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Up-Regulation of Local TGF-β Contributes to a Decrease in Renal Tubular Na^+-K^+ ATPase and Hyperkalemia in a Mouse Model of Crush Syndrome

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Abstract

Hyperkalemia is one of the most important risk factors in patients suffering from crush syndrome with rhabdomyolysis. Glycerol-injected animals have been used as an experimental model of rhabdomyolysis-induced acute kidney injury (AKI), but little information is available for the onset and molecular mechanism of hyperkalemia. In our murine model, plasma potassium levels increased after a single injection of 50%-glycerol solution (10 ml/kg, i.m.) during the progression of muscular and renal injuries. Renal tubular Na^+-K^+-ATPase functions as ion-exchange pomp for potassium clearance from blood into renal tubular epithelial cells. Renal histochemistry revealed an apparent decrease in the tubular Na^+-K^+-ATPase expression, especially at 24 hours post-glycerol challenge in our AKI model. In contrast to the loss in active Na^+-K^+-ATPase, there was a significant increase in the renal levels of transforming growth factor-β (TGF-β) that is known to suppress Na^+-K^+-ATPase production *in vitro*. When anti-TGF-β antibody was administered in mice after the glycerol challenge, the suppression of renal Na^+-K^+-ATPase activity was partially restored. As a result, hyperkalemia was improved in the TGF-β-neutralized AKI mice, associated with a significant decrease in plasma potassium concentration. Taken together, we predict that endogenous TGF-β is a key regulator for inhibiting Na^+-K^+-ATPase production and, in part, enhancing hyperkalemia during progression of rhabdomyolysis-induced AKI. This is, to our knowledge, the first report to determine a critical role of endogenous TGF-β in renal potassium metabolism during crush syndrome.

Keywords

Rhabdomyolysis, AKI, Hyperkalemia, Na^+-K^+-ATPase, TGF-β

1. Introduction

Crush syndrome (CS) is clinically characterized by the primary muscular injury and subsequent acute kidney injury (AKI), often associated with an increase in plasma potassium levels [1] [2]. These pathological conditions are caused by muscular traumatic stresses, as noted in traffic accident or earthquake. The clinical findings are induced by destruction of muscle tissues and leakage of the contents of myocytes into blood. Myoglobin is toxic to renal epithelial cells [3], and occlusion of renal tubular fluid flow by cell debris leads to enhancement of AKI via a pathological circuit, such as oxidant stress and local inflammation, common to AKI after renal ischemia [4]. Especially, post-traumatic AKI often occurs in patients suffering from CS in the aftermath of major earthquake. For example, more than 350 patients were estimated to die due to post-traumatic AKI in the disaster of Hanshin Awaji earthquake that struck Hyogo Prefecture of Japan in 1995 [5]. In addition, a total of 149 patients (8.2%) were diagnosed with CS in Wenchuan earthquake of China in 2008 [6].

Cardiac arrhythmia and arrest are a clinical hallmark of severe hyperkalemia during CS [1]. Given that kidney tissue plays a central role in regulating plasma potassium levels, it is important to elucidate an intrinsic mechanism to inhibit or minimize the intractable hyperkalemia after rhabdomyolysis. For example, Na^+-K^+-ATPase is located in a basolateral site of renal tubular epithelium and functions as an ion-exchange pump for potassium clearance from blood to renal cells [7], thus suggesting a compensatory role of Na^+-K^+-ATPase for minimization of hyperkalemia. Inversely, loss of this enzyme is associated with several types of AKIs, caused by ischemia, mercury chloride and cisplatin in rats [8] [9] [10]. With regard to this, emerging evidence indicates that transforming growth factor-β (TGF-β) plays a crucial role in renal homeostasis and AKI [11] [12] [13]. Nevertheless, less information is available about the cytokine-based mechanism of hyperkalemia in animal models of rhabdomyolysis-induced AKI.

So far, glycerol-treated rats have been used as a model of rhabdomyolysis-related AKI, since they manifested morphological and functional abnormalities in a stable fashion [14] [15]. However, there are no reports showing the changes in plasma potassium levels in mice treated with glycerol. In the present study, we determined the natural course of renal and muscular injuries in mice after an injection of 50%-glycerol. Here, we provide evidence that hyperkalemia occurs in glycerol-injected mice, possibly via a decrease in renal Na^+-K^+-ATPase and that these pathological events are, in part, dependent on up-regulation of renal TGF-β.

