Simulated microgravity decreases circulating iron in rats: role of inflammation-induced hepcidin upregulation

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Running Title: Role of hepcidin in microgravity-induced iron metabolism alteration

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What is the central question of this study?

Although microgravity is well known to reduce circulating iron in astronauts, the underlying mechanism is still unknown. We investigated whether hepcidin, a key hormone regulating iron metabolism, could be involved in this deleterious effect.

What is the main finding and its importance?

We show that hindlimb suspension, a model of microgravity, stimulates the production of hepcidin in liver of rats. In agreement with the biological role of hepcidin, we found a decrease of circulating iron and an increase of spleen iron content in hindlimb unloaded rats. Consequently, our study supports that hepcidin could play a role in the alteration of iron metabolism parameters observed during spaceflight.

Abstract

During spaceflight, humans exposed to microgravity exhibit an increase of iron storage and a reduction of circulating iron. Such perturbations could promote oxidative stress and anemia in astronauts. The mechanism by which microgravity modulates iron metabolism is still unknown. Herein, we hypothesized that microgravity up-regulates hepcidin, a hormone produced by the liver that is the main controller of iron homeostasis. To test this hypothesis, rats were submitted to hindlimb unloading (HU), the reference model to mimic the effects of microgravity in rodents. After seven days, the mRNA level of hepcidin was increase in the liver of HU rats (+74%, p=0.001). In agreement with the biological role of hepcidin, we found an increase of spleen iron content (+78%, p=0.030) and a decrease of serum iron concentration (-35%, p=0.002) and transferrin saturation (-25%, p=0.011) in HU rats. These findings support a role of hepcidin in microgravity-induced iron metabolism alteration. Furthermore, among the signaling pathways inducing hepcidin mRNA expression, we found that only the interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) axis was activated by HU as shown by the increase of phospho-STAT3 (+193%, p<0.001) and of the hepatic mRNA level of haptoglobin (+167%, p<0.001), a STAT3-inducible gene, in HU rats. Taken together, these data supports that microgravity may alter iron metabolism through an inflammatory process up-regulating hepcidin.

Introduction

In the future, the number and duration of manned spaceflights will inexorably increase. Space agencies plan to send astronauts to Moon, asteroids and Mars; whereas private companies develop a commercial exploitation of space travel by proposing future services such as “space taxis” and space tourism. In this context, the effect of microgravity on human physiology needs to be better understood. In addition to the well-known loss of skeletal muscle and bone mass (Hargens & Vico, 2016), recent findings highlighted that microgravity affects iron metabolism (Zwart *et al.*, 2013).

Iron plays a key role in numerous biological functions including oxygen transport, enzyme activity or energy production. Human body contains 3-5 g of iron: 65-75% being linked to hemoglobin; 10-20% is stored in the liver, associated with the iron-storage protein ferritin; 10% is found in myoglobin of striated muscles (Zhang, 2010) and the remaining localized in the different cell types. Humans exposed to microgravity exhibit a decrease of the circulating level of iron and transferrin (Smith *et al.*, 2005; Zwart *et al.*, 2013). Additionally, an elevation of iron store has been reported in astronauts of the International Space Station (Zwart *et al.*, 2013). These findings raise concern since iron excess in tissues can impair bone and muscle metabolism, disrupt immune function and increase the sensitivity to radiation injury (Reardon & Allen, 2009; Tsay *et al.*, 2010). On the other hand, a reduction in circulating iron may promote anemia, a deleterious effect already observed during space missions and known as "spaceflight anemia"(Tavassoli, 1982). This phenomenon could limit the duration of space travels. Although the effects of microgravity on iron metabolism are identified, the molecular mechanism driving such regulation remains unexplored.

Iron metabolism is mainly controlled by hepcidin, a peptide hormone secreted by the liver that reduces iron export through degradation of the iron exporter ferroportin (Zhang, 2010). In addition, hepcidin reduces iron absorption by the duodenal enterocytes. Consequently, high levels of hepcidin induce both a decrease of serum iron and an increase of iron store in cells expressing ferroportin, especially macrophages (Ganz, 2011).

