Scientific Research Publishing

Pharmacology & Pharmacy, 2018, 9, 547-558
http://www.scirp.org/journal/pp
ISSN Online: 2157-9431
ISSN Print: 2157-9423

TNF Type-I Receptor Inhibitor, R-7050 Attenuates Acute Kidney Injury in a Mouse Model of Crush Syndrome

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How to cite this paper: Mizuno, S., Osaki, E. and Ohnishi, H. (2018) TNF Type-I Receptor Inhibitor, R-7050 Attenuates Acute Kidney Injury in a Mouse Model of Crush Syndrome. *Pharmacology & Pharmacy*, **9**, 547-558.
https://doi.org/10.4236/pp.2018.912043

Received: December 6, 2018
Accepted: December 26, 2018
Published: December 29, 2018

Abstract

Crush syndrome (CS) is caused by severe and extensive muscular skeletal damages, and acute kidney injury (AKI) with hyperkalemia is one of the most lethal factors of this syndrome. Especially under natural disasters of earthquake, many persons die due to AKI and hyperkalemia-induced cardiac arrest, but there has been no pathogenesis-based drugs for preventing CS-induced AKI. Pro-inflammatory cytokines, such as TNF-α and IL-6, play a critical role for induction of AKI during CS development. Glycerol-injected mice are used as an experimental tool for reflecting pathological events of human CS. Using this popular model, we provide evidence to show that TNF type-I receptor (TNFR1) inhibitor, R-7050 significantly attenuates the onset of AKI after the muscular destruction. In this process, R-7050 treatment suppressed the NF-κB activation in the affected kidney, and this was associated with a decrease in blood IL-6, a downstream target of NF-κB. As a result, renal tubular apoptosis became milder in the R-7050-treated CS mice. These findings suggest that induction of IL-6 via sequential events of TNF-α → TNFR1 → NF-κB is contributable for renal tubular apoptosis, a histological hallmark of AKI. Thus, TNFR1-selective inhibition can be a pharmacological strategy to attenuate the onset of AKI immediately after the clinical manifestation of rhabdomyolysis.

Keywords

AKI, Hyperkalemia, IL-6, Rhabdomyolysis, TNF-α

1. Introduction

Crush syndrome (CS) is clinically characterized by muscular injury and subse-

quent acute kidney injury (AKI), associated with an increase in plasma potassium levels [1]. These pathological conditions are caused by muscular traumatic damages, as noted in traffic accident or natural disasters of earthquake. The clinical findings are induced by destruction of muscle tissues and leakage of the contents of myocytes into circulation. Myoglobin is toxic to renal epithelial cells [1], and occlusion of 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 [2]. 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 [3]. In addition, a total of 149 patients (8.2%) were diagnosed with CS in Wenchuan earthquake of China in 2008 [4].

Hyperkalemia is defined as a serum potassium concentration greater than approximately 5.0 - 5.5 mEq/L in adults. This symptom can be a lethal factor of AKI, because higher levels of potassium often induce cardiac arrhythmia and arrest [1]. Needless to say, renal tissue plays a major role in regulating plasma potassium levels through excretion of potassium from distal tubules into urine. Thus, it is important to elucidate an intrinsic mechanism to initiate and promote intractable hyperkalemia during AKI development. Several lines of evidence indicate that endogenous TNF-α plays a key role for the onset and progression of AKI, caused by various stresses or injuries [5] [6] [7]. Of importance, TNF-α blocking antibody suppressed the pathological conditions in a rat model of CS-inducing AKI [8], thus delineating a key role of endogenous TNF-α in pathogenesis of AKI, especially after severe muscular damage.

There is now emerging evidence to show that ligand TNF-α can utilize type-I receptor (TNFR1) or Type-II receptor (TNFR2), and pathological phenotype depends on TNFR1 or TNFR2 signaling cascades [9] [10]. Recently, regulatory T cells (*i.e.*, Treg cells) are shown to be necessary for muscular regeneration [11] and renal repairs [12]. With regard to this, TNF-α-TNFR2 signaling pathway plays a central role for Treg cell activation and expansion [13], thus suggesting a contraindication of TNFR2 signaling arrest, due to the possible repair arrest or retardation. Thus, we hypothesize that TNFR1-selective inhibitors, rather than TNFR2 antagonists, may be recommendable for prevention or attenuation of CS-associated AKI, but it remains unclear which TNFR1 or TNFR2 pathway is critically involved in the progression of AKI with hyperkalemia post-CS stages.

