

# Molecular Changes Induced in Rat Liver by Hemorrhage and Effects of Melanocortin Treatment

Caterina Lonati, Ph.D.,\* Andrea Sordi, Ph.D.,\* Daniela Giuliani, Ph.D.,† Luca Spaccapelo, M.D.,‡ Patrizia Leonardi, B.Sc.,§ Andrea Carlin, B.Sc.,§ Alessandra Ottani, Ph.D.,† Maria Galantucci, Ph.D.,# Paolo Grieco, Ph.D.,|| Anna Catania, M.D.,\*\* Salvatore Guarini, Ph.D.††

## ABSTRACT

**Background:** Melanocortin peptides improve hemodynamic parameters and prevent death during severe hemorrhagic shock. In the present research we determined influences of a synthetic melanocortin 1/4 receptor agonist on the molecular changes that occur in rat liver during hemorrhage.

**Methods:** Controlled-volume hemorrhage was performed in adult rats under general anesthesia by a stepwise blood withdrawal until mean arterial pressure fell to 40 mmHg. Then rats received either saline or the synthetic melanocortin 1/4 receptor agonist Butir-His-D-Phe-Arg-Trp-Sar-NH<sub>2</sub> (Ro27-3225; n = 6–8 per group). Hemogasanalysis was performed throughout a 60-min period. Gene expression in liver samples was determined at 1 or 3 h using quantitative real-time polymerase chain reaction.

\* Postdoctoral Fellow, Center for Surgical Research, \*\* Director, Center for Preclinical Investigation, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milano, Italy. † Assistant Professor of Pharmacology, ‡ Postgraduate Student, # Postdoctoral Fellow, †† Full Professor of Pharmacology, Department of Biomedical Sciences, Section of Pharmacology, University of Modena and Reggio Emilia, Modena, Italy. § Research Assistant, Department of Internal Medicine, University of Milano, Milano, Italy. || Associate Professor of Pharmaceutical Chemistry, Department of Pharmaceutical Chemistry and Toxicology, University of Napoli "Federico II," Napoli, Italy.

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Address correspondence to Dr. Guarini: Department of Biomedical Sciences, Section of Pharmacology, University of Modena and Reggio Emilia, Modena, Italy. [salvatore.guarini@unimore.it](mailto:salvatore.guarini@unimore.it). Information on purchasing reprints may be found at [www.anesthesiology.org](http://www.anesthesiology.org) or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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## What We Already Know about This Topic

- Melanocortin molecules, including adrenocorticotrophic hormone, may protect organs against hemorrhagic shock-induced injury
- This study in rats investigates if a synthetic melanocortin may alleviate hemorrhagic-induced systemic and hepatic injury

## What This Article Tells Us That Is New

- The melanocortin receptor agonist Ro27-3225 restores mean arterial pressure; attenuates metabolic acidosis; and inhibits upregulation of interleukin-6, acute phase protein A2m, and several other injury mediators in the liver after a hemorrhagic challenge

**Results:** At 1 h, in saline-treated shocked rats, there were significant increases in activating transcription factor 3 (Atf3), early growth response 1 (Egr1), heme oxygenase (cycling) 1 (Hmox1), FBJ murine osteosarcoma viral oncogene homolog (Fos), and jun oncogene (Jun). These changes were prevented by Ro27-3225 (mean ± SEM: Atf3 152.83 ± 58.62 vs. 579.00 ± 124.13,  $P = 0.002$ ; Egr1 13.21 ± 1.28 vs. 26.63 ± 1.02,  $P = 0.001$ ; Hmox1 3.28 ± 0.31 vs. 166.54 ± 35.03,  $P = 0.002$ ; Fos 4.36 ± 1.03 vs. 14.90 ± 3.44,  $P < 0.001$ ; Jun 6.62 ± 1.93 vs. 15.07 ± 2.09,  $P = 0.005$ ; respectively). Increases in alpha-2-macroglobulin (A2m), heat shock 70kD protein 1A (Hspa1a), erythropoietin (Epo), and interleukin-6 (Il6) occurred at 3 h in shocked rats and were prevented by Ro27-3225 treatment (A2m 6.90 ± 0.82 vs. 36.73 ± 4.00,  $P < 0.001$ ; Hspa1a 10.34 ± 3.28 vs. 25.72 ± 3.64,  $P = 0.001$ ; Epo 0.49 ± 0.13 vs. 2.37 ± 0.73,  $P = 0.002$ ; Il6 1.05 ± 0.15 vs. 1.88 ± 0.23,  $P < 0.001$ ; respectively). Further, at 3 h in shocked rats treated with Ro27-3225 there were significant increases in

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tight junction protein 1 (Tjp1;  $27.30 \pm 2.43$  vs.  $5.03 \pm 1.68$ ,  $P < 0.001$ ) and nuclear receptor subfamily 4, group A, member 1 (Nr4a1;  $91.03 \pm 16.20$  vs.  $30.43 \pm 11.0$ ,  $P = 0.01$ ) relative to sham animals. Treatment with Ro27-3225 rapidly restored blood pressure, hemogasanalysis parameters, and lactate blood levels.

