Evidence for sustained renal hypoxia and transient hypoxia adaptation in experimental rhabdomyolysis-induced acute kidney injury

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Abstract

Background. Indirect evidence suggests that hypoxia contributes to the pathophysiology of rhabdomyolysis-induced acute kidney injury (AKI). However, the cellular location and kinetics of hypoxia, as well as potential hypoxia adaptation are unclear.

Methods. Rhabdomyolysis was induced in rats by IM glycerol (GLY) injection, which largely recapitulates the full clinical syndrome. Additional rats received IV myoglobin (MYO), in order to assess the contribution of MYO per se. We performed immunohistochemistry for hypoxia markers [pimonidazole (PIM) adducts and hypoxia-inducible factors (HIFs)] and the cell-protective HIF target gene heme oxygenase-1 (HO-1). Furthermore, we sought a potential negative feedback loop to terminate HIF activation, driven by HIF prolyl-hydroxylase-2 (PHD-2).

Results. In GLY, progressive tubular injury, mainly of proximal tubules (PT), developed over time, but its extent was heterogeneous. PIM, HIFs and HO-1 were all absent in controls, but strongly positive in GLY, with a specific spatiotemporal pattern. In PT, (a) PIM was detectable throughout the study with a maximum at 6 h, (b) HIF was activated only at 3 h and (c) HO-1 and PHD-2 appeared at 6 h and persisted at a lower level at 24 h. Apart from tubular cast formation, MYO did not cause overt tissue damage, but led to strong activation of HIFs, in a pattern similar to 3 h of GLY.

Conclusions. Our data suggest that renal hypoxia occurs in rhabdomyolysis, and that MYO, at least partly, contributes to hypoxia generation. Since in the most affected tubules transcriptional hypoxia adaptation is transient and inhomogeneous, pharmacologic HIF enhancement holds the potential to improve outcome in rhabdomyolysis-induced AKI.

Keywords: acute renal failure; heme oxygenase-1; HIF prolyl hydroxylase-2; hypoxia-inducible factors; pimonidazole

Introduction

Rhabdomyolysis-induced acute renal injury (AKI), also named pigment nephropathy or crush kidney injury, develops following skeletal muscle trauma related to physical, thermal, ischemic, infective, metabolic or toxic causes, and is associated with high morbidity and mortality [1–4].

The pathophysiology of AKI in rhabdomyolysis is likely complex and incompletely understood [3]. Myoglobin (MYO) has been shown to bind to Tamm-Horsfall protein, especially in an acidic milieu, forming tubular casts [5]. Cast-bound MYO can scavenge nitric oxide (NO) [6] and generate reactive oxygen species (ROS) [7–12], causing tubular toxicity and vasoconstriction. Muscle swelling, intravascular volume depletion, reduced cardiac output [13] and lactic acidosis, all probably contribute to renal injury. In experimental models, renal cortical blood flow is markedly reduced [14]. The pathology in humans is remarkable for pigmented casts in distal tubules and collecting ducts with varying degrees of injury in other nephron segments [15,16]. In animals, glycerol (GLY) injection into the hind limbs produces renal changes resembling that observed in the human [17].

Using oxygen microelectrodes, we have previously shown an acute reduction of renal parenchymal oxygenation during the infusion of MYO [18]. Alterations in renal parenchymal oxygenation have not been assessed in the more clinically relevant GLY model.

Hypoxia adaptation is largely conferred through the so-called hypoxia-inducible transcription factors (HIFs) [19–21]. HIFs are heterodimers composed of a constitutive β-subunit and one of at least two different
oxygen-dependent α-subunits (HIF-1α and -2α). HIF is mainly regulated by oxygen-dependent proteolysis of the α-subunits. The key enzymes of HIFα degradation are HIF prolyl-hydroxylases (PHDs) [22,23]. PHDs require molecular oxygen for substrate and their $K_m$ is within the range of ambient oxygen concentrations [24]. Therefore, PHDs can be considered cellular oxygen sensors. HIFs are ubiquitous, instantaneously activated upon hypoxia and short-lived after reoxygenation. HIFα immunohistochemistry can serve to detect hypoxia within tissues. It is noteworthy that non-hypoxic HIF activation by various growth factors and cytokines has been demonstrated in vitro [25,26]. However, since the latter is caused by enhanced HIFα transcription/translation, it likely takes longer to establish than hypoxic HIF activation, which is due to impaired HIFα degradation. Heme oxygenase-1 (HO-1) is one of the most prominent HIF target genes with well-proven cell protective properties [27]. Importantly, since it generates carbon monoxide, a vasodilator, and biliverdin, a potent radical scavenger, HO-1 has the potential to counterbalance at least some factors suspected to cause rhabdomyolysis-induced AKI.