To test our hypothesis, we used a TNFR1-selective inhibitor for an animal model of CS. Glycerol-injected rodent is now used as a popular model of CS-induced AKI [14] [15]. R-7050 is a small chemical compound that selectively inhibits TNFR1 signaling pathway [16]. Using this agent in the murine model, we provide evidence to show that R-7050 is effective for preventing AKI during CS via attenuation of local inflammation. This is, to our knowledge, the first report to delineate the usefulness of TNFR1 intracellular inhibition for inhibiting

AKI post-rhabdomyolytic stage.

2. Materials and Methods

2.1. Preparation of a Murine Model of CS with AKI

Specific pathogen-free ICR mice (6 - 8 weeks old, female) were purchased from SLC (Hamamatsu, Japan), because female mice in this strain were free of fighting when they were housed in groups (3 mice per cage) [15]. We induced muscular injury-induced AKI in mice, as reported [15]. In brief, mice were treated with 50%-glycerol (in distilled water) at a dose of 10 ml/kg (i.m.). To determine the natural course of post-traumatic AKI, 30 mice were subjected to autopsy at 0, 6, 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 [17]. All animal experiments were carried out according to the Guideline for Experimental Animal Care issued by the Prime Minister's Office of Japan and approved by the Committee on Animal Experimentation of Osaka University School of Medicine.

2.2. Blood Chemistry

To detect the rhabdomyolysis in mice, plasma creatinine kinase (CK) levels were determined with a commercial kit (test Wako CK, Wako Pure Chem., Osaka, Japan) [15]. To assess the loss of renal functions, blood urea nitrogen (BUN) and creatinine (Cre) levels were measured, using kits (urea nitrogen-B test Wako and creatinine test Wako, WakoPure Chem.) [17]. Plasma potassium levels were determined, using a dry-chemistry kit (Fuji Drychem 800 V, Fuji, Tokyo, Japan), as reported [15].

2.3. Histology and Morphological Scores

At the scheduled necropsy, renal tissues 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 methyl green for histological examination. To detect apoptosis, an *in situ* end-labeling method was applied on renal sections, using a kit (ApoTag; Intergen, Purchase, NY) [17]. Renal tubular apoptosis was checked in >15 chosen × 200 fields, and its degrees were expressed as an average of TUNEL-positive cells per this magnification field, as reported [18].

2.4. Enzyme-Linked Immunosorvent Assay (ELISA)

Plasma TNF-α and IL-6 levels were measured at 0, 12, 24 and 36 hours post-glycerol challenge, using a commercial kit (R & D systems, Minneapolis, MN, USA), as reported [19]. Renal tissues were homogenized and active NF-κB levels in the renal extraction were measured, using the TransAM NF-κB kit (Active Motif, Rixensart, Belgium) [17].

2.5. R-7050 Treatment in Mice

R-7050 (Tocris, Bristol, UK) is a synthetic agent to inhibit assembly of TRADD to TNFR1 intracellular domain [16] and improves neural function post-intracerebral hemorrhage in mice [20]. To evaluate the role of TNFR1 pathway in AKI progression during CS, we administered R-7050 (10 mg/kg, i.p.), based on the previous report [20], or saline (vehicle control), into mice at 0.5 and 12 hours post-glycerol challenge. All mice were sacrificed at 24 hours after the start of this treatment. Blood chemistry and histological examination were performed on R-7050-treated and control mice, as described above.

2.6. Statistical Analysis

All data were expressed as mean ± standard deviation (SD). A Student's *t*-test was used to compare group means, with $P < 0.05$ accepted as significant.

3. Results

3.1. Characterization of Rhabdomyolysis-Induced AKI in Mice Post-Glycerol Challenge

We first characterized the time course of AKI in mice, based on the biochemical analysis. Indeed, plasma CK levels increased within 12 hours post-glycerol injection (**Figure 1(A)**). Consistently with the muscular damage, there was an apparent increase in blood potassium levels, especially at 24 hours post-muscular injury (**Figure 1(B)**), thus suggesting the clinical onset of rhabdomyolysis and hyperkalemia, as reported [14] [15].

