Renal effect of severe hypoxia evaluated By NGAL measurements: An in vivo and in vitro study

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Abstract

Purpose: To investigate possible renal damage in healthy men exposed to extreme hypobaric hypoxia, using urinary Neutrophil Gelatinase-Associated Lipocalin (NGAL) concentration as biomarker. The value of NGAL as a biomarker of proximal tubular cell damage under hypoxic conditions was also tested in vitro experiments.

Methods: NGAL was assayed in a cohort of air cadets ($n = 16$) exposed to hypobaric hypoxia in a hypobaric chamber during their training program. In all subjects, urine creatinine (Cr) and urinary NGAL levels were measured immediately before, 3, and 24 h after hypobaric environment exposure. Three in vitro experiments using proximal tubular cell cultures were also performed to measure NGAL gene expression, NGAL secretion in the culture medium and to evaluate apoptosis under two cycles of hypoxia and reoxygenation.

Results: In the in vivo study, geometric means of urinary NGAL/Cr ratio measured 24 h after hypobaric hypoxia in the hypobaric chamber were significantly lower than baseline values (13.4 vs 25.9 ng/mg, $p = 0.01$). In cell cultures, hypoxia down-regulated NGAL gene expression without significantly changing NGAL secretion in the culture medium. Hypoxia significantly increased the percentage of apoptotic/necrotic cells, especially after the second hypoxia-reoxygenation cycle.

Conclusions: Exposure to hypobaric-hypoxic environments does not cause significant and irreversible renal tubular injury in vivo and in vitro, except than in a late stage. The hypoxic insult does not seem to be mirrored by an increase of urinary NGAL in healthy men nor of NGAL gene expression in HK-2 cell culture or secretion in the culture medium in the in vitro conditions reported in the present study.

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Introduction

Hypoxia may induce changes in renal function and during acute hypoxia renal blood flow increases 8%–20%.[1–3] As a whole, physiological studies have not disclosed any negative effect of hypoxia, whether hypobaric or normobaric, on a number of tubular and glomerular functions.[4] Nevertheless, ischemia is one of the most important causes of acute kidney injury (AKI).[5]

In recent years many potential biomarkers of AKI have been identified, such as Neutrophil Gelatinase-Associated Lipocalin (NGAL), kidney injury molecule-1, and tissue inhibitor metalloproteinases-2, that could allow to investigate in depth whether transient episodes of hypoxia are really safe for the kidney.

Urinary NGAL is one of the most interesting biomarkers of renal function impairment being able to allow for an early detection of acute kidney injury (AKI) before any rise in serum creatinine.[6–8]

NGAL is mainly synthetized by neutrophils where it is stored in granules and by many different tissues including renal tubular cells. However, while neutrophils mainly release homodimeric NGAL, renal tubular cells predominantly secrete the monomeric form.[9]

The expression of NGAL is very low in human tissues, however, its production could be induced significantly in epithelial cells, such as those in the kidney, playing a protective role against the ischemic damage.[6]

Previous studies based on proteomics showed that ischemic and nephrotoxic AKI induces the production of NGAL in the kidney and the protein can be identified in urine.[10–13]

No study in the physiology setting has excluded any negative effect of hypoxia, either in hypobaric or in normobaric condition. To give a contribution in the field, the present study was aimed to investigate possible renal damage in air cadets exposed to extreme hypobaric hypoxia during their training program, using urine NGAL concentration as biomarker. The value of NGAL as biomarkers of proximal tubular cell damage under hypoxic conditions was also tested by in vitro experiments.

Materials and methods

Since April 2016 to May 2016, we prospectively enrolled male air cadets exposed to hypobaric hypoxia in a hypobaric chamber.

The data were collected non-invasively during training that the subjects would have undertaken.

To avoid potential confounding effect by gender in this pilot study with planned low sample size, the inclusion criteria were male gender, and fit for flight. Exclusion criteria were renal colic episodes (less than 12 months before), kidney impairment (Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula estimated glomerular filtration rate <60 ml/min/1.73 m^2), use of potentially nephrotoxic drugs (nonsteroidal anti-inflammatory drugs

or antibiotics) within 4 weeks before the study. The study has been conducted according to the World Medical Association Declaration of Helsinki.

Hypobaric hypoxia protocol

Equipment. The hypobaric chamber was a model developed by AMST Systemtechnik GmbH—Ranshofen (Austria). It was operated according to the instructions released by the factory and in compliance with the safety requirements regarding pressure driven systems laid down by the European Union and Italy Health Authorities.