From these data, we hypothesized that alteration of iron metabolism parameters during microgravity could be linked to hepatic hepcidin overexpression induced by iron-related and/or inflammatory signals. To test this hypothesis, we submitted rats to hindlimb unloading (HU), the reference model to simulate the effects of microgravity in rodents (Globus & Morey-Holton, 2016), and we analyzed whether hepatic hepcidin expression and the related signal transduction pathways are modulated.

Materials and methods

Ethical Approval

The protocol and procedure used in this study were in accordance with the local committee on Ethics in Research of Rennes (authorization 01259.03) and approved by the French Ministry of Higher Education.

Animals and experimental procedures

Male Wistar rats (10-weeks old, Janvier Labs) were randomly assigned to 2 groups: non-suspended (Control, n=8) and hindlimb unloaded (HU, n=8) for 7 days. HU was performed by using the Morey's tail-suspension model (Morey-Holton & Globus, 2002). Rats were housed with food and water *ad libitum*. At the end of the protocol, rats were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg). Liver, spleen and gastrocnemius were dissected, weighed, frozen in liquid nitrogen and stored at -80 °C. Blood was collected from the *vena cava* and the animals killed by exsanguination. Hemoglobin concentrations were determined using HemoCue® 201+. Blood was centrifuged at 1500 × *g* for 10 min, serum was removed and stored at -80 °C.

Biochemical methods

Serum iron and unsaturated iron-binding capacity (UIBC) were measured in the biochemistry laboratory (Rennes Pontchaillou Hospital) on Cobas 8000 analyzer Roche® (Cobas® reagents 03183696 122 and 04536355 190, respectively). Serum transferrin saturation was calculated as (serum iron/(serum iron + UIBC)) × 100. Liver and spleen iron concentrations were quantified as previously described (Barry & Sherlock, 1971). Serum IL-6 concentrations were determined using a rat IL-6 detection kit according to the manufacture's protocol (BioLegend, San Diego, CA, USA).

Histological analysis

Spleen and liver samples were fixed in 4% paraformaldehyde fixative (PFA) for 24 h and paraffin embedded. Serial transverse sections of 4 µm were obtained from each sample using a LEICA microtome and were mounted on glass slides. To visualize ferric iron deposits, deparaffinized tissue sections were stained with Perls' Prussian blue.

RNA extraction and quantitative Real-time PCR

RNA extraction and quantitative real-time PCR were performed as previously described (Pierre *et al.*, 2013). Analysis was done using the $2^{-\Delta CT}$ method with HPRT (liver and spleen) or RPL19 (gastrocnemius) as a reference gene. The following primer sequences were used: IL-6: (F: TCCGCAAGAGACTTCCAGC and R: GTCTGTTGTGGGTGGTATCCTCT); Hepcidin (F: CTGCAGCCTTGGCATGG and R: CAGCAGCGCACTGTCATCA); Haptoglobin (F: AACACTTGGTTCGTTATCGCTG and R: CCACACACTGCCTCACACTTG); HPRT (F: CTGATTATGGACAGGACTGAAAGAC and R: CCAGCAGGTCAGCAAAGAACT); RPL19 (F:

CAATGCCAACTCTCGTCAACAG and R: CATCCAGGTCACCTTCTCGG).

Western Blotting

Protein extraction and western blots were performed as previously described (Salaun *et al.*, 2016). The following antibodies were used: ERK1-2(1:1000, Cell Signaling), p-ERK1-2-Thr^{202}Tyr^{204} (1:1000, Cell Signaling), p-STAT3-Tyr^{705} (1:1000, Cell Signaling), p-SMAD1/5/8-Ser$^{463/465}$ (1:1000, Abcam), SMAD5 (1:1000, Cell Signaling), and α-actin (1:700, Sigma Aldrich).

Statistical analysis

Normality and equality of variances were checked using a Kolmogorov-Smirnov and Fischer test, respectively. If one of these two tests failed we checked the significance using a Mann-Whitney test and data were presented with a box plot. When normality and equal variance were met, we used the unpaired Student's t-test and data were presented as mean ± SD. The level of significance was set at $p<0.05$.