Pimonidazole (PIM) is a bioreductive agent which, after in vivo delivery, binds to tissues at pO$_2$ below 10 mmHg, where it can be visualized with the help of commercially available antibodies [28,29]. In previous studies from our lab immunohistochemistry for HIFα or PIM was undetectable in normal kidneys, but strongly positive after various hypoxic stimuli [30–33]. Thus, HIFα and PIM most likely detect those pathologic conditions in which oxygen availability becomes limiting, ensuing either cell injury or adaptive responses [34].

The present study employs high amplification immunohistochemical techniques to seek evidence of renal hypoxia in rhabdomyolysis-induced AKI, to define its cellular location, kinetics, and relationship to tubular injury, as well as potential adaptive responses. Our investigation includes events shortly after the initiation of AKI, when tubular injury is sublethal and potentially reversible—a time never examined in human material. Indeed, our work suggests that hypoxia contributes to tubular injury in a clinically relevant model of rhabdomyolysis-induced AKI.

Methods

Animals and materials

Male Sprague Dawley (SD) rats (345 ± 6 g) were used, fed regular chow with free excess to water. Chemicals were purchased from Sigma (St. Louis, MO, USA). Experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

GLY model

Under ether anaesthesia rats were injected with 50% GLY, 8 ml/kg, applied in divided volumes into four sites at the hind limb muscles. Dipyrone (50 mg/kg IP) was given for pain management.

MYO infusion model

As previously detailed [18], under ketamine anaesthesia (100 mg/kg) and following catheterization of the femoral vein, MYO was infused over 45 min (380 mg/kg, dissolved in 5 ml saline).

Functional studies

Rats were kept in metabolic cages (Nalge, Rochester, NY, USA) for 24 h urine collections. Blood sampling from the femoral vein was obtained under anaesthesia at baseline, and during sacrifice in the 24 h experiments. Plasma and urine samples were processed for the determination of creatinine, urea, sodium and potassium, and creatinine phosphokinase (in a Roche/Hitachi Modular [30], Grenzach-Wyhlen, Germany). Creatinine clearance, fractional sodium reabsorption and potassium excretion were calculated.

Determination of renal morphology

Under pentobarbital anaesthesia (60 mg/kg), the kidneys were perfusion-fixed selectively through the abdominal aorta with 1.25% glutaraldehyde (for plastic embedded semi-thin sections) or with 3% paraformaldehyde (for paraffin-embedded sections for immunohistochemistry), as previously detailed [31,33]. Semiquantitative assessment of cortical tubular damage was performed, using a 96-field grid, superimposed on a representative low power figure (magnification 90×) of a 3-µm paraffin section (haematoxylin and eosin staining, comparable with Figure 4A and B). To each field of the grid a tubular injury score was assigned, based on the most severe tubular damage observed therein: 0 = no significant tubular damage (corresponding with ‘P’ in Figure 1), 1 = moderate damage (‘P’ in Figure 1), 2 = severe damage (‘P’ in Figure 1). Field scores were added and divided by 96.

Immunohistochemistry

PIM (HypoxyProbe, Pharmacia International, Belmont, MA, USA), which binds to tissues with pO$_2$ levels below 10 mmHg [28], was injected in vivo (60 mg/kg IV) 1 h before the kidneys were perfusion-fixed. The following primary antibodies were used as previously reported [30–33,35,36]: mouse-anti-human HIF-1α (α67, Novus Biologicals, Littleton, CO, USA, 1:10 000), rabbit-anti-mouse HIF-2α (PM9, gift from Patrick Maxwell, Hammersmith Hospital, Imperial College, London, UK, 1:10 000), rabbit-anti-rat HO-1 (Stressgen, Victoria, Canada, 1:60 000), rabbit-anti-mouse HIF prolyl-hydroxylase-2 (Novus Biologicals, Littleton, CO, USA, 1:10 000), mouse-anti-PIM (1:1000 Hypoxyprobe; Natural Pharmacia International, Belmont, MA, USA). Immunostaining was assessed semiquantitatively according to the following score: 0 = no signals, 1+ = staining in <5% of tubular profiles or IC/EC, 2+ = 5–20%, 3+ = 20–33%; 4+ = 33–50%.