Procedure. Before each profile a brief ascent up to 5000 ft (1524 m) followed by return to ground level was performed in order to assess the ability of the trainees to balance the middle ear and sinus pressure changes (sinus check). Subjects who did not pass the sinus check were discharged.

Each session started with 30 min of denitrogenation with 100% oxygen breathing before ascent in order to minimize the risk of decompression sickness. The climb rate was set at 4000 ft min^{-1} (1219.2 m min^{-1}) and the descent rate at 2500 ft min^{-1} (762 m min^{-1}) for all profiles in order to reduce the risk of barotrauma while maintaining the need to balance the pressure changes. Criteria for terminating the hypoxia session were: two or more symptoms of hypoxia reported by the trainees, blood saturation of 55%, or time spent in hypoxia of 4 min at 25,000 ft (7620 m) and 15 min at 18,000 ft (5486.4 m).

After the sinus check, the profile proceeded with ascent up to 25,000 ft (7620 m). At this altitude, the trainees removed their masks and performed a set of tasks until two or more symptoms of hypoxia were detected. Then they donned the mask and restored the oxygen supply. After descent to 18,000 ft (5486.4 m), the lights were attenuated, and the trainees removed their masks again for demonstration of night vision impairment. Finally, the profile concluded with return to ground level.[14]

Urinary NGAL determination

In all subjects, urine was collected immediately before, 3, and 24 h after hypobaric environment exposure. After urinalysis performed by dipstick (Aution Stick 10PA/Menarini Diagnostics®), the samples were centrifuged for 10 min at 2000 g/min and the supernatants were stored in small aliquots at −80°C until assayed.[10] All specimens were thawed and analyzed on the same day for NGAL, using Architect NGAL chemiluminescent assay (Abbott Laboratories, North Chicago, Architect c4000®) and for Cr (enzymatic method, kit ROCHE—Cobas 8000®). The measuring interval of the NGAL kit is 10–1500 ng/ml and in terms of imprecision, the intra- and inter-assay coefficients of variation were <4%. Urine NGAL concentrations were normalized to urinary Cr and reported as NGAL/Cr ratio expressed in ng/mg to account for variable hydration status.[10]
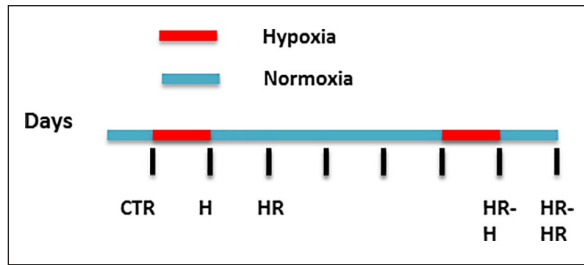
Figure 1. Schematic representation of cell treatments. CTR: control; H: hypoxia; HR: reoxygenation after hypoxia; HR-H: hypoxia after HR; HR-HR: 24h reoxygenation after second hypoxia.

Table 1. Urine NGAL/Cr values in air cadets at different time points after exposition to hypobaric hypoxia.

Baseline	3 h	24 h
25.9 (10.3, 41.4)	19.1 (7.6, 30.5)	13.4 (5.4, 21.5)*

Values are expressed as geometric means and 95% CI of NGAL/Cr (ng/mg). *Statistically significant ($p = 0.01$).

Cell culture and treatments

The human renal proximal tubular cells HK-2 (human kidney 2) were maintained in DMEM-F12 medium with 17.5 mM glucose (EuroClone), supplemented with 10% fetal bovine serum (FBS, Biochrom AG), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.[15] Hypoxic conditions were established incubating cells in serum free medium for 24 h using a hypoxia incubator chamber (STEMCELL) and a hypoxia indicator test (Sigma–Aldrich). The cells were then cultured under normoxic conditions and complete medium for a reoxygenation phase. A second hypoxia was induced 4 days later.[16] The cells were tested immediately after hypoxia and 24 h after the beginning of reoxygenation (Figure 1). Control cells were grown under normoxic conditions in the serum free medium for the same time periods.

NGAL gene expression and secretion analysis

Total RNA was extracted from cells Trizol reagent (Invitrogen) according to the manufacturer's instructions.[15] RNA yield and purity were checked using a Nanodrop spectrophotometer (EuroClone) and total RNA from each sample was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Real-time PCR was performed on an ABI-Prism 7500 using Power SYBR Green Master Mix 2X (Applied Biosystems). The comparative Ct method ($\Delta\Delta$Ct) was used to quantify gene expression and the relative quantification was calculated as $2^{-\Delta\Delta Ct}$. The presence of non-specific amplification products was excluded by melting curve analysis.[15] Data were normalized to GAPDH expression. NGAL was measured in cell culture conditioned media using Architect NGAL assay Abbott/Architect c4000® (Abbott Laboratories, North Chicago).