Results

HU induces iron misdistribution. Firstly, we aimed to check the disruption of iron metabolism induced by our model of microgravity. We found that available iron is reduced in HU rats as shown by the decrease of serum iron concentration (-35%, p=0.002) and transferrin saturation (-25%, p=0.011) (Fig. 1A and 1B). This reduction of systemic iron was not associated with changes in hemoglobin concentration (15.8 ± 0.4 vs. 16.6 ± 0.30 g/dL, p=0.153, Fig. 1C). To better characterize the effect of HU on iron metabolism, we measured iron concentration in liver and spleen, the main organs responsible for iron storage. Iron content increased in spleen of HU animals (+78%, p=0.030), whereas liver iron concentration was not significantly modified (p=0.217) (Fig. 1D and 1E). These results are supported by histological analyses, where iron deposits are more visible in spleen of HU animals (Fig. 1F). It is noteworthy that spleen weight was not reduced in HU condition (0.25 ± 0.04 vs. 0.23 ± 0.03 g/100g of body weight, p=0.437, Table 1), thus dismissing an increase of spleen iron concentration related to spleen weight modification. Taken together, our results indicate that simulated microgravity induces an increase of iron storage in the spleen associated with a reduction of the circulating iron.

HU upregulates hepatic hepcidin expression via the IL-6/STAT3 signaling pathway. To

investigate the mechanism by which HU induces iron misdistribution, we measured in liver the expression of hepcidin, the master regulator of iron metabolism. We found that HU increased hepcidin mRNA level (+74%, p=0.001, Fig. 2A), supporting thereby a role of this hormone in the alteration of iron homeostasis observed in our experiment. Hepcidin expression is known to be upregulated by BMP6/SMAD, ERK1/2 and IL-6 /STAT3 signaling pathways (Zhao *et al.*, 2013). Therefore, we investigated whether these three signaling pathways were activated in the liver of HU animals. We observed that HU increased STAT3 phosphorylation state (+193%, p<0.001, Fig. 2C and 2F), whereas HU modulated neither phospho-ERK1-2 nor phospho-SMAD1/5/8 levels (Fig. 2C, 2D and 2E). STAT3 is a transcription factor well known to regulate genes of the acute phase response such as haptoglobin (Alonzi *et al.*, 2001). Herein, we observed an elevation of the mRNA level of haptoglobin (+167%, p<0.001, Fig. 2B) in the liver of HU rats. Taken together, these results support that HU induces STAT3 pathway activation in the liver. Consequently, we propose that stimulated microgravity promotes a transcriptional regulation of hepcidin through the IL-6/STAT3 signaling pathway.

IL-6 mRNA increases in skeletal muscle of HU rats. IL-6 being the main activator of STAT3, we analyzed its mRNA level in liver, spleen, and gastrocnemius. We found that IL-6 mRNA level increased in gastrocnemius of HU animals (+135%, p=0.028, Fig. 3C), whereas these was not the case in spleen (p=0.444, Fig. 3B) and liver (p=0.657, Fig. 3A). The increase of IL-6 mRNA in gastrocnemius was likely related to the atrophic process. Indeed, HU rats exhibit a 12% (p=0.004, Table 1) loss of gastrocnemius mass, a deleterious effect well known to be associated with inflammation. The serum concentrations of IL-6 remained below the limit of sensitivity of the assay in both control and HU animals.

Discussion

By using the HU model, we demonstrated that simulated microgravity induces hepcidin upregulation, the master regulator of iron metabolism. This finding highlights that hepcidin could play a key role in the alteration of iron metabolism induced by microgravity. Furthermore, our data support that HU-induced hepcidin upregulation through the inflammatory signaling pathway IL-6/STAT3. This study constitutes a first step toward a better understanding of the mechanism by which microgravity alters iron metabolism.