In this pathological process, BUN and plasma Cre levels increased in mice between 12 and 24 hours post-glycerol injection (**Figure 1(C)** and **Figure 1(D)**). These findings suggest the successful preparation of murine model for mimicking rhabdomyolysis-associated AKI conditions, as observed in humans [21].

3.2. Changes in Blood TNF-α and IL-6 Levels during AKI

Inflammatory cytokines, such as TNF-α and IL-6, are known to be a key mediator(s) for induction of AKI under pathological conditions [10] [22]. In our murine model, plasma TNF-α levels markedly increased, especially from 12 to 24 hours after the glycerol injection in mice (**Figure 2(A)**). Likewise, plasma IL-6 levels were markedly elevated throughout the present experimental protocol, with a peak value of IL-6 (24 hours: 309.7 ± 52.1 pg/ml) (**Figure 2(B)**). Consistently with BUN levels, plasma TNF-α and IL-6 levels became lower in the recovery phase of 36 hours. Thus, we hypothesized that endogenous TNF-α-IL-6 axis play a central role for AKI progression in our AKI model.

3.3. Effect of R-7050 on Rhabdomyolysis-Induced AKI

To test this hypothesis, we injected a chemical agent of R-7050, an inhibitor to arrest TNFR1-specific signaling cascade [16]. Indeed, treatment of mice with R-7050 failed to suppress rhabdomyolysis, because there was no significant

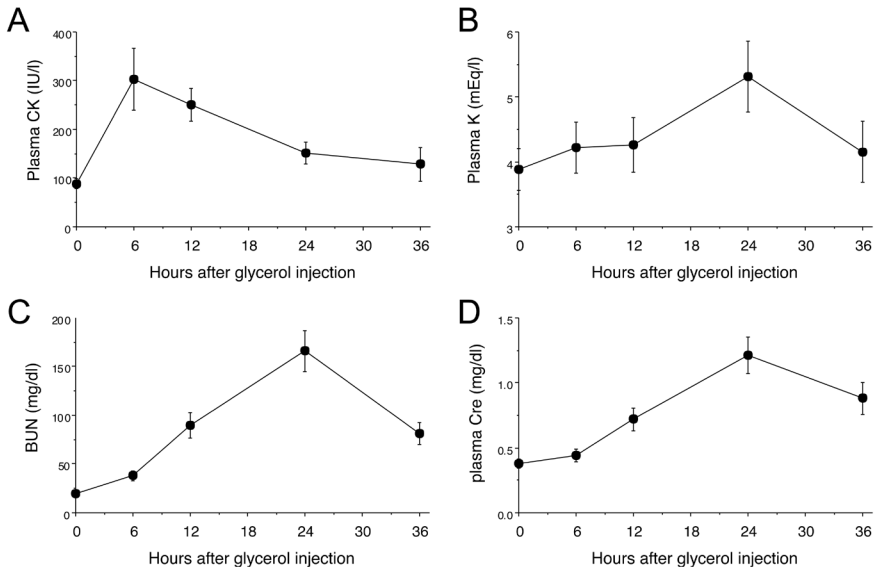

Figure 1. Characterization of muscular and renal injuries after 50%-glycerol injection in mice. Changes in plasma creatinine kinase (CK) (A), potassium (B), blood urea nitrogen (BUN) (C) and creatinine (Cre) (D). Data are shown as mean ± SD (n = 6).

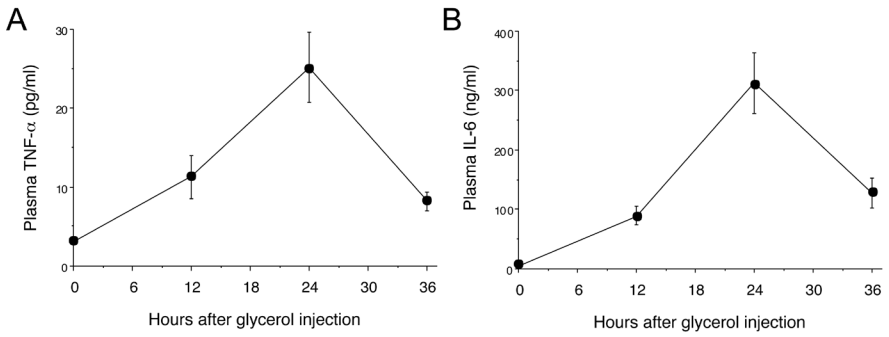

Figure 2. Time-course of plasma TNF-α (A) and IL-6 levels (B) in glycerol-induced mice. Data are shown as mean ± SD (n = 6).