**Conclusions:** Melanocortin treatment significantly prevents most of the systemic and hepatic detrimental changes induced by hemorrhage.

**H**EMORRHAGIC shock triggers a systemic inflammatory response and oxidative stress. Blood loss, oxidative stress, and the adrenergic outflow start signaling events that promote induction of genes involved in stress and heat shock response, inflammatory reaction, and apoptosis. These processes can cause systemic inflammation and organ damage. In the most severe cases, multiple organ dysfunction syndrome occurs as a serious complication associated with high morbidity and mortality.<sup>1–8</sup> Redistribution of cardiac output and persistent gut ischemia after apparently adequate resuscitation can lead to hemorrhagic shock.

Treatment of hemorrhagic shock includes rapid operative resuscitation with fluids, blood transfusions, and vasopressor agents to limit activation of the inflammation mediator systems and abort the microcirculatory changes.<sup>9</sup> However, these approaches may not be sufficient to prevent organ failure. Therefore, therapies against this pathologic condition should modulate multiple cellular events induced by hemorrhage.

Melanocortin peptides, the collective name for adrenocorticotrophic hormone and  $\alpha$ -,  $\beta$ -, and  $\gamma$ -melanocyte stimulating hormone, exert protective influences on the host during local and systemic injury.<sup>10</sup> Effects of melanocortins are mediated by activation of five melanocortin (MC<sub>1</sub> through MC<sub>5</sub>) receptors that belong to the class A of G-protein-coupled seven transmembrane receptors.<sup>10</sup> The melanocortin 4 (MC<sub>4</sub>) is the prevalent melanocortin receptor within the central nervous system, where it is highly expressed in the hypothalamus, spinal cord, vagus nuclei, and cortex.<sup>11</sup> The MC<sub>1</sub> subtype is expressed in a wide range of peripheral tissues,<sup>12</sup> including the human liver.<sup>13</sup>

In previous research, we found that melanocortin peptides have a life-saving activity in experimental and clinical hemorrhagic shock.<sup>14–18</sup> Indeed, early treatment with adrenocorticotrophic hormone-(1–24) in a rat model of hemorrhagic shock prolonged survival and extended by some hours the time-limit for blood reinfusion to be effective.<sup>17</sup> The melanocortin-induced reversal of hemorrhagic shock was associated with mobilization of the residual blood, that, in shock conditions, is trapped in capillaries and large blood reservoirs, including the liver and spleen.<sup>19</sup> Furthermore, the survival rate in patients with acute type-A aortic dissection treated with adrenocorticotrophic hormone-(1–24) was significantly increased.<sup>18</sup> The synthetic MC<sub>1</sub>/MC<sub>4</sub> receptor agonist Ro27-3225 prevented death in rats subjected to lethal

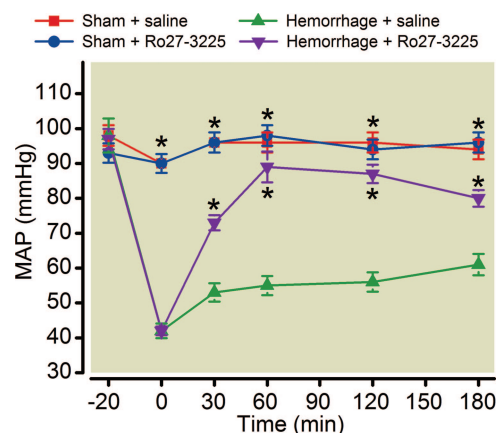
hemorrhagic shock.<sup>14</sup> Such life-saving effect was associated with improved cardiovascular and respiratory functions, reduced concentration of plasma free radicals, and prevention of multiple organ damage. These results prompted further investigations on molecular effects of this molecule in hemorrhagic shock. Research was focused on the liver, which is highly susceptible to ischemic damage,<sup>20</sup> and inflammatory cells accumulated in the liver during shock should be sources of inflammatory mediators that greatly contribute to multiple organ dysfunction.<sup>14–16</sup>

The aim of the present study was to determine effects of melanocortin treatment on gene expression profile in the liver during hemorrhage. Transcript selection was made to examine class of representative molecules that could be significant in local and systemic injury after hemorrhage. A controlled volume nonlethal hemorrhage was used in order to perform prolonged analysis of gene expression. Ro27-3225 was chosen because of its selective affinity for the MC<sub>1</sub> and MC<sub>4</sub> receptor subtypes. Indeed, these two receptors mediate most of peripheral (MC<sub>1</sub>) and brain-mediated (MC<sub>4</sub>) antiinflammatory effects of melanocortins.<sup>10</sup>

## Materials and Methods

### Animals

Wistar rats (Harlan, Milan, Italy) were kept in air-conditioned colony rooms (temperature  $21 \pm 1^\circ\text{C}$ ; humidity 60%) on a natural light/dark cycle, with food in pellets and tap water available *ad libitum*. Body weight (270–300 g) was similar in all groups ( $n = 6–8$  per group;  $P > 0.05$ ). Housing conditions and experimental procedures were in strict accordance with the European Community regulations on the use and care of animals for scientific purpose (CEE Council 89/609; Italian D.L.22-1-92 No. 116), and were approved by the Committee on Animal Health and Care of Modena and Reggio Emilia University (Comitato Etico per



**Fig. 1.** Treatment with the melanocortin MC<sub>1</sub>/MC<sub>4</sub> receptor agonist Ro27-3225 significantly restored mean arterial pressure values in rats subjected to controlled-volume hemorrhage. Values are means  $\pm$  SEM ( $n = 6$ ). \*  $P < 0.01$  versus hemorrhage + saline. MAP = mean arterial pressure.