Apoptosis detection

Apoptosis was detected by Annexin V—FITC/Propidium Iodide staining using an apoptosis detection kit (Immunostep) according to the manufacturer's protocol.[15] Briefly, cells were harvested by trypsin, washed twice with temperate PBS, and suspended in 1X Annexin Buffer at a concentration of 1×10^6 cells/ml. One hundred microliter of cell suspension were added with 5 µl of Annexin V-FITC and 5 µl of Propidium Iodide (PI). Cells were incubated at room temperature for 15 min in the dark. Subsequently, 400 µl of 1X Annexin Buffer were added to each sample. Samples were analyzed by flow cytometry.

Statistical analysis

For the purpose of the in vivo study, urine NGAL concentrations were normalized by urinary Cr and reported as NGAL/Cr ratio expressed in ng/mg to account for variation in hydration status. Univariate analyses of NGAL/Cr at each time point revealed a log-normal distribution, hence NGAL/Cr values were log-transformed prior to statistical analysis. Differences in log-transformed NGAL/Cr over time were analyzed with a mixed linear model with a random intercept for each patient, as previously described. Statistical significance was set at p-value $\leqslant 0.05$. Results were reported as geometric means (GM) and 95% confidence intervals (CI).

For the purpose of the in vitro experiments, means and standard deviation (SD) of the real-time PCR data were calculated by Rest2009 software (Qiagen, Germantown, MD, USA). Differences between treated and untreated cells were compared by a two-tailed Student's t-test. Statistical significance was set at p-value $\leqslant 0.05$. Gene expression differences were analyzed by linear regression models with groups as categorical variables. A Bonferroni-corrected p-value $\leqslant 0.05$ was considered statistically significant. All statistical analyses were performed with Stata version 13.1 (Stata Corp LP®, Texas, USA).

Results

In vivo experiment

Sixteen air cadets were enrolled in the present study. None of them was discharged with all successfully passing the sinus check. Mean age was 28.8 ± 9.2 years.

Urinalysis showed no significant differences in specific gravity at three time points; hemoglobin and leukocyte esterase were undetectable in all specimens and microscopic examination confirmed neither significant presence of erythrocytes nor leukocytes in all subjects.

NGAL/Cr showed a statistically significant decrease at 24 h compared with baseline (13.4 vs 25.9 ng/mg, $p = 0.01$)

(a)

(b)
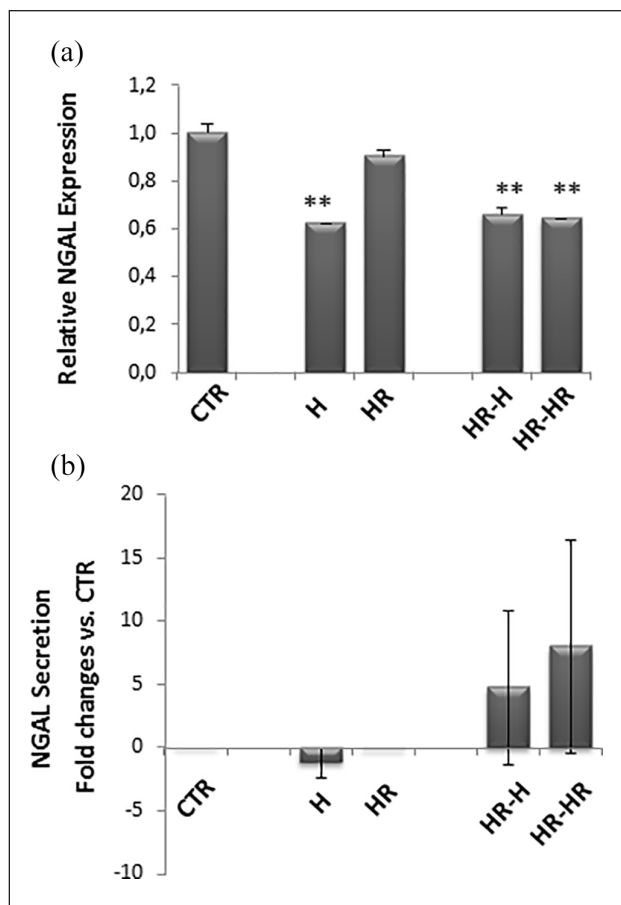
Figure 2. NGAL expression and secretion. NGAL expression and secretion were evaluated in HK-2 cells exposed to sequential hypoxia and reoxygenation cycles: (a) the expression of NGAL was evaluated by real-time PCR. Data were normalized to GAPDH expression. **$p < 0.01$ versus CTR and (b) NGAL was assayed in cell culture conditioned media by a turbidimetric KIT. Values are expressed as fold changes versus control.

whilst being decreased without reaching the statistical significance at 3 h assessment (Table 1). These findings were confirmed after repeating the analyses on NGAL expressed as concentration (unpublished data).