Statistics

Data are represented as means ± SEM. Paired Student’s t-test was used for the comparison of functional parameters.
Results

A total of 42 animals were included into the study: 5 untreated controls, 6 with IV MYO infusion, sacrificed at 3 h, and 31 with IM GLY injection (10, 6 and 15 for the 3, 6 and 24 h time points, respectively). Rhabdomyolysis was confirmed by an increase in serum creatine phosphokinase in a subset of three animals at 3 h after GLY injection: 1210, 1560 and 970 units/l, compared with baseline levels of 44, 62 and 34 units/l. A 33% mortality was noted only in GLY-treated animals kept for 24 h.

*Functional data: acute renal failure in GLY*

At 24 h GLY led to a significant reduction in creatinine clearance, fractional sodium reabsorption, and to a significantly enhanced serum creatinine, serum urea and fractional excretion of potassium (Table 1).

### Table 1. Functional data

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Day 1</th>
<th>P value</th>
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<tbody>
<tr>
<td>Urine volume (ml/h)</td>
<td>0.27 ± 0.03</td>
<td>0.45 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td>PCr (µmol/l)</td>
<td>51 ± 3</td>
<td>177 ± 14</td>
<td>0.01</td>
</tr>
<tr>
<td>CICr (ml/min)</td>
<td>0.66 ± 0.05</td>
<td>0.12 ± 0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>ClCr (ml/min/100 g)</td>
<td>0.20 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>PUrea (mmol/l)</td>
<td>8.2 ± 0.4</td>
<td>42.6 ± 2.6</td>
<td>0.01</td>
</tr>
<tr>
<td>TRNa (%)</td>
<td>99.27 ± 0.08</td>
<td>97.57 ± 0.45</td>
<td>0.05</td>
</tr>
<tr>
<td>FEK (%)</td>
<td>9.0 ± 1.5</td>
<td>71.5 ± 8.0</td>
<td>0.01</td>
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Functional parameters in rats subjected to Rh-AK at baseline (day 0) and at 24 h later (n = 10).

**PIM-adducts are detectable in GLY but not in MYO**

To test if profound renal hypoxia occurs in GLY, and whether its location predicts tubular injury, we searched for PIM-adducts, which accumulate in tissues at oxygen tensions below 10 mmHg [28]. Indeed, while control animals all were negative for PIM, in GLY, strongest staining occurred in PT (mostly S2 and S3) and preceded the development of tubular damage, suggesting hypoxic genesis of injury (Figures 2A and B). In distal nephron segments, morphology was relatively well preserved throughout the study, albeit tubular casts and cellular debris were seen (Figure 1). In MYO, tubular damage was minimal (Figure 3), although tubular casts and capillary congestion were comparable to 3 h of GLY.

**Morphologic findings: proximal tubular injury in GLY, minimal structural alterations in MYO**

In GLY, the major morphologic injury developed in proximal tubules (PT), with the formation of casts in the distal nephron, observations in line with previous studies [15–17]. PT injury progressed over time (Table 2, Figure 1), mainly appearing in the cortex (mostly S2 segments) and occasionally in the outer stripe (S3 segments). Alterations ranged from mild (some loss of brush border, cytoplasmic vacuoles, but generally preserved epithelial structure) to severe (complete destruction of tubular architecture and eventually necrosis) (Figure 1). Perfusion fixation allowed us to discriminate between open tubules (presumably belonging to filtering nephrons) and collapsed tubules (presumably non-filtering nephrons). In general, morphology was heterogeneous within the same section, with groups of open and collapsed tubules side by side, but the number of collapsed tubules increased over time, suggesting a reduced number of filtering nephrons (Figure 2A and B). In distal nephron segments, morphology was relatively well preserved throughout the study, albeit tubular casts and cellular debris were seen (Figure 1). In MYO, tubular damage was minimal (Figure 3), although tubular casts and capillary congestion were comparable to 3 h of GLY.