HU model is widely used since decades to understand the effects of microgravity observed

during spaceflight. This model presents the advantage of exploring the organ-specific responses to microgravity, a key characteristic of spaceflight environment, and excludes the concomitant effects of astronaut's exposure to radiation or hyperoxia. The neuroendocrine stress occurring during the first days, depending on the experiment, is the main weakness recognized for HU model since it could mask or amplify adaptive responses to microgravity (Globus & Morey-Holton, 2016). However, as highlighted by Globus and Morey-Holton, this acute stress in rodents may mimic the acute stress occurring in astronaut crews during launch and the first days in spaceflight (Stowe *et al.*, 2011; Mehta *et al.*, 2014).

As observed during space missions, microgravity reduces serum iron concentration and increases iron storage in human (Smith *et al.*, 2005). Our model of microgravity, HU, reproduces those effects and constitutes *de facto* an appropriate experimental design to study the effect of spaceflight on iron metabolism. In our experiment, we found that simulated microgravity increased iron storage in the spleen and reduced iron availability as shown by the reduction of circulating iron and transferrin saturation. In other words, the increase of iron storage by HU occurred to the detriment of the blood iron content, a regulation which looks like an effect of hepcidin. Indeed, this hormone promotes a reduction of the circulating iron through induction of iron sequestration in cells expressing ferroportin such as the spleen resident macrophages (Zhao *et al.*, 2013). Remarkably, we observed such effect in HU rats and this was associated with an increase of hepcidin mRNA level. Although this result does definitively not prove a causal link between hepcidin upregulation and iron misdistribution, it supports a potential role of this hormone in the alteration of iron metabolism induced by microgravity. However, one study reported that, despite a clear alteration of iron metabolism, astronauts did not exhibit any change of the circulating level of hepcidin (Zwart *et al.*, 2013). Various confounding factors related to space travels including a disturbance of circadian rhythms (Schaap *et al.*, 2013) and hyperoxia, as well as the low number of subjects, could explain the lack of significant differences reported in this study. Alternatively, we cannot exclude that HU and microgravity alone exert a distinct effect on hepcidin production.

To go further in the understanding of hepcidin regulation by microgravity, we investigated the signaling pathways modulating hepcidin expression. The BMP/SMAD signaling pathway is the main positive regulator of hepcidin (Zhao *et al.*, 2013). More specifically, it has been demonstrated that both increase in transferrin saturation and liver iron content promote BMP/SMAD activation (Core *et al.*, 2014). Here, we showed that hepatic phospho-SMAD1/5/8 levels were not affected by HU, whereas serum transferrin saturation was

decreased and liver iron content was unchanged. Thus, our data did not support an implication of the BMP/SMAD axis in HU-induced hepcidin upregulation. In addition to the BMP/SMAD pathway, evidences from *in vitro* studies and *Tfr2*/*Hfe* null mice support that the ERK1/2 signaling pathway is a positive regulator of hepcidin (Ramey *et al.*, 2009; Poli *et al.*, 2010). Here, we observed that 7 days of HU did not affect phospho-ERK1/2 levels, thus showing that ERK1/2 signaling pathway is not involved in HU-induced hepcidin upregulation. Finally, we explored the IL-6/STAT3 signaling pathway known to be regulated by inflammation (Zhao *et al.*, 2013). Interestingly, we observed that HU increased the phosphorylation state of STAT3 and the mRNA level of haptoglobin, a STAT3-inducible gene. All together, these data indicate that HU probably stimulates hepcidin synthesis through STAT3 activation.

The inflammatory cytokine IL-6 is well known to activate STAT3 which in turn promotes hepcidin production (Wrighting & Andrews, 2006). IL-6 being a well-known myokine released by atrophied muscle, we analyzed its mRNA level in gastrocnemius. In agreement with others (Caron *et al.*, 2009; Kwon *et al.*, 2015), we found that IL-6 mRNA level increased in the gastrocnemius of HU animals. Since HU rats exhibit a 12% loss of gastrocnemius mass, HU-induced IL-6 upregulation is most likely related to inflammation associated with the atrophic process. Indeed, atrophied myotubes produces IL-6, an effect which is mediated by the nuclear factor-kB (NF-κB) inflammatory pathway (Yamaki *et al.*, 2012). Moreover, our laboratory and other studies demonstrated that NF-κB signaling pathway was early activated in skeletal muscle from unloaded rats (Judge *et al.*, 2007; Salaun *et al.*, 2016).