**Table 1.** Effect of Intravenous Injection of Ro27-3225 (90  $\mu\text{g/kg}$ ) on Venous pH,  $\text{Po}_2$ ,  $\text{Pco}_2$ ,  $\text{HCO}_3^-$ , SBE,  $\text{SO}_2$ , and Lactate Levels in Hemorrhage Shocked Rats

Treatment	Time of Sampling	Rats (n)	pH	$\text{Po}_2$ (mmHg)
Saline	Before bleeding	8	$7.32 \pm 0.03$	$54.20 \pm 3.64$
—	After bleeding	7	$7.21 \pm 0.01^*$	$26.09 \pm 0.81^*$
—	15 min after treatment	8	$7.15 \pm 0.03^*$	$22.64 \pm 0.51^*$
—	60 min after treatment	6	$7.12 \pm 0.02^*$	$23.56 \pm 0.53^*$
Ro27-3225	Before bleeding	8	$7.30 \pm 0.02$	$53.16 \pm 2.98$
—	After bleeding	6	$7.18 \pm 0.02^*$	$25.69 \pm 0.78^*$
—	15 min after treatment	7	$7.14 \pm 0.02^*$	$38.02 \pm 0.24^{*\dagger}$
—	60 min after treatment	8	$7.28 \pm 0.01^\dagger$	$45.39 \pm 1.28^{*\dagger}$

Values are means  $\pm$  SEM.

\*  $P < 0.05$ , versus the corresponding value before bleeding.  $^\dagger P < 0.001$ , versus the corresponding value after bleeding.

$\text{HCO}_3^-$  = bicarbonate;  $\text{Pco}_2$  = carbon dioxide partial pressure;  $\text{Po}_2$  = oxygen partial pressure; SBE = standard base excess;  $\text{SO}_2$  = oxygen saturation values.

la Sperimentazione Animale, Modena, Italy). Moreover, all procedures conformed to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington D.C., 1996). Every effort was made to minimize the number of animals used and their suffering.

### Surgical Procedures, Shock Induction, and Treatments

Surgical procedures were performed under general anesthesia (urethane, 1.25 g/kg intraperitoneally). Urethane was chosen because it provides long-lasting and stable anesthesia with minimal interference with neurally mediated cardiovascular and respiratory regulatory functions.<sup>14–17,21</sup> Animals were spontaneously ventilating. After heparinization (heparin sodium, 600 U/kg intravenously), rats were instrumented with indwelling polyethylene catheters in a common carotid artery to record arterial blood pressure and in iliac vein for treatments and bleeding. The arterial catheter was connected to a pressure transducer coupled to a polygraph (Mortara-Rangoni, Bologna, Italy). Hemorrhage was induced by a stepwise withdrawal of about 40% of circulating blood (1.4–1.6 ml/100 g body weight;  $n = 6–8$  per group;  $P > 0.05$ ), until mean arterial pressure (MAP) fell to and stabilized at 40 mmHg or fewer. The procedure was completed within 20 min. Sham hemorrhage rats were subjected to all surgical procedures of hemorrhage animals, but were not bled. All the procedures were performed in sterile conditions. Animals were randomly assigned to the following intravenous bolus treatments: 1) the  $\text{MC}_1/\text{MC}_4$  receptor agonist Butir-His-D-Phe-Arg-Trp-Sar- $\text{NH}_2$  (Ro27-3225) administered to hemorrhage rats, at the dose of 90  $\mu\text{g/kg}$  dissolved in a volume of 1 ml/kg saline, 5 min after termination of the bleeding procedure; 2) hemorrhage and 3) sham hemorrhage rats received as control injection an equal volume of saline (1 ml/kg); 4) a further group of sham hemorrhage animals received Ro27-3225 (90  $\mu\text{g/kg}$  dissolved in 1 ml/kg saline). Ro27-3225 was synthesized in our laboratory by conventional solid phase chemistry, purified by reversed phase high-performance liquid chromatography, and checked for proper molecular weight by mass spectroscopy, as previously reported.<sup>14</sup> Ro27-3225 was used at a

fixed dose previously found to be effective in reversing severe hemorrhagic shock in rats in our laboratory.<sup>14</sup>

For gene expression study, rats treated with Ro27-3225 dissolved in saline (group 1: hemorrhage + Ro27-3225), or control rats treated with saline alone (group 2: hemorrhage + saline; and group 3: sham hemorrhage + saline), were sacrificed under deep anesthesia at 1 h ( $n = 6$  per group) or 3 h ( $n = 6$  per group) after treatment. Six normal (naïve) rats were sacrificed under deep anesthesia at time 0 and received no injections (baseline control for gene expression study). In all assessments, each animal was used for a single sample. Livers were immediately harvested and tissue samples were snap-frozen in liquid nitrogen for RNA extraction. Arterial blood pressure was monitored in all animals until sacrifice at scheduled intervals. The MAP values over time were compared among experimental groups 1–4 considering only animals that were sacrificed at 3 h ( $n = 6$  per group).