difference in CK values between control and tested groups at 24 hours post-challenge (vehicle: 193.1 ± 35.1 IU/l vs. R-7050: 212.8 ± 38.1 IU/l, p = 0.43). However, this TNFR1 inhibitor significantly suppressed the elevations of BUN and plasma Cre levels (**Figure 3(A)** and **Figure 3(B)**). Consistently, R-7050 was shown to improve the degree of hyperkalemia (**Figure 3(C)**), as evidenced by plasma potassium levels. Furthermore, R-7050 suppressed the leukocyte infiltration round the renal tubules in our model (not shown). These findings indicate that treatment of R-7050 is useful for attenuating AKI-associated events without changing the muscular damages.

3.4. Attenuation of Apoptosis in Renal Tubules by R-7050

TNFR1 signaling is causative for inducing apoptotic cell death [9] [10]. Thus, we next examined whether R-7050 modified the possible apoptotic event in our model of AKI. Immunohistochemistry of TUNEL staining revealed that tubular

apoptosis became evident in the renal cortex at 24 hours post-glycerol challenge (Figure 4(A)). In contrast, early treatment of R-7050 suppressed the onset of tubular apoptosis (Figure 4(B)). Indeed, there was a significant difference in the number of apoptotic cells between saline- and R-7050-treatedgroups at this time-point (vehicle: 5.85 ± 0.64 vs. R-7050: 3.22 ± 0.38, $p < 0.01$) (Figure 4(C)). Thus, inhibition of renal apoptosis may be responsible for the beneficial effect of R-7050 in our AKI model.

3.5. Effect of R-7050 on Cytokines and NF-kB during AKI in Mice

We finally checked the effect of R-7050 on inflammatory events during AKI. Treatment with R-7050 did not alter the blood levels of TNF-α at 24-hours post-glycerol challenge (Figure 5(A)). However, R-7050 significantly suppressed the renal NF-κB activation, as determined by ELISA (Figure 5(B)). Consistently, this inhibitor significantly suppressed the elevation of plasma IL-6 levels at 24 hours post-glycerol challenge (Figure 5(C)).

4. Discussion

Several lines of evidence indicate that TNF-α is a key mediator for the pathogenesis

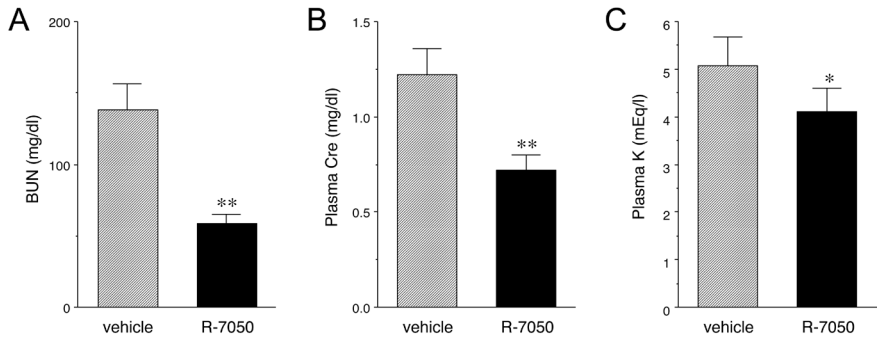

Figure 3. Effect of TNFR1-selective inhibitor (R-7050) on glycerol-induced AKI in mice. Preventive role of R-7050 in blood levels of BUN (A), Cre (B) and potassium (C) (mean ± SD, n = 6). Statistical analysis: *, $p < 0.05$ or **, $p < 0.01$ vs. vehicle group.