Although we showed an increase of the mRNA level of IL-6 in the gastrocnemius of HU rats, we were not able to detect IL-6 in the blood. Unfortunately, serum IL-6 levels remained undetectable in both control and HU animals. This sensitive issue is frequently observed for IL-6 due to the moderate inflammation induced by microgravity (Xu *et al.*, 2013; Crucian *et al.*, 2014). The minimum detectable level of IL-6 with the ELISA kits, included those used in this study, is generally between 5 to 10 pg/ml, whereas *in vitro* studies demonstrated in hepatocytes that small amounts of IL-6 (< 2 pg/ml) were able to induce a 30-fold increase of the hepcidin mRNA level (Memoli *et al.*, 2010). In our study, the impossibility to detect the circulating level of IL-6 does not mean that this cytokine has no role on hepcidin expression. Dedicated studies are needed to determine the implication of IL-6 in HU-induced hepcidin upregulation.

The alteration of iron metabolism induced by microgravity raises concern since iron accumulation in tissues could promote oxidative damages through the Fenton reaction. It has been proposed that such mechanism could accelerate loss of bone mass in astronauts, a population already exposed to a higher degree of oxidative damages due to cosmic radiation (Zwart *et al.*, 2013). In view of our results, the role of hepcidin in microgravity-induced oxidative damages needs to be investigated. On the other hand, our result suggests that hepcidin could contribute to "spaceflight anemia". Indeed, the reduction of available iron observed in HU rats could reduce erythropoiesis. In the present study, we did not report differences in hemoglobin concentration between control and HU animals. This result is in accordance with previous studies showing that microgravity did not reduce hemoglobin concentration, it decreases red blood cell mass and plasma volume, thus maintaining hemoglobin concentration (Tavassoli, 1982). Consequently, appropriate markers are needed to study spaceflight anemia, this was not the goal of the present study.

In human, microgravity is simulated through bedrest, a situation which also alters iron metabolism (Zwart *et al.*, 2009b). Studies showed that bedrest induces a reduction of the circulating concentration of transferrin (Zwart *et al.*, 2009a; Zwart *et al.*, 2009b; Morgan *et al.*, 2012). In a similar manner than HU, bedrest is an immobilization model where skeletal muscle wasting occurs rapidly. This atrophic process is associated with an elevation of the circulating level of IL-6 (Bosutti *et al.*, 2008; Mutin-Carnino *et al.*, 2014). As already mentioned this cytokine stimulates the production of hepcidin and could therefore alter iron metabolism. To the best of our knowledge, no studies measured hepcidin in bedridden patients. As a consequence, it is unknown whether hepcidin contributes to the iron metabolism alteration observed in bedridden patients. In future studies, particular attention must be paid to hepcidin in bedridden patients; inappropriate modulation hepcidin could have multiple deleterious effects such as oxidative stress and anemia.

In summary, similarly to spaceflight, we found that HU induces an increase of iron storage in the spleen and a decrease of circulating iron. These results being associated with an elevation of hepcidin in the liver of HU animals, we propose that hepcidin could be involved in the effects of microgravity on iron metabolism. Furthermore, we show that the IL-6/STAT3 signaling pathway seems responsible for HU-induced hepcidin upregulation. This study provides new directions to investigate the mechanism by which microgravity alters iron metabolism.

Author Contributions

F.D. and O.L. conceived and designed the study. T.C., N.P., K.N., C.A., M.R. and F.D. performed the experiments. N.P. and K.N. analyzed the data and prepared the figures. T.C., N.P., F.D. and O.L. drafted the manuscript. All authors approved the final version of the manuscript.

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Disclosure of Conflicts of Interest

The authors declare no competing financial interests regarding this work.

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Table 1. Effect of hindlimb unloading (HU) on gastrocnemius and spleen weights.