### Hemogasanalysis and Lactic Acid Measurement

Hemogasanalysis and lactic acid measurements were performed in experimental groups 1–4, in 6–8 rats per group and per time point. The recorded values over time of group 1 (hemorrhage + Ro273225) and group 2 (hemorrhage + saline) were compared. In all assessments, each animal was used for a single sample to avoid possible alterations of parameters that were recorded, induced by repeated blood sampling. Blood samples (0.2 ml) were taken from the venous catheter just before bleeding (basal conditions), immediately before treatment, and 15 and 60 min after treatment, and analyzed for pH, oxygen partial pressure, carbon dioxide partial pressure, bicarbonate, standard base excess, and oxygen saturation by using a System 1302 pH/blood gas analyzer (Instrumentation Laboratory, Milan, Italy). Lactate was measured by means of an enzymatic test (Ortho Clinical Diagnostics, Rochester, NY).

### RNA Isolation and Real-time Polymerase Chain Reaction

Gene expression was evaluated as described previously.<sup>22</sup> In brief, total RNA was isolated on an ABI Prism 6100 Nucleic

Table 1. Continued

Pco <sub>2</sub> (mmHg)	HCO <sub>3</sub> <sup>-</sup> (mequiv/l)	SBE (mequiv/l)	SO <sub>2</sub> (%)	Lactate (mmol/l)
46.01 ± 1.64	22.45 ± 0.62	-4.02 ± 0.49	84.01 ± 1.19	5.85 ± 0.51
52.39 ± 1.25*	19.01 ± 0.96*	-8.05 ± 0.72*	30.26 ± 1.18*	10.12 ± 1.01*
56.69 ± 1.95*	16.64 ± 1.12*	-12.24 ± 1.12*	19.64 ± 1.24*	11.01 ± 1.31*
58.32 ± 2.01*	15.91 ± 1.16*	-15.38 ± 1.32*	16.12 ± 0.84*	11.83 ± 1.28*
48.64 ± 1.31	24.01 ± 0.26	-3.86 ± 0.38	82.19 ± 1.91	5.69 ± 0.62
53.84 ± 1.61*	19.89 ± 0.62*	-8.09 ± 0.84*	29.18 ± 1.68*	10.31 ± 1.05*
64.32 ± 1.98*	18.84 ± 0.29*	-10.96 ± 0.49*	51.32 ± 1.28*†	10.84 ± 1.26*
47.39 ± 2.03	22.87 ± 0.98	-4.19 ± 0.83†	72.84 ± 3.64†	6.02 ± 0.58†

Acid PrepStation using Total RNA Chemistry (Applied Biosystems, Carlsbad, CA). RNA was checked for integrity by electrophoresis on denaturing agarose-formaldehyde gels and quantified by optical density measurement at 260 nm. Real-time polymerase chain reaction analysis was based on TaqMan chemistry. Two micrograms of total RNA were reverse transcribed to single-stranded complementary DNA using the High-Capacity Complementary DNA Archive Kit (Applied Biosystems). Polymerase chain reactions were performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Assay IDs for target and reference genes are reported in Supplemental Digital Content 1, <http://links.lww.com/ALN/A812>, which is a table listing all the genes investigated in this study. Fluorescence intensities were converted in threshold cycles (Ct) using ABI Prism SDS 2.3 software (Applied Biosystems). To optimize accuracy and precision, baseline and threshold were set automatically by the software. Only genes with Ct lower than 35 were considered significantly expressed; two genes (Chrna7, Il10) were excluded based on this criterion. Relative quantification was performed by means of the comparative Ct method ( $\Delta$ Ct), using reverse-transcribed Rat Universal Reference Total RNA (Clontech, Palo Alto, CA) as calibrator sample. Polymerase chain reaction efficiencies ( $\eta$ ) were evaluated by reference complementary DNA standard curves covering a 4 to 5 log dynamic range. Relative quantities were normalized using a normalization factor calculated by the public algorithm geNorm VBA applet version 3.4 for Excel.<sup>23</sup> Three independent polymerase chain reaction amplification experiments were performed for each transcript. The relative expression was calculated as the mean of normalized quantities in the replicates.