**Fig. 1. Fine morphology in rhabdomyolysis-induced AKI.** AKI was induced in rats by IM GLY injection. Asterisk = medullary thick ascending limb (mTAL); CD = collecting duct; DT = distal tubule (discrimination between distal nephron segments was impossible in this material); P = proximal tubule (largely intact); P = moderately injured P; S3 = S3 portion of the proximal tubule; T = thin limb; VB = vascular bundles. Proximal tubular injury increases from 3 h (A) to 6 h (C) and 24 h (E). At 24 h in the outer medulla tubular injury mainly occurs in S3 (B), whereas in the inner stripe mTAL are filled with casts (D). In the papilla morphology is preserved, apart from tubular casts, which at 24 h mainly occur in thin limbs (F). Magnification 800×.

ANOVA with post hoc Student’s t-test was applied for between-group comparisons of morphology and immunohistochemistry. Statistical significance was set at *P* < 0.05.
had no major damage, suggesting that hypoxia developed in filtering nephrons. To test if MYO per se had induced renal hypoxia in GLY, PIM was investigated 3 h after the beginning of a 45-min MYO infusion, but no signals were detected (with the rare exception of some faint signals in cortical PT, not shown).

Early activation of HIFα in GLY and MYO

Results of HIFα immunohistochemistry are outlined for GLY (Figure 5 and Table 2) and MYO (Figure 3 and Table 2). Control animals were negative for either HIFα isoform. With occasional exceptions (intensity of papillary immunostaining), the two main isoforms, HIF-1α and -2α, were upregulated in a largely comparable pattern at 3 h of GLY and at 3 h of MYO, suggesting that MYO per se induces regional renal hypoxia and contributes to hypoxia generation in GLY. This study largely confirms the previously described cell-type specificity of the HIF isoforms [30–33]: HIF-1α mainly in tubules and in papillary interstitial cells, and HIF-2α exclusively in endothelial and interstitial cells. But exceptionally, in both GLY and MYO, additional HIF-1α occurred in tubulo-interstitial capillaries (mostly in medullary rays and in the outer medulla, arrow in Figure 3E), pointing to a complex pathophysiology of GLY/MYO. This study confirms that CD and the papillary cells hold a high potential for hypoxia adaptation. By contrast, in PT, the most severely injured tubular segment, HIF, was only detectable at 3 h, when morphologic changes were moderate. Later on, as severe injury developed, PT became negative for HIF, suggesting defective/exhausted hypoxia adaptation. Interestingly, at 6 h PIM staining was maximal (Figure 2C), but HIFα was negative for all renal cell types (Table 2).

Expression of the cell-protective HIF target gene HO-1 after 6 h of GLY

To test whether HIF activation was accompanied by upregulation of HIF target genes, we performed immunohistochemistry for HO-1. In controls and at 3 h after GLY, no renal HO-1 was detectable (not shown), but strong tubular HO-1 signals occurred at 6 h after GLY (Figures 2E and 6A–D, Table 2) in cell types with previous HIF-1α activation (compare Figure 5), suggesting hypoxic induction: cortical PT (Figures 2E and 6A), S3 in the outer stripe (Figure 6B) and CD in all renal zones (Figure 6B–D). At 24 h after GLY, HO-1 signals were less abundant and intense (Figure 2F), which is consistent with previous mRNA data in the same model [37]. Interestingly, at the time point of maximum HO-1 (6 h) HIF was undetectable (Table 2). Moreover, careful assessment of parallel sections showed that in individual tubules coincident staining for HO-1, HIF and PIM were extremely rare, if ever detectable (compare rectangles in Figure 2). Taken together, such data suggest a sequential rather than concomitant expression of PIM, HIF and HO-1 in the nephron.