In vitro experiments

Expression and secretion of NGAL under hypoxic conditions. Gene expression analysis showed a statistically significant reduction of NGAL expression in proximal tubular cells under hypoxic conditions. NGAL levels returned to basal level after 24 h of reoxygenation. A second hypoxic insult reduced NGAL expression at comparable levels of the first hypoxia but its expression remained reduced 24 h after reoxygenation (Figure 2(a)).

The secretion of NGAL in the culture medium remained stable during the first exposure to hypoxia and in
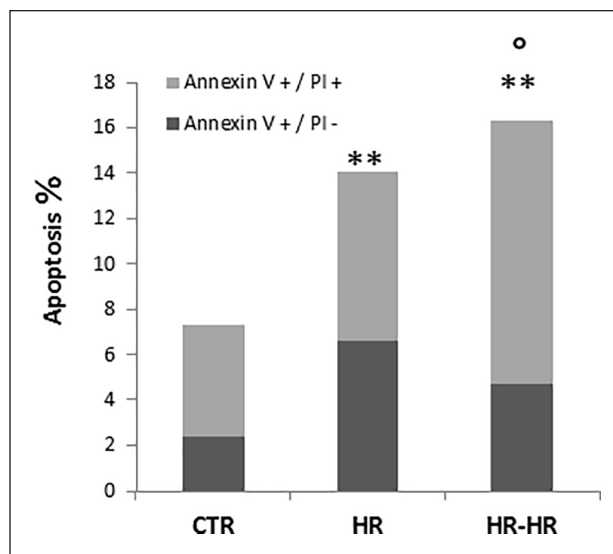
Figure 3. Regulation of apoptosis.
The percentage of early apoptotic (Annexin V$^+$/PI$^-$) and late apoptotic/necrotic (Annexin V$^+$/PI$^+$) was measured in HK-2 cells exposed to sequential hypoxia and reoxygenation cycles. **$p < 0.01$ versus CTR; °$p = 0.06$ versus HR.

subsequently 24 h of reoxygenation. A slight not statistically significant increase in NGAL secretion was observed after the second cycle of hypoxia-reoxygenation 4 days later (Figure 2(b)).

Apoptosis analysis. Results showed that both the first and the second cycle of hypoxia/reoxygenation significantly increased the percentage of early apoptotic (Annexin V$^+$/PI$^-$) and late apoptotic/necrotic (Annexin V$^+$/PI$^+$) cells compared to controls. Compared to the first cycle, the percentage of early apoptotic (Annexin V$^+$/PI$^-$) cells after the second cycle of hypoxia/reoxygenation was significantly reduced, while the percentage of late apoptotic/necrotic (Annexin V$^+$/PI$^+$) cells was significantly increased, suggesting a more severe injury (Figure 3).

Discussion

In recent years, NGAL has been one of the most studied renal damage biomarkers, since it is able to pinpoint acute kidney injury (AKI) before any rise in serum creatinine.[11] Plasma NGAL is filtered in the glomerulus and largely reabsorbed by the proximal tubule. It is the synthesis of NGAL protein in the distal nephron and its secretion in urine that give the largest contribution to NGAL in urine.[11,12] Therefore, an abnormal NGAL excretion in urine occurs when NGAL reabsorption is deranged in the proximal tubule or distal NGAL synthesis is increased. A fast and substantial rise of NGAL mRNA in the ascending Henle's loop and in the collecting tubule has been demonstrated in previous gene expression studies in AKI.[11,12]

The primary goal of the present study was to capture a possible renal damage induced by hypobaric hypoxia by detecting an increase in urine concentration of NGAL, which is known to quickly rise (as early as 1–2 h) in urine after nephrotoxic and ischemic damages. Total urine NGAL was detected by dedicated kits, including homodimeric and monomeric NGAL forms. Urinalysis and microscopic assessment ruled out the presence of leukocytes, excluding that the measured NGAL derived from leukocytes.[10] In our pilot in vivo experiment, we were unable to observe any increase in urine NGAL concentration. Conversely, we found that urinary NGAL concentration was significantly decreased at 24 h compared with baseline, whilst being decreased but not to a statistically significant extent at 3 h assessment. These results suggest that no transient kidney damage followed a single short exposure to hypobaric hypoxia, however a subtle injury with possible transient increase in urine NGAL concentration might have occurred at earlier time point.