### Data Analysis and Statistical Evaluation

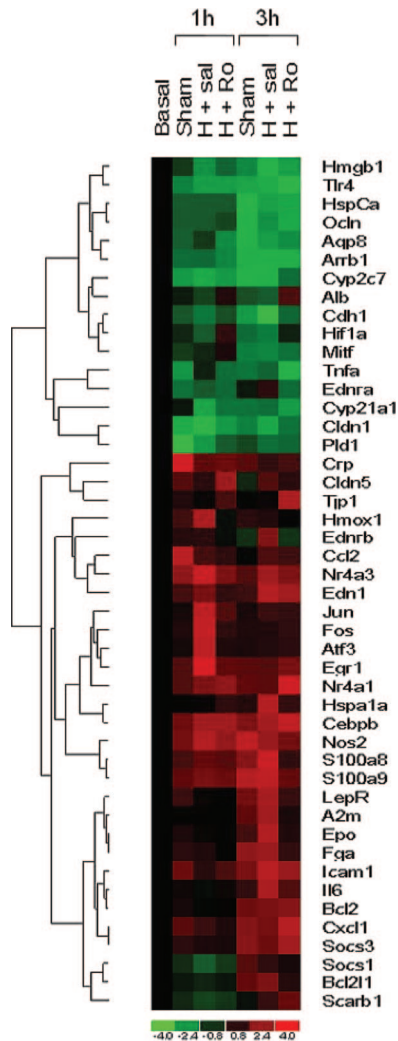
Analysis was performed blind to the treatment. Agglomerative hierarchical cluster analysis was performed on relative quantities of target genes using DNA-chip analyzer soft-

ware.<sup>‡‡</sup> In the analysis of genes listed in Supplemental Digital Content 1 (see <http://links.lww.com/ALN/A812>), which play a role in hemorrhagic shock<sup>1,4-9,24-30</sup>), Spearman rank correlation and average linkage were used as similarity metric and clustering technique, respectively. Here, Spearman rank correlation and average linkage don't test for significance the results; instead, they are used to show a global trend of gene expression across all the samples. Briefly, Spearman rank correlation, a nonparametric similarity measure, clusters genes whose expression levels may be very different, and it is robust against outliers. The average linkage method, used as distance metric, computes the distance between two gene clusters as the average of the distances between all the points in those clusters. Statistical evaluation of gene expression was performed on selected genes considered of relevant interest in our experimental conditions, on biologic basis.<sup>1,4-9,24-30</sup> Comparison of MAP values was performed using two-way repeated measures ANOVA and *post hoc* Bonferroni correction for comparisons of means. Differences in hemogasanalytic data, lactate levels, body weight, and blood withdrawn to induce shock were analyzed using one-way ANOVA followed by Bonferroni multiple comparison test. Differences in relative expression of individual transcripts were analyzed using two-way ANOVA followed by Bonferroni multiple comparison test. We assessed whether the different experimental groups differ among them in terms of the parameters we considered, such as MAP, gene expression, hemogasanalysis parameters, and lactate levels. We, therefore, set  $\alpha$  (level of statistical significance) equal to 0.05 (two-tailed). Results with  $P < \alpha$  were considered as statistically significant. All the statistical tests were performed using SigmaStat software, version 3.5 (Systat Software Inc, San Jose, CA).

### Results

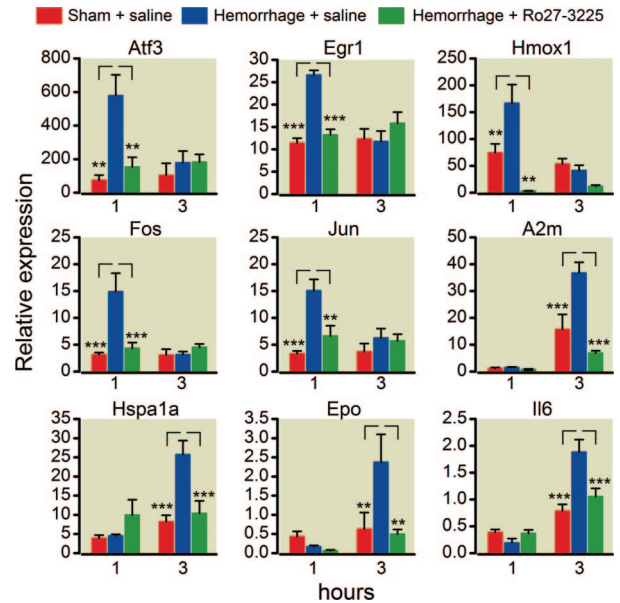
MAP values after hemorrhage and the effects of Ro27-3225 treatment on such values are reported in figure 1. Ro27-3225 ( $n = 6$ ) significantly enhanced MAP values relative to untreated animals ( $n = 6$ ) during the whole observation period. Bleeding caused a significant alteration in pH, oxygen partial

‡‡ [www.dChip.org](http://www.dChip.org); version 2010.01. Accessed July 30, 2011.



**Fig. 2.** Agglomerative hierarchical clustering heatmap of gene expression in livers of naïve, sham-hemorrhage, hemorrhage + saline, and hemorrhage + Ro27-3225 rats. Gene expression data obtained by real-time polymerase chain reaction are shown using dCHIP software (www.dChip.org; version 2010.01; accessed July 30, 2011). Samples are displayed in columns and genes in rows. Differences in gene expression are represented as a color normalized across each row, with *brighter red* for higher expression compared to basal sample, and *brighter green* for lower. Gene symbols are listed on the right. H + Sal = hemorrhage + saline; H + Ro = hemorrhage + Ro27-3225.