	Control	HU
Body weight (g)	301 ± 23	272 ± 26***
Gastrocnemius weight-to-body weight ratio (g/100 g)	530.6 ± 40.8	459.3 ± 42.8**
Liver weight-to-body weight ratio (g/100 g)	4.09 ± 0.36	3.59 ± 0.39***
Spleen weight-to-body weight (g/100 g)	0.25 ± 0.04	0.23 ± 0.03

Values are means ± SD. Significance was checked using unpaired t-test. **p<0.01, ***p<0.001

Figures legends

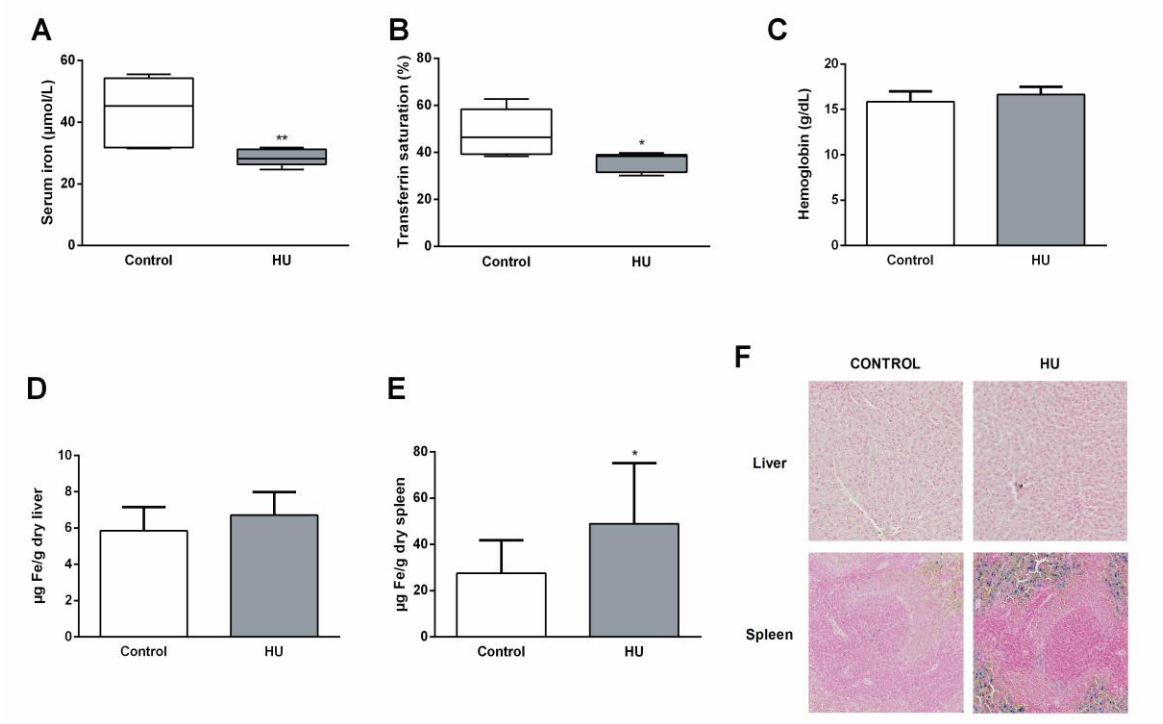

Figure 1. Hindlimb unloading induces iron misdistribution. *A:* Serum iron concentration. *B:* transferrin saturation. *C:* hemoglobin concentration. *D:* liver iron concentration. *E:* spleen iron concentration. *F:* histological detection of iron deposits in liver and spleen sections by Perls' iron staining. Significance was checked using Mann-Whitney (A and B) or unpaired t-test (C, D, E). *p<0.05, **p<0.01.