2. Materials and Methods

2.1. Preparation of a Murine Model of CS with AKI

Specific pathogen-free ICR mice (6 weeks old, female, 20 - 24 g) were purchased from SLC (Hamamatsu, Japan). We attempted to induce rhabdomyolysis in mice, according to a previous report [16]. In brief, mice were treated with 50%-glycerol (diluted in distilled water) (Nacalai, Kyoto, Japan) at a dose of 10 ml/kg (i.m.). To determine the natural course of post-traumatic AKI, 24 mice were subjected to autopsy at 0, 12, 24 and 36

hours post-glycerol challenge (n = 6 per group). For the scheduled autopsy, glycerol-injected mice were anesthetized by an injection of ketamine chloride (80 mg/kg, i.p.) and xyladine sulfate (8 mg/kg, i.p.), and then sacrificed by blood collection from the submaxillary artery, as reported [17]. All experiments were carried out according to the Guideline for Experimental Animal Care issued by the Prime Minister's Office of Japan.

2.2. Blood Chemistry

To detect the possible rhabdomyolysis in mice, plasma creatinine kinase (CK) levels were determined with a kit (test Wako CK, Wako Pure Chem., Osaka, Japan) [18]. To assess the loss of renal functions, blood urea nitrogen (BUN) levels were measured, using a kit (urea nitrogen-B test, Wako), as reported [17]. Plasma potassium levels were determined, using a dry-chemistry kit (Fuji Drychem 800V, Fuji-film, Tokyo, Japan).

2.3. Histology

At the scheduled necropsy, femoral muscular tissues and kidney were collected, fixed in neutral buffered formalin (pH 7.4) and embedded in paraffin. These tissues were cut at a thickness of 4 μm, de-waxed and then stained with hematoxylin and eosin (H.E.) for histological examination.

2.4. Renal Histochemistry

Renal Na^+-K^+-ATPase activity was visualized, as reported [19]. In brief, renal tissues were perfused with 2% polyvinyl pyrrolidone, 0.8 mM $CaCl_2$ and 100 mM histidine (pH 7.3) for 20 min and refrigerated in dry ice acetone. Frozen section was cut at 5 μm and incubated at 37C for 2 hours in a reaction solution [30 mM KCl, 5 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate and 70 mM 2-amino-2-methyl-1-propanol buffer (pH 9.0) with the addition of 1 mM ouabain] and then exposed to 2% $CoCl_2$.

2.5. Morphological Score

To quantify the expression of Na^+-K^+-ATPase, renal sections were examined under a light microscopy and the degree of renal expression was represented as a mean value of the following scales: 0 = no staining; 0.5 = trace staining; 1 = light staining; 2 = moderate staining; and 3 = intense staining, as checked in > 15 randomly chosen x200-field, as reported [17]. These analyses were all made in a blinded fashion.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA) of TGF-β

Renal tissues were homogenized and TGF-β1 levels in the renal extraction were measured, using a commercial kit (TGF-β1 quantikine ELISA, R&D systems, Minneapolis, MN, USA), as reported [17].

2.7. Neutralization of TGF-β *in Vivo*

Anti-pan TGF-β rabbit antibody (AB-100NA, R & D systems) is known to react with

mouse TGF-β and neutralize its function in murine models of organ diseases [20] [21]. To determine the role of TGF-β during rhabdomyolysis, we administered anti-pan TGF-β rabbit IgG (60 μg/head/time, i.p.), or normal IgG (*i.e.*, placebo control), into mice at 3 and 16 hours post-glycerol challenge. These mice were sacrificed at 24 hours after the start of this treatment. Blood was collected from tail veins at 6 hours post-induction under light anesthesia, and BUN and potassium levels were measured, as described above. The kidneys were sampled and subjected to Na^+-K^+-ATPase staining, as mentioned above.

2.8. Statistical Analysis

Data were expressed as mean ± S.D. A Student's t-test or ANOVA analysis was used to compare the group means and a value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Chronology of Rhabdomyolysis and AKI in Mice

Glycerol-injected mouse has been used as an animal model of muscular regeneration [22] [23], with a focus on myo-tubular differentiation in a late phase of rhabdomyolysis (*i.e.*, 2 weeks post-trauma). However, there is no report describing an "initial" phase of muscular injuries, especially prior to the onset of AKI in mice. Thus, we first determined whether the intramuscular injection of 50%-glycerol (10 ml/kg) induced the rhabdomyolysis. As expected, hypertonic glycerol solution induced the myotubular destruction and atrophy between 3 and 12 hours post-injection, as evidenced by H.E. staining of muscular tissues (**Figure 1(a)**). The destructed areas of myotubules were replaced with infiltrated leucocytes (such as macrophages) and interstitial edema, in particular, at 36 hours post-glycerol challenge. Being consistent with the early onset of histological damage, plasma CK levels rapidly increased within 3 hours, reaching a peak at 6 hours after the glycerol injection (**Figure 1(b)**), thus suggesting the successful onset