pressure, carbon dioxide partial pressure, bicarbonate, standard base excess, and oxygen saturation values, as well as in lactate levels ( $n = 6-8$ ; table 1); treatment with Ro27-3225 almost completely restored the basal conditions throughout the 60-min observation period ( $n = 6-8$ ; table 1). Treatment of sham animals with intravenous injection of Ro27-3225 or a control injection of an equal volume or saline (1 ml/kg) did not affect MAP ( $n = 6$  per group; fig. 1), hemogasanalysis parameters, and lactate levels ( $n = 6$  per group; not shown). Gene expression in the liver during hemorrhage was considerably altered (fig. 2). Indeed, out of the 46 transcripts examined, expression of nine genes was significantly

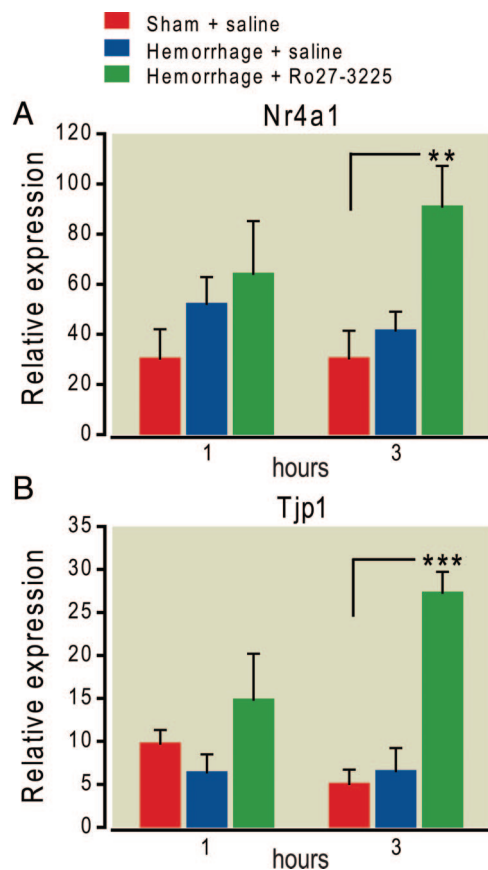


**Fig. 3.** Effects of Ro27-3225 treatment on expression of target genes evaluated using real-time polymerase chain reaction in rat livers. Ro27-3225 treatment reduced expression of activating transcription factor 3, early growth response 1, heme oxygenase (decycling) 1, FBJ murine osteosarcoma viral oncogene homolog, and jun oncogene at 1 h; expression of alpha-2-macroglobulin, heat shock 70kD protein 1A, erythropoietin, and interleukin-6 was significantly reduced by treatment at 3 h. Bars denote mean  $\pm$  SEM ( $n = 6$ ). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . A2m = alpha-2-macroglobulin; Atf3 = activating transcription factor 3; Egr1 = early growth response 1; Epo = erythropoietin; Fos = FBJ murine osteosarcoma viral oncogene homolog; Hmox1 = heme oxygenase (decycling) 1; Hspa1a = heat shock 70kD protein 1A; Il6 = interleukin-6; Jun = jun oncogene.

different in saline-treated shocked animals relative to sham rats at either 1 or 3 h. At 1 h, in saline-treated hemorrhage animals, there were significant increases in activating transcription factor 3 (Atf3), early growth response 1 (Egr1), heme oxygenase (decycling) 1 (Hmox1), FBJ murine osteosarcoma viral oncogene homolog (Fos), and jun oncogene (Jun) ( $n = 6$ ; fig. 3). Significant increases in expression of alpha-2-macroglobulin (A2m), heat shock 70kD protein 1A (Hspa1a), erythropoietin (Epo), and interleukin-6 (Il6) were observed at 3 h ( $n = 6$ ; fig. 3). All these changes were prevented when Ro27-3225 was given after hemorrhage ( $n = 6$  per group; fig. 3). Further, in Ro27-3225-treated hemorrhage rats there was a significant increase in expression of tight junction protein 1 (Tjp1 or Zo1) and nuclear receptor subfamily 4, group A, member 1 (Nr4a1) at 3 h ( $n = 6$ ; fig. 4). These transcripts were not induced in saline-treated hemorrhage animals ( $n = 6$ ; fig. 4). In all experimental groups, survival was 100% throughout the 3-h observation period.

## Discussion

The present research on hemorrhagic shock shows that administration of the MC<sub>1</sub>/MC<sub>4</sub> receptor agonist Ro27-3225



**Fig. 4.** Effects of Ro27-3225 treatment on expression of target genes evaluated using real-time polymerase chain reaction in rat livers. Ro27-3225 treatment significantly induced expression of (A) nuclear receptor subfamily 4, group A, member 1 and (B) tight junction protein 1 in livers of hemorrhage rats. Expression remained at baseline values in untreated hemorrhage animals. Bars denote mean  $\pm$  SEM ( $n = 6$ ). \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ . Nr4a1 = nuclear receptor subfamily 4, group A, member 1; Tjp1 = tight junction protein 1.

restores MAP, reverses the marked metabolic acidosis and hypoxemia, and prevents the upset in gene expression that occurs in rat liver after hemorrhage. Melanocortin treatment, associated with current therapies, could limit damage in this dire condition.

