R, Alam HB, et al. Valproic acid salvages hemorrhage associated lethality and affects the modification pattern of cardiac histones. Shock 2006;25:1—8.
13. Champion HR. Combat fluid resuscitation: introduction and overview of conferences. J Trauma 2003;54:S7—12.
14. Rhee P, Koustova E, Alam HB. Searching for the optimal resuscitation method: recommendations for the initial fluid resuscitation of combat casualties. J Trauma 2003;54:S52—62.
15. Chambers LW, Rhee P, Baker BC, et al. Initial experience of US Marine Corps forward resuscitative surgical system during operation Iraqi freedom. Arch Surg 2005;140:26—32.
16. Patel TH, Wenner KA, Price SA, Weber MA, Leveridge A, McAtee SJA. U.S. army forward surgical team's experience in operation Iraqi freedom. J Trauma 2004;57(2):201—7.
17. Rush RM, Stockmaster NR, Stinger HK, et al. Supporting the global war on terror: a tale of two campaigns featuring the 25th Forward Surgical Team (Airborne). Am J Surg 2005;189:564—70.
18. Remmers DE, Wang P, Cioffi WG, Bland KI, Chaudry IH. Testosterone receptors blockade after trauma-hemorrhage improves cardiac and hepatic functions in males. Am J Physiol 1997;273:H2919—25.
19. Wassarman PM, Wolffe AP. Chromatin (Methods in Enzymology), 304. Academic Press; 1999. p. 715—25.
20. Kline JA, Thornton LR, Lopaschuk GD, Barbee RW, Watts JA. Heart function after severe hemorrhagic shock. Shock 1999;12:454—61.
21. Khorasanizadeh S. The nucleosome: from genomic organization to genomic regulation. Cell 2004;116:259—72.
22. Grunstein M. Histone acetylation in chromatin structure and transcription. Nature 1997;389:349—52.
23. Agalioti T, Chen G, Thanos D. Diciphering the transcriptional histone acetylation code for human gene. Cell 2002;111:381—92.
24. Rahman I, Marwick J, Kirkham P. Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation NF-kappaβ and pro-inflammatory gene expression. Biochem Pharmacol 2004;68:1255—67.
25. Turner BM. Cellular memory and the histone code. Cell 2002;111:285—91.
26. Imhof A, Wolffe AP. Transcription: gene control by targeted histone acetylation. Curr Biol 1998;8:R422—4.
27. Saito T, Matsumoto I, Goto S, Kamada N, Motoki R, Wilce PA. The differential induction of two immediate early genes, c-fos and c-jun, after systemic hypovolemic shock/resuscitation in the rat liver and kidney. Surg Today 1998;28(6): 608—17.
28. Sng JC, Taniura H, Yoneda Y. A tale of early response genes. Bio Pharm Bull 2004;27:606—12.
29. Berger SL. An embarrassment of niches: the many covalent modifications of histones in transcriptional regulation. Oncogene 2001;20:3007—13.
30. Soloaga A, Thomson S, Wiggin GR, et al. MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of Histone H3 and HMG-14. EMBO J 2003;22:2788—97.
31. Martens JH, Verlaan M, Kalkhoven E, Zantema A. Cascade of distinct histone modicifications during collegenase gene activation. Mol Cell Biol 2003;23:1808—16.
32. Alam HB, Austin B, Koustova E, Rhee P. Resuscitation induced pulmonary apoptosis and Intracellular Adhesion Molecule-1 expression in rats are attenuated by the use of ketone Ringer's solution. J Am Coll Surg 2001;193:255—63.
33. Koustova E, Rhee P, Hancock T, et al. Ketone and pyruvate Ringer's solutions decrease pulmonary apoptosis in a rat model of severe hemorrhagic shock and resuscitation. Surgery 2003;134:267—74.
34. Shires GT, Browder LK, Steljes TP, Williams SJ, Browder TD, Barber AE. The effects of shock resuscitation fluids on apoptosis. Am J Surg 2005;189:85—91.