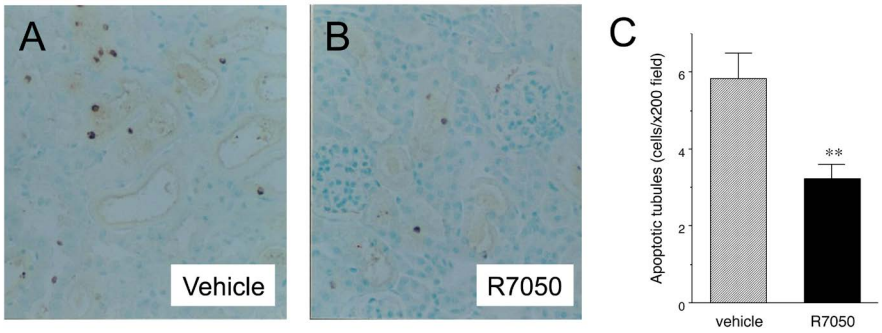

Figure 4. Typical findings of renal tubular apoptosis in control group (A) and R-7050-treated group (B) of mice at 24 hours post-glycerol challenge (mean ± SD, n = 6). Apoptotic score are expressed as an average of TUNEL-positive tubular cells per x200 field (C) (mean ± SD, n = 6). Statistical analysis: **$p < 0.01$ vs. vehicle group.

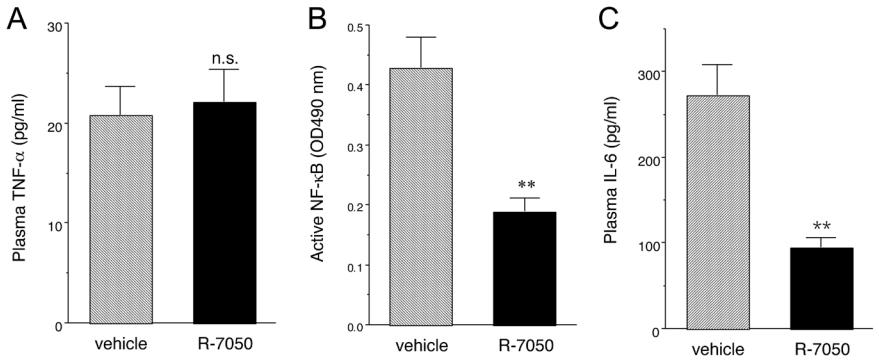

Figure 5. Effects of R-7050 on plasma TNF-*α* (A), renal NF-κB (B) and plasma IL-6 levels (C) of mice at 24 hours post-glycerol injection. Data are shown as mean ± SD (n = 6). Statistical analysis: **, $p < 0.01$ vs. vehicle group.

of AKI, caused by various types of renal stresses [5] [6] [7], including CS-like conditions [8]. In this process, soluble type TNF-*α* can utilize both TNFR1 and TNFR2, and the final pathological outcomes depend on TNFR1 or TNFR2 signaling cascades [9] [10]. However, it is still unclear which TNFR1 or TNFR2 is responsible for the onset of AKI post-rhabdomyolysis. Using the murine model of human CS, we found that TNFR1 downstream pathway is critical for the onset of AKI, at least in part, through inducing renal apoptosis and inflammation. We will discuss the pharmacological inhibition of TNFR1 for anti-AKI therapy post-CS status.

In our model, a peak of plasma TNF-*α* levels predicted the degree of AKI, as evaluated by BUN levels, being similar to other types of AKI models [23] [24]. Furthermore, clinical reports described that blood TNF-*α* level can be a biomarker to predict AKI in human patients [25]. With regard to this, ligand TNF-*α* transduces several types of biological functions through two types of receptors. One is a type-I receptor (*i.e.*, TNFR1) that leads to onset of apoptosis, and another is type-II receptor (*i.e.,* TNFR2) for cell survival [10]. For example, TNFR1 is necessary for septic AKI [26], but is protective for tunicamycin-induced renal toxicity [27]. Of interest, TNFR2 is critical for onset of glomerulonephritis [28], hence suggesting a distinct role(s) of these receptors among several types of AKIs. At least in our AKI model, TNFR1-selective inhibitor attenuated the renal phenotypes, caused by rhabdomyolysis.