Liver biology is highly susceptible to ischemic injury associated with hemorrhagic shock<sup>20</sup> and impairment in liver function widely affects other organs. In view of the consequences of hemorrhage-related liver injury, the present study investigated expression of representative genes whose induction seemingly triggers crucial signaling pathways during hemorrhage. The data showed marked up-regulation of genes related to immediate/early response (Jun, Fos, Atf3), acute phase response (A2m, Fga), response to oxidative stress (Hmox1, Epo, endothelin1 [Edn1], Hspa1a), and inflammation (Egr-1, Il-6, s100 calcium binding protein A8, calgranulin A [s100a8], s100 calcium binding protein A9, calgranulin B [s100a9], chemokine [C-C motif] ligand 2 [Ccl2]). Treatment with the MC<sub>1</sub>/MC<sub>4</sub> agonist Ro27-3225

was associated with impressive stability in gene expression. Indeed, several genes that were enhanced by hemorrhage showed only marginal changes when animals were treated with Ro27-3225. Gene profile in these livers was remarkably similar to the profile of organs from sham-shocked rats. It appears, therefore, that MC<sub>1</sub>/MC<sub>4</sub> stimulation can prevent inflammatory responses induced by hemorrhage. This action could be very important to reduce subsequent organ dysfunction.

Melanocortin treatment prevented induction of key transcripts related to acute phase reaction and inflammatory response. The cytokine IL-6 promotes induction of acute phase proteins and its importance in posthemorrhage inflammation and organ damage/dysfunction is well established.<sup>24,25</sup> Therefore, the observation that Ro27-3225 prevented IL-6 induction in the liver is particularly significant. The acute phase protein A2m was likewise raised by hemorrhage and inhibited by Ro27-3225. This observation reinforces the idea that hepatic acute phase reaction, a critical event in hemorrhage-induced systemic inflammatory response,<sup>3-5</sup> is modulated by melanocortin treatment. In the present research, we found significant up-regulation of Egr-1 in hemorrhage rats; such increase was prevented by Ro27-3225 administration. Based on its multiple effects, the control of Egr-1 activation could be critical to protect liver tissue. This gene is induced by a variety of acute cellular stresses and is a transcription factor for several key mediators of inflammation, coagulation, vascular permeability, and injury.<sup>26-29</sup> Consistently, recent research found that Egr-1 messenger RNA was rapidly up-regulated in the liver during hemorrhagic shock, where it promoted local and systemic inflammatory reactions.<sup>3</sup> Of interest, deficiency in Egr-1 resulted in a blunted inflammatory response, thus providing evidence that Egr-1 is an inducer of the early inflammatory response to shock that subsequently leads to systemic injury.<sup>3</sup>

In evaluation of the effects of Ro27-3225 on gene expression in the hemorrhage liver, it should be considered that melanocortins participate in central regulation of cardiovascular functions. For example,  $\alpha$ -melanocyte-stimulating hormone acutely increased blood pressure and heart rate through central stimulation of sympathetic nervous outflow.<sup>30</sup> This action of the peptide was mediated by stimulation of MC<sub>4</sub> receptors within the brain. Other research found that intracerebroventricular injections of  $\alpha$ -melanocyte-stimulating hormone increased arterial pressure and renal sympathetic nerve activity in rabbits.<sup>31</sup> However, in our previous investigations,<sup>14,16,17,32</sup> and in the present study, nanomolar concentrations of melanocortins did not affect MAP in sham (unshocked) animals.

Liver protection was not restricted to prevention of inflammation. In livers from hemorrhage rats treated with Ro27-3225 there was no up-regulation of immediate-early genes (Jun, Fos) and stress-induced genes (Atf3, Hmox1, and Hspa1a) that were conversely enhanced in untreated animals. The up-regulation of these transcripts in untreated

hemorrhage animals likely represents a compensatory mechanism in hepatic tissue to restrain damage. Therefore, it is not surprising that hemorrhage livers treated with Ro27-3225, in which MAP rapidly increased, lacked these adaptive responses. Finally, two transcripts, Nr4a1 (Nur77) and Tjp1 (Zo1), were induced in hemorrhage liver only when animals were treated with Ro27-3235, whereas they remained at baseline expression in untreated hemorrhage animals. NR4A are immediate-early genes broadly expressed in metabolically demanding tissues including skeletal muscle, adipose, and heart cells.<sup>33</sup> Nr4a1 is expressed in the liver, where it modulates metabolic processes including apoptosis, gluconeogenesis, and lipogenesis.<sup>34,35</sup> The present observation that melanocortin administration promotes expression of NR4a1 in hemorrhage liver is particularly interesting in that this effect could be part of a broad protective effect. Melanocortin-based treatment could sustain liver function through Nr4a1 up-regulation, thus preventing metabolic dysfunction after hemorrhage. Indeed, several observations indicate that hemorrhagic shock is complicated by hypoglycemia, which occurs after an early hyperglycemic phase, and, therefore, preservation of gluconeogenesis appears crucial for survival.<sup>36,37</sup> Tjp1 is a key element in organization of tight junction protein complexes, regulation of epithelial and endothelial cell interactions, and tissue permeability.<sup>38</sup> Inflammatory stimuli alter Tjp1 expression and reduce barrier function.<sup>39</sup> In several pathologic conditions including hemorrhage, alteration in vascular permeability is associated with diminished expression of Tjp1.<sup>40,41</sup> Therefore, the increased production of Tjp1 induced by Ro27-3225 could contribute to preserve barrier function in hepatocytes.