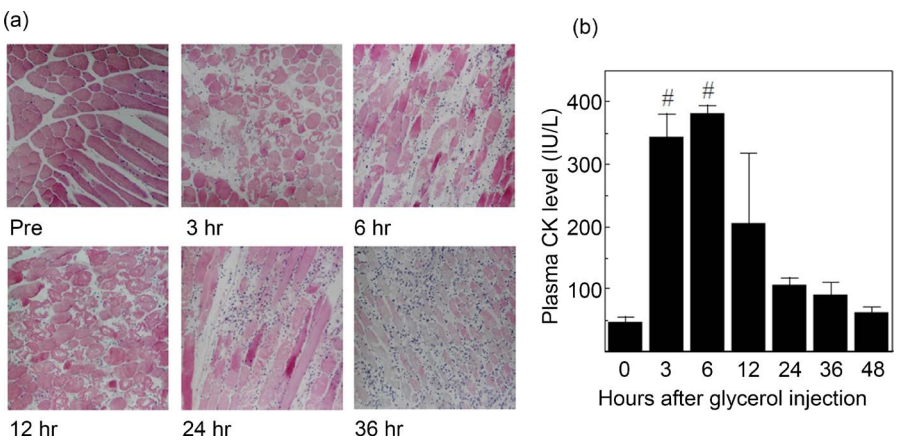

Figure 1. Change in muscular damage after a single injection of 50%-glycerol in mice. (a) Representative findings of muscular tissues (H.E. staining, original magnification: ×400). (b) Time course of plasma CK levels during the muscular damages (mean ± S.D., n = 6). Statistical analysis: #, $p < 0.01$ vs. pretreatment group.

of rhabdomyolysis in an "initial" phase after the glycerol injection.

We next determined when rhabdomyolysis induces AKI in the glycerol-treated mice. In a peak time of plasma CK levels (*i.e.*, 3 - 6 hours), renal tubular and glomerular structure was still normal, as evidenced by histological examinations (**Figure 2(a)**), and this was also convinced by blood biochemistry of BUN (**Figure 2(b)**), implying a time-lag (*i.e.*, latent phase) between rhabdomyolysis and AKI. Follow-up study revealed that there were severe lesions (such as tubular dilatation with luminal debris, cast occlusion and peri-tubular inflammation), especially from 24 hours post-challenge (**Figure 2(a)**). These lesions were followed by the regenerative response (*i.e.*, appearance of basophilic tubular epithelium in **Figure 2(a)**) till 36 hours post-challenge. Biochemical data were consistent with these histological changes: BUN levels reached a peak at 24 hours and then returned near the basal level till 36 hours post-challenge (**Figure 2(b)**). Little is known about a relationship between rhabdomyolysis and AKI in mouse models of CS. Our results clearly demonstrated the pathological sequence (*i.e.*, muscular injuries to AKI), being similar to that of human patients suffering from CS [1] [4].

3.2. Decrease in Na^+-K^+-ATPase and Up-Regulation of TGF-β in Mice

Hyperkalemia is an important life-threatening factor of CS [1] [4], but little information is available about the potassium abnormality in mice undergoing rhabdomyolysis. Thus, we determined the time course of plasma potassium levels. As a result, there was an increase in plasma potassium at 24 hours, then reversed near the basal value within 48 hours post-injury (**Figure 3(a)**), suggesting a transient hyperkalemia in the glycerol-treated mice. Renal tubular Na^+-K^+-ATPase activity is known to determine the predisposition to hyperkalemia [24]. Thus, we next checked the possible alternation in this ion-exchanging pomp enzyme, using an *in situ* histochemistry. As a result, we found that Na^+-K^+-ATPase activity was evident in distal and collecting tubules of the renal medulla in control mice (**Figure 3(b)**). In contrast, renal tubular Na^+-K^+-ATPase activities became faint in glycerol-injected mice, especially from 6 to 24 hours post-challenge.