The effect of hypoxia was already investigated by Mellor et al.[17] in a study on 14 subjects who underwent a 3-h exposure to normobaric hypoxia (FiO$_2$ 11.6%, equivalent to 4800 m altitude) in a hypoxic chamber. This exposure included a 5-min step test (step height of 25 cm, one complete step every 2 s) at 95 min. NGAL was assayed at baseline and after 180 min of hypoxic exposure and the values were not significantly different. Our data at the third hour after the hypobaric hypoxia stress test were similar, with no significant variation in urine NGAL. However, surprisingly, NGAL/Cr values after 24 h were significantly lower compared with baseline values. The pathophysiological meaning of such a decrease in NGAL is unknown. The simplest deduction is that it rules out a tubular damage in both proximal tubule and distal nephron.

In proximal tubule cell cultures we showed that NGAL gene expression was significantly reduced by hypoxia but recovers to basal levels by 24 h of reoxygenation. As expected, 24 h after hypoxia, the percentage of apoptotic and necrotic cells was increased. On the other hand, the secretion of NGAL in the culture medium remained substantially stable during hypoxia and in subsequently 24 h of reoxygenation. Based on these results, it appears that hypoxic conditions actually down-regulate the expression of NGAL. The process seems to be reversible after a transient hypoxic insult, but not after a second hypoxia incubation of the tubular cells that induced a major apoptotic/necrotic response and an irreversible decrease in NGAL expression. It is possible that the experimental conditions created specifically for the purpose of the study, by causing apoptosis and necrosis, reduced the NGAL gene expression. On the other hand, the extensive apoptotic/necrotic response induced by the second hypoxia incubation of the tubular cells was likely responsible for the increased NGAL secretion.

The NGAL overexpression immediately after injury in AKI has been hypothesized to be part of a protective mechanism. In the kidney, NGAL is filtered by glomeruli and then internalized by binding to the megalin–cubilin receptor complex and 24p3 in proximal or distal tubules, respectively.[18] By this mechanism, NGAL is able to reduce the iron availability and limit the bacterial proliferation.[19,20] Furthermore, in the proximal tubular epithelial cells, the most injured compartment after ischemia/reperfusion and AKI, the NGAL overexpression/administration reduces apoptosis.[21] Following this suggestion, we speculated that the lower urinary excretion of NGAL observed after 24 h of hypobaric hypoxia, rather than mirroring a tubular injury, is a biomarker of the hypoxic preconditioning of the kidney. This is a phenomenon by which mild episodes of hypoxia, preceding a more severe one, enhance cell survival, and differentiation after the severe hypoxia–ischemia event.[22,23]

To test for this hypothesis, we performed a second hypoxia-reoxygenation cycle in the cell culture model. When tubular cells were exposed to a second hypoxic insult, NGAL gene expression was again reduced but its expression remained reduced also after 24 h of reoxygenation. After the second ischemia, the percentage of total apoptotic cells was comparable to the first hypoxia/reoxygenation, whereas the relative percentage of necrotic cells significantly increased suggesting that the second hypoxic insult had more severe effects on the cells. This likely led to an even far lower expression of NGAL. While these results rule out a preconditioning effect, they lead to support the protective role of NGAL against hypoxia/reoxygenation-induced cell death discovered by Zang et al.[21] in NRK-52E cells, another proximal tubule cell line.

The link between the apoptotic damage and the expression pattern is yet to be investigated. We hypothesize that hypoxia/reoxygenation downregulates NGAL expression and induces tubular cell death. The amount of plasma NGAL filtered by glomeruli and reabsorbed by tubular cells via megalin complexes could then exerts an anti-apoptotic effect protecting tubular cells from subsequent ischemic events.

The present study only represents a pilot experience to pioneer further pre-clinical and clinical studies. We acknowledge some limitations such as the low sample size and the inclusion of male subjects only in the in-vivo study. Our results are limited to the evaluated time points. The reader should note that reducing the experimental hypoxic conditions and the related induction of apoptosis to a lower level, could have potentially shown different results, such as an initial increase of NGAL expression. Furthermore, in vitro experiments need to be validated on other cell lines.

Conclusions

Exposure to hypobaric-hypoxic environments does not cause significant and irreversible renal tubular injury in

vivo and in vitro, except than in a late stage. The hypoxic insult does not seem to be mirrored by an increase of urinary NGAL in healthy men, nor of NGAL gene expression in HK-2 cell culture or secretion in the culture medium in the in vitro conditions reported in the present study. More work on different experimental conditions and extended time points is needed to further investigate the clinical implications of our findings.

Declaration of conflicting interests

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