The HIF target gene PHD-2 is activated after 6 h of GLY

To test if a negative feedback loop could be responsible for the lack of HIF expression at 6 and 24 h of GLY, we performed immunohistochemistry for PHD-2, which is the main renal isoform of the key HIF degradation enzyme [38],...
and itself an HIF target gene. In controls, PHD was detected in virtually all cells of the papilla and inner stripe, in distal tubules of the outer stripe and medullary rays (not shown), as well as in distal tubules of the labyrinth (Figure 7C). This expression pattern is in line with previous mRNA and immunohistochemical [38] data. At 6 h of GLY, additional nuclear immunostaining appeared in glomeruli and in PT of the labyrinth (Figure 7A and B), medullary rays (Figure 7A) and outer stripe (not shown). Thus, de novo PHD-2 activation clearly occurred in formerly HIF-positive cell types, which is consistent with a negative feedback loop to stop HIF activation. This staining pattern persisted at 24, but less intensely and less abundantly in PT.

**Discussion**

This is a proof of concept study providing immunohistochemical evidence for hypoxia and hypoxia adaptation in rhabdomyolysis-induced AKI. Our data suggest that (a) renal hypoxia and hypoxia adaptation occur early in experimental rhabdomyolysis-induced AKI caused by IM GLY injection; (b) MYO per se contributes to hypoxia generation; (c) the extent of tubular hypoxia relates to cell damage and (d) insufficient hypoxia adaptation in proximal tubular cells may explain their marked injury in the GLY injection model.
Fig. 4. Immunohistochemistry for PIM adducts at 3 h of rhabdomyolysis-induced Rh-AKI. AKI was induced by IM GLY injection. Arrowhead = interstitial cell (IC); asterisk = medullary thick ascending limb (mTAL); CD = collecting duct; PT = proximal tubule; S3 = S3-portion of the proximal tubule. In (D) the renal pelvis and parapelvine portions of the kidney were artificially covered with grey for a better distinction of the papilla. Deep hypoxia was detectable in the superficial cortex (A, PT and CD), in S3 of the outer stripe (B), in mTAL and CD of the inner stripe (C), as well as in the interstitial compartment of the papilla (D, E). Magnification: A–D: 600×; E: × 90×.

Theoretically, the validity of hypoxia detection techniques used herein may be questioned, but a considerable amount of evidence backs the concept that, indeed, in this study PIM and HIF immunostaining correspond with cellular hypoxia. First, elegant studies have proven that PIM binding to tissues is highly reliable at a pO2 below 10 mmHg and largely independent of pH and redox state [28,29]. Second, HIF activation was quite rapid (within 3 h), within the time frame previously reported for hypoxic induction in whole animals [32]. To the best of our knowledge, non-hypoxic HIF activation has not been demonstrated in whole animals, so far, but most likely would require more time to establish than hypoxic HIF activation. In cell cultures, hypoxic HIF activation is instantaneous [39] and based on impaired HIFα degradation, whereas non-hypoxic HIF upregulation has been shown after hours, and relies on increased HIF-1α synthesis via activation of the phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathways ([26,40] and references therein).

Fig. 5. Immunohistochemistry for HIFs at 3 h of rhabdomyolysis-induced AKI. AKI was induced by IM GLY injection. Arrow = endothelial cell (EC); arrowhead = interstitial cell (IC); asterisk = medullary thick ascending limb (mTAL); CD = collecting duct; DT = distal tubule (discrimination between distal nephron segments was impossible in this material); PT = proximal tubule; S3 = S3 portion of the proximal tubule; T = thin limb; VB = vascular bundles. HIFα occurred in all renal zones, HIF-1α mainly in tubular profiles and in papillary IC and HIF-2α exclusively in EC/IC. Some HIF-2α signals clearly located in EC, but in most cases distinction between IC/EC was impossible. Tubular HIF-1α mainly located in PT and CD of the superficial cortex (A), in S3 of the outer stripe (C), in CD and mTAL of the inner stripe (E), and in CD of the papilla (G). Magnifications: A–F and inset in H: 400×; G and H: 90×.