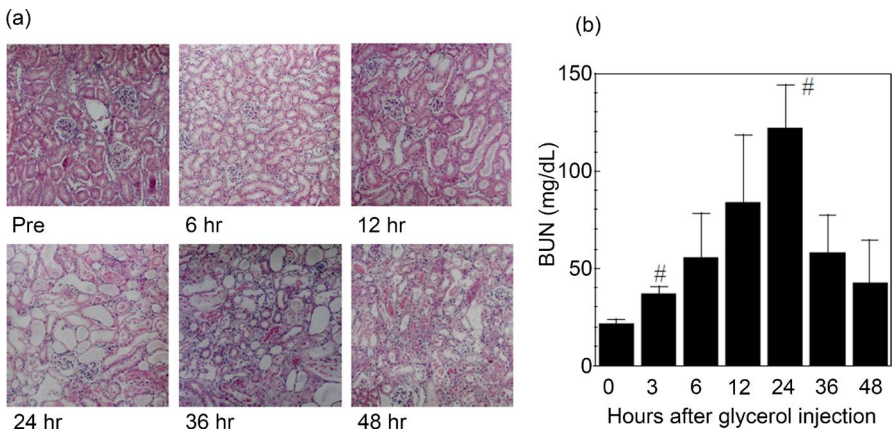

Figure 2. Natural course of AKI after glycerol challenge in mice. (a) Typical microphotograph of kidney tissues (H.E. staining, original magnification: ×40). (b) Changes in BUN levels during the renal damages (mean ± S.D., n = 6). #: $p < 0.01$ vs. pretreatment group.

Indeed, there was a significant difference in Na^+-K^+-ATPase staining score between control and 24 hr groups (in **Figure 3(b)**, $p < 0.05$). With regard to this, TGF-β is shown to inhibit Na^+-K^+-ATPase activation in culture of renal epithelial cells [25]. Thus, we checked the change in TGF-β1 levels, using an ELISA kit. As expected, there was a difference in renal TGF-β1 between control and glycerol groups (Pretreatment: 166.3 ± 27.1 ng/g vs. 24 hours: 251.3 ± 38.1 ng/g, $p < 0.05$) (**Figure 3(c)**).

3.3. Contribution of TGF-β to a Decrease in Na^+-K^+-ATPase Activity

The change in renal TGF-β1 correlated inversely with that of Na^+-K^+-ATPase activity *in vivo*. Thus, we hypothesized that endogenous TGF-β1 play a key role in CS-associated pathological conditions. To test this hypothesis, we injected anti-pan TGF-β antibody into mice at 3 and 12 hours after glycerol challenge. Interestingly, the glycerol-induced loss in renal Na^+-K^+-ATPase activity (*i.e.*, 24 hours post-challenge) was partially but significantly restored by the anti-TGF-β1 antibody treatment (**Figure 4(a)**), with a significant difference in staining score (control: 2.11 ± 0.24 vs. TGF-β1 neutralization: 2.55 ± 0.27, $p < 0.01$). Consistently with the reversed expression of renal tubular Na^+-K^+ATPase, plasma potassium levels were decreased in the TGF-β1-neutralized group than in control group (**Figure 4(b)**). There was a tendency to attenuate AKI by TGF-β1 neutralization, but there was no significant difference in BUN levels between both groups (control: 108.5 ± 18.3 mg/dl vs. TGF-β1 neutralization: 88.3 ± 17.2 mg/dl, $p = 0.22$) (**Figure 4(c)**).