The present data confirm the idea that melanocortin-induced shock reversal is associated with a gradual correction of severe metabolic acidosis, as indicated by recovery of venous pH, standard base excess, lactate, and bicarbonate. Although in the present study we did not assess hemogasanalysis parameters in arterial blood, such analysis has been performed in a previous investigation on hemorrhagic shock.<sup>21</sup> This paper shows that changes in arterial and venous hemogasanalysis parameters follow a very similar pattern.

With regard to the mechanism(s) underlying the protective action of Ro27-3225 treatment in hemorrhage, concomitant activation of central MC<sub>4</sub> and peripheral MC<sub>1</sub> pathways likely contributed to the effects. Central neurogenic antiinflammatory signals activated by melanocortins consist of both adrenergic<sup>42,43</sup> and cholinergic<sup>16,44</sup> pathways. It appears that these central modulatory effects depend on activation of MC<sub>4</sub> receptors within the brain. Through an efferent vagal pathway, central melanocortins protected rats against myocardial ischemia/reperfusion injury, ischemic stroke, and hemorrhagic shock.<sup>16,44</sup> Further, research on acute inflammation in the mouse indicated that  $\alpha$ -melanocyte-stimulating hormone can act solely within the brain to inhibit nuclear factor- $\kappa$ B activation in peripheral tissues.<sup>43,44</sup> This latter effect appeared to be mediated by sympathetic signals

that require a peripheral  $\beta_2$  adrenergic receptor. MC<sub>1</sub> receptor subtypes are expressed in many peripheral tissues.<sup>10</sup> A function for this receptor during inflammation and immunoregulation is now supported by a large number of studies.<sup>10</sup> The broad effects of melanocortins on inflammatory mediator production partly depends on the capacity of these peptides to inhibit I $\kappa$ B $\alpha$  phosphorylation and, consequently, nuclear factor- $\kappa$ B translocation to the nucleus.<sup>12</sup> The many inflammatory mediators that are under transcriptional control of nuclear factor- $\kappa$ B are, therefore, inhibited. Based on these observations, Ro27-3225 likely exerted its protective influences through a combination of central and peripheral effects *via* activation of MC<sub>4</sub> receptors within the brain and MC<sub>1</sub> receptors in the liver.

In conclusion, liver overproduction of inflammatory mediators affects other organs, and this could account for multiple organ failure. Treatment with the MC<sub>1</sub>/MC<sub>4</sub> agonist Ro27-3225 rapidly restores blood pressure, gradually reverses metabolic acidosis, prevents detrimental gene expression changes, and causes induction of genes with a protective function. Because blood replacement alone does not inhibit hemorrhagic shock-induced systemic inflammation and tissue injury, our previous<sup>14,16–18</sup> and present data suggest that a melanocortin-based treatment could exert substantial beneficial effects. Notably, our experimental model of hemorrhagic shock is characterized by a marked hypoxemia. The present data on venous oxygen partial pressure and oxygen saturation indicate a melanocortin-induced improvement in respiratory and circulatory functions, whereas those on carbon dioxide partial pressure, lactate, pH, bicarbonate, and standard base excess are consistent with mobilization of blood from hypoxic tissues and consequent inflow of acid metabolites into the systemic circulation, followed by a gradual reversal of metabolic acidosis. Indeed, in hemorrhage-shocked rats melanocortin treatment doubled the volume of circulating blood that maintained a normal composition, including hemoglobin levels.<sup>19</sup> Therefore, in shock conditions melanocortins could be helpful drugs for a safe, non-toxic treatment able to rapidly improve cardiovascular function and tissue perfusion for some hours, as previously reported.<sup>17,18</sup> By blocking the main pathophysiological mechanisms of organ damage, melanocortins would considerably extend the effective time-limit for blood reinfusion.<sup>17,18</sup> The antishock effect of melanocortins in experimental and clinical conditions have been recently highlighted by Corander *et al.*<sup>45</sup> in an authoritative review aimed at spreading these relevant findings of basic science to clinicians.

Some weakness of the present results for a potential clinical use of melanocortins in shock conditions deserve consideration. The animal model used in this research is closer to spontaneous bleeding in humans, and urethane, used as anesthetic, cannot be employed in the clinical setting. Therefore, the actual possibility to translate the present results to trauma patients in hemorrhagic shock conditions should be

verified in further animal models. Furthermore, the melanocortin-induced improvement in circulatory condition is transient, and requires volume restoration within a few hours.<sup>17,18</sup> Conversely, the short half-life of peptide molecules, including natural melanocortins, that are broken down readily in the body fluids, should not represent a limitation as several of the generated fragments are biologically active.<sup>10</sup> Finally, passage through the blood-brain barrier<sup>10,12,44,45</sup> and lack of receptor selectivity of melanocortins should not be problematic in short-term, acute treatments such as in hemorrhagic shock.

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