PIM staining suggests a more severe/protracted renal hypoxia in GLY when compared with MYO, which is in line with much less tubular damage [18] in the latter. To our knowledge, the two experimental models have not been compared directly, so far. Theoretically, the extent and kinetics of myoglobinuria should be different. Moreover, cofactors like intravascular volume depletion and acidosi likely aggravate hypoxia in GLY. It is noteworthy that some authors [5] have shown that acidosis enhances the tubulotoxicity of MYO. Jiang et al. have shown that HIF activation is half maximal at 1.5–2% oxygen [41], which corresponds with 11–15 mmHg, whereas Gross et al. have reported that nitroimidazole binding to tissue occurs below
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Fig. 6. Immunohistochemistry for the cell-protective HIF target gene HO-1 at 6 h of rhabdomyolysis-induced AKI (higher power). AKI was induced by IM GLY injection. CD = collecting duct; G = glomerulus, PT = proximal tubule (probably S2); S1 = S1 portion of the proximal tubule; S3 = S3 segment of the proximal tubule. In this model at 6 h, HO-1 induction is maximal, and predominantly locates in nephron segments which had upregulated HIF-1α at 3 h (compare Figure 2): in the cortex (A) in PT including S1, in the outer stripe in S3 and CD (B), in the inner stripe (C) and in the papilla (D) in CD. Magnification: 250×.

Fig. 7. Immunohistochemistry for the HIF target gene HIF prolyl-hydroxylase-2 at 6 h of rhabdomyolysis-induced AKI. AKI was induced by IM GLY injection. Halfed arrows point to medullary rays; DT = ‘distal tubules’, which were not further characterized, potentially distal convoluted tubules, connecting tubules, thick ascending limbs or collecting ducts; G = glomerulus; PT = proximal tubules; (B) is a higher power of the region outlined by the rectangle in (A). In the cortex of control kidneys (C) cytoplasmic signals are detectable in ‘distal tubules’, but proximal tubules and glomeruli are negative. In AKI (A, B), ‘distal tubules’ remain positive, but additional nuclear signals appear in glomeruli and in proximal tubules. Magnification: A: 100×, B, C: 250×.

10 mmHg [28]. We believe that in MYO, renal hypoxia was more moderate, below the threshold for PIM detection.

We show that the cell-protective HIF target gene HO-1 appears de novo and that HIF precedes HO-1 by several hours in the same cell types, which is consistent with hypoxic upregulation of HO-1. The kinetics of HO-1—with highest expression at 6 h after induction of injury—are in line with a previous mRNA study [37]. Interestingly, in the cortex of rats with rhabdomyolysis-induced AKI Ishizuka et al. have only found HO-1 immunostaining in what they called ‘distal tubules’ [42], which at least from the photographs, in our opinion were collecting ducts. The fact that, in addition, we detected HO-1 in PT may reflect a higher sensitivity of our staining technique. Obviously, at least some PT are able to mount an adaptational response to hypoxia.

We report considerable variation of tubular damage, PIM, HIF and HO-1 among different nephrons, as well as between different nephron segments. Nephron segment-specific HIF activation has been demonstrated in several previous studies with different hypoxic stimuli [30–33]. As to differences between nephrons, the most likely explanation is that filtering and non-filtering nephrons exist side by side, as has been revealed by micropuncture studies [43,44]. We have previously shown that functioning freshly engrafted human kidney grafts activate HIF, whereas non-functioning grafts are HIF-negative [36]. Possibly, glomerular filtration and energy consumption for tubular salt reabsorption are prerequisites for the development of tubular hypoxia and HIF activation.

Despite of the proximity of PIM and HIF signals, there was virtually no colocation within individual cells. This is in line with previous [36] and [30] reports that HIF activation is maximal at moderate hypoxia, whereas PIM adducts occur at more severe hypoxia. Thus, both methods of hypoxia detection are complementary rather than redundant. As to the missing overlap between HIF and HO-1, HIF is short-lived and its peak activity precedes that of its target genes by several hours. It is noteworthy that PHD-2 is an HIF target gene capable of shutting off HIF activation [45]. Such a negative feedback loop might explain the lack of HIF in PT at later stages of Rh-AKI.

In conclusion, in experimental rhabdomyolysis-induced AKI, location and kinetics of overt cell injury on the one
hand and immunohistochemical markers on the other hand suggest that hypoxia occurs within the kidney and that it contributes to cell damage. Transcriptional adaptation to hypoxia seems to be limited to certain cells and to a relatively short period of time. Therefore, extending such adaptational responses might be a reasonable therapeutic approach in rhabdomyolysis.

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Conflict of interest statement. None declared.

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