4. Discussion

Hyperkalemia is a common hallmark of AKIs and occurs via dysregulation of

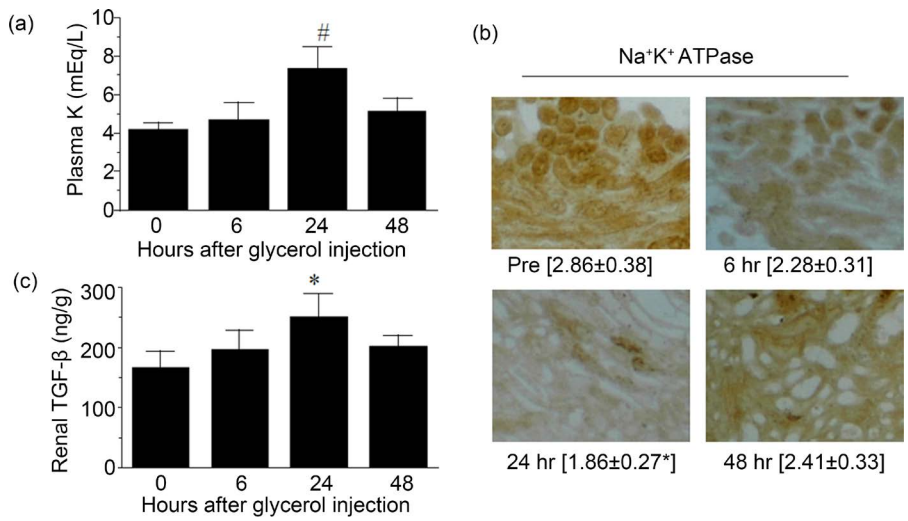

Figure 3. Change in hyperkalemia-related pathological events during rhabdomyolysis-induced AKI in mice. (a) Changes in plasma potassium levels. (b) Representative images of Na^+-K^+ ATPase activity in the renal tissues. [ ]: Na^+-K^+ ATPase staining score per each time-point. (c) Change in renal TGF-β concentration, as determined by ELISA [17]. Data are expressed as mean ± S.D. (n = 6). *: $p < 0.05$ or #: $p < 0.01$ vs. pretreatment group.

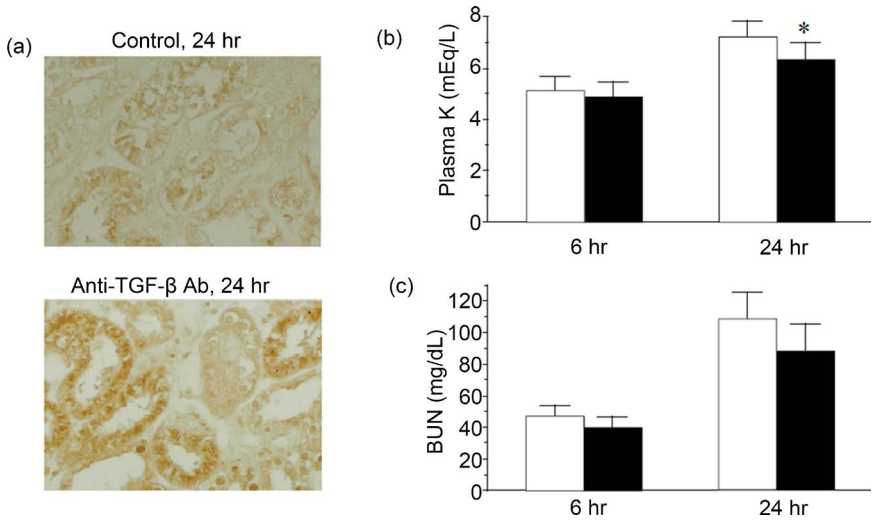

Figure 4. Effects of anti-TGF-β antibody (Ab) on AKI-associated phenotypes. (a) Typical findings of Na^+-K^+ ATPase activity in the kidneys between control and anti-TGF-β Ab groups. (b) & (c) Comparison of plasma potassium levels (b) and BUN levels (c) between control (open bar) and neutralization (closed bar) groups. Data are expressed as mean ± S.D. (n = 6). Statistical analysis: *, $p < 0.05$ vs. control group (24 hr).

potassium metabolism. Experimentally, hyperkalemia is inducible in rodents after renal ischemia and re-perfusion [26], sepsis [27] and chemical exposure [28], and this was associated with acute tubular destruction or dysfunction. In a rat model of muscular compression, hyperkalemia becomes evident post-muscular decompression or damage [29] [30], but molecular mechanism of hyperkalemia is not yet fully understood. In the present study, we obtained evidence that plasma potassium levels increased during the rhabdomyolysis-induced AKI in mice. This was concomitant with the loss in Na^+-K^+-ATPase activity in the affected kidney. We discuss the possible molecular events (including cytokine's role), leading to hyperkalemia, as followed.

Na^+-K^+-ATPase is located in a basolateral site of renal tubules and plays a key role as an ion-exchange pump for intake of blood potassium by renal epithelial cells. Activation levels of this pomp likely determine a predisposition to hyperkalemia. For example, cyclosporin-A suppresses Na^+-K^+-ATPase production in a culture of renal tubular cells [31], while forced induction of this pump by thyroxine leads to the attenuation of hyperkalemia in a rat model of cyclosporin-A-induced AKI [19]. Inversely, inhibition of this pump by drugs underlies the mechanism of their toxicity, including hyperkalemia [32] [33]. These previous reports prompted us to determine whether renal Na^+-K^+-ATPase governs a degree of rhabdomyolysis-induced AKI. Using a murine model of CS, we found that: (i) Na^+-K^+-ATPase expression levels rapidly declined from 6 hours post-challenge; and (ii) there was an inverse correlation between plasma potassium and renal Na^+-K^+-ATPase, hence suggesting a definitive role of this pump in potassium handling under CS.