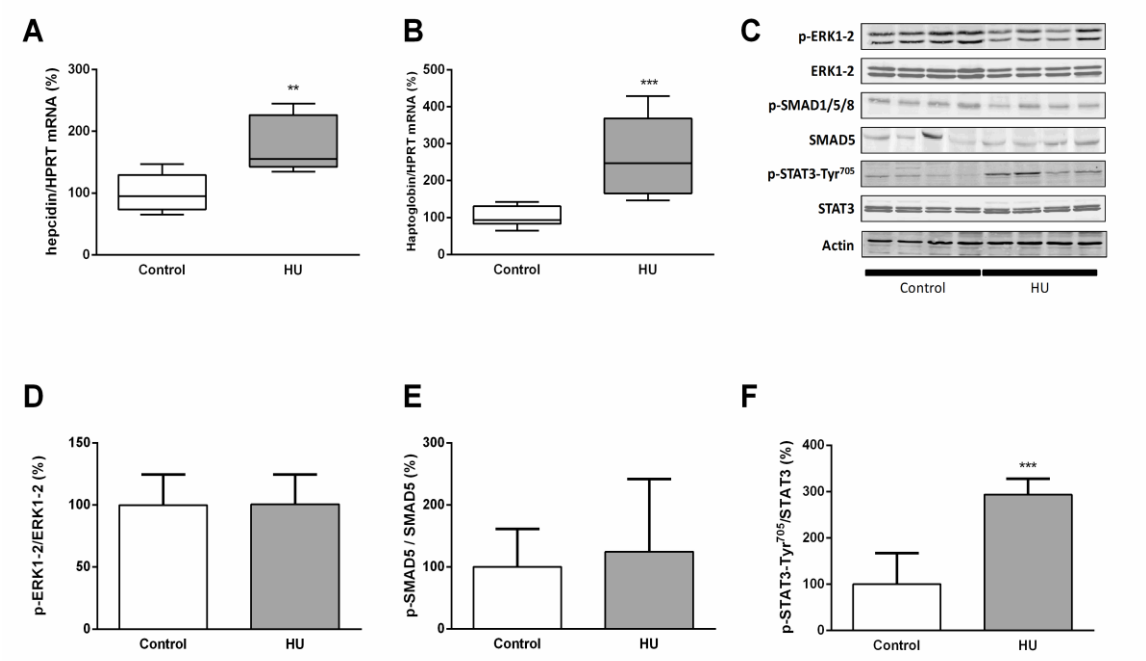

Figure 2. Hindlimb unloading induces hepatic hepcidin upregulation via the IL-6/STAT3 signaling pathway. *A:* Hepatic hepcidin mRNA level. *B:* hepatic haptoglobin mRNA level. *C:* representative blots of p-ERK1-2-Thr^{202}/Tyr^{204}, ERK1-2, p-SMAD1/5/8 Ser$^{463/465}$, SMAD5, p-STAT3-Tyr^{705}, STAT3 and actin. *D:* hepatic ERK1-2 activation. *E:* hepatic SMAD1/5/8 activation. *F:* hepatic STAT3 activation. Significance was checked using Mann-Whitney (A and B) or unpaired t-test (D, E and F). **p<0.01, ***p<0.001.

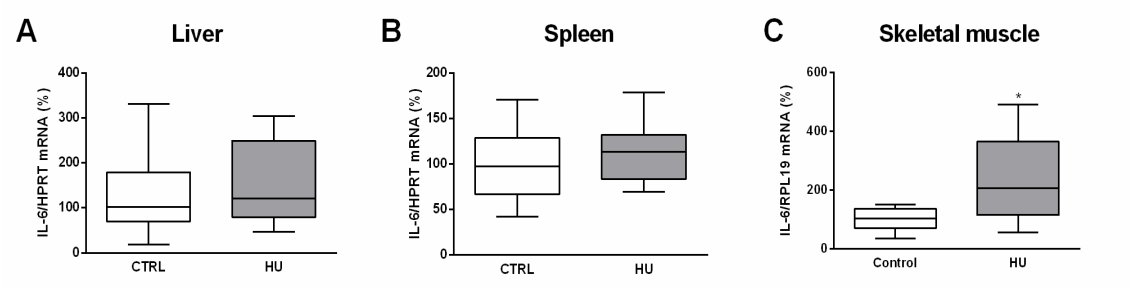

Figure 3. IL-6 mRNA increases in skeletal muscle of HU rats. *A:* IL-6 mRNA level in liver. *B:* IL-6 mRNA level in spleen. C: IL-6 mRNA level in gastrocnemius. Significance was

checked using Mann-Whitney. *$p<0.05$