The forced restoration of Na^+-K^+-ATPase activation may be useful for determining its physiological function in our CS model. Thus, we attempted to elucidate the mole-

cular mechanism whereby renal Na^+-K^+-ATPase activity is impaired under such an AKI-associated pathological condition. Our attention was paid to the dynamism of TGF-β, because of the following reasons: (i) TGF-β is up-regulated in a rat model of glycerol-induced AKI [34] [35]; (ii) this cytokine can inhibit production and activation of Na^+-K^+-ATPase *in vitro* [25] [36]; and (iii) renal phenotype of AKI (such as repair or fibronesis) depends on local TGF-β expression levels [37] [38]. In our murine model, TGF-β expression levels became higher, especially around 24 hours post-challenge, as evidenced by ELISA. In this time-point, TGF-β was detected in the interstitial cells (not sown), as reported in a rat model of glycerol nephropathy [37].

One of the most highlighted findings is that the lowered activity of Na^+-K^+-ATPase is restored by TGF-β neutralization. When anti-TGF-β polyclonal antibody was injected in mice post-glycerol challenge, renal Na^+-K^+-ATPase staining was significantly enhanced. Of interest, this was concomitant with the significant decrease in plasma potassium level. Thus, we predict that interstitial cell-secreted TGF-β is, in part, responsible for the enhancement of hyperkalemia, via inhibiting Na^+-K^+-ATPase activity in renal tubules. The effect of anti-TGF-β antibody was significant, but 'marginal' on hyperkalemia, thus implying the possible involvement of "other" mechanism in renal potassium metabolism. Pro-inflammatory cytokines (such as TNF-α and IL-1β) are known to be up-regulated during glycerol-induced AKI in rats [39] [40] [41]. Notably, these pro-inflammatory cytokines are able to suppress production of Na^+-K^+-ATPase in a culture of renal tubular epithelium [42]. Thus, we predict that not only TGF-β but also pro-inflammatory cytokines (such as TNF-α) likely induce or accelerate hyperkalemia.

We finally discuss the possible functions of TGF-β during AKI. TGF-β induces apoptosis and growth arrest in renal tubular cells [43] [44]. TGF-β is also important for enhancing collagen synthesis in renal epithelium [45]. Of note, TGF-β plays a key role in epithelial-to-mesenchymal transition [46], resulting in a decrease in Na^+-K^+-ATPase production [36], and all of these events contribute to progression of AKI-to-chronic kidney disease. These data suggest a beneficial effect of anti-TGF-β antibody on AKI. Indeed, there was a tendency to show that TGF-β neutralization enhanced a recovery from the renal dysfunction in our mouse model. In addition, anti-TGF-β antibody or chemical peptides are shown to suppress ischemic AKI in animals [47] [48]. The potential clinical application of TGF-β-antagonists in the attenuation of AKIs warrants further attention.

In summary, TGF-β-induced decrease in Na^+-K^+-ATPase activity is, at least in part, contributable for hyperkalemia soon after rhabdomyolysis. Based on the present results, we conclude that glycerol-injected mouse can be a conceptual model to elucidate the molecular mechanism of hyperkalemia during CS. Maintenance or re-induction of Na^+-K^+-ATPase may be a pharmacological target for inhibiting AKI-based lethal events. Our model may be useful for development of anti-hyperkalemic agents (including cytokine-antagonists). We are now under the process of screening candidate drugs, with a focus on roles of growth factors.

5. Conclusion

Previous reports describe that a loss in renal Na^+-K^+ ATPase activity is responsible for hyperkalemia. Using a mouse model of crush syndrome, we demonstrated that an increase in renal TGF-β levels contributes to the loss in Na^+-K^+ ATPase activity in the tubules of affected kidney. Indeed, anti-TGF-β antibody restored the loss in this pomp enzyme, leading to attenuation of hyperkalemia in mice. Thus, we conclude that endogenous TGF-β can be a pharmacological target to suppress hyperkalemia during rhabdomyolysis-induced AKI.

Acknowledgements

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Abbreviations

TGF-β, transforming growth factor-β; CS, Crush syndrome; AKI, Acute kidney injury; BUN, blood urea nitrogen; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.