How R-7051 produced the preventive effect against AKI should be discussed. TNF-*α* induces apoptosis in various types of cells via TNFR1-transduced signaling [29]. In this process, intracellular domain of TNFR1 interacts with, adaptor molecule, TRADD, followed by sequent recruitment of FADD and caspase-8 (*i.e.*, complex-II or DISC formation). Under such a caspase-8-activated condition, TNF-*α*-stimulated cells enter apoptotic phases [9] [10]. Of note, R-7050 is permeable and is incorporated into cells to inhibit molecular assembly of TRADD (or of RIP1) with TNFR1 C-terminal (*i.e.*, intracellular) tails [16]. Indeed, this drug is known to delay the progression of renal tubular damage in a

murine model of crystal nephropathy, and this result is also associated with anti-apoptotic outcomes *in vivo* [30].

It is now widely accepted that NF-κB inactivation can be an anti-inflammatory strategy in numerous organs. R-7050 was originally developed by pharmaceutical researchers, based on its inhibitory effect on NF-κB-targeted molecules, such as ICAM-1 [16]. Renal NF-κB activation is responsible for AKI, and this pathway seems to be a target as an anti-AKI therapy, even after rhabdomyolysis [31]. Thus, we next evaluated the anti-inflammatory effect of R-7050, with a focus on NF-κB activation. Indeed, early treatment of glycerol-injected mice with R-7050 resulted in the attenuation of renal NF-κB activation, without changing blood TNF-*α* levels. Furthermore, R-7050 did not modify the muscular damages. Thus, R-7050 was considered to be effective for reducing NF-κB activation under persistent rhabdomyolysis with systemic storm of TNF-*α*.

It is important to discuss the potential downstream target of NF-κB in AKI-related pathogenic events during CS. In addition to TNF-*α*, IL-6 is known to be a key molecule to accelerate AKI [32]. Indeed, anti-IL-6 antibody attenuated the several types of AKI in rodents [33] [34]. Using a mouse model of acute hepatitis, Yamada *et al.* demonstrated that IL-6 production is upregulated, in response to TNFR1-NF-κB pathways [35]. In our AKI model, R-7050 reduced not only blood IL-6 levels but also renal NF-κB activation, as seen in the hippocampus of rats after R-7050 treatment [36]. Of note, IL-6-induced neutrophil infiltration is contributable for renal inflammation and apoptosis [33]. In our

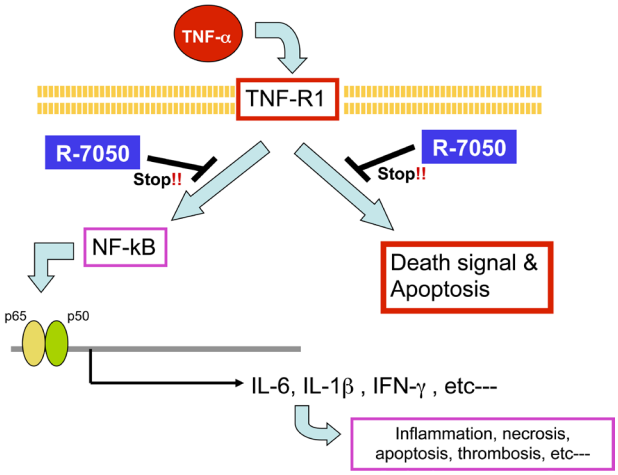

Figure 6. During progression of CS, TNF-*α* predominantly utilizes TNFR1 receptor to activate NF-κB → IL-6 axis, followed by the onset and progression of AKI. Actually, TNF-*α*-derived TNFR1 signaling pathway enhances the nuclear location of NF-κB, a key transcriptional switch for induction of inflammatory cytokines, such as IL-6 and IL-1β. These downstream cytokines elicit inflammation, thrombosis, necrosis and apoptosis in the affected kidney. Furthermore, TNF-*α* directly induces apoptotic cell death as a death ligand. Thus, "intracellular" arrest of TNFR1 downstream pathway by chemical compounds, including R-7050, can be a reasonable strategy for preventing AKI, even after the onset of rhabdomyolysis.

model, R-7050 repressed leukocyte infiltration, associated with the decreased IL-6 levels. In a viewpoint of pathogenesis, it is possible that an increase in blood IL-6 via TNFR1 → NF-κB axis contributes to enhancement of AKI during CS. Thus, we predict that TNFR1 inhibitors, such as R-7050, are available as an "anti-IL-6 therapy" for preventing AKI during CS. TNFR1 inhibition also leads to interception of TNF-*α*-primed death signaling cascades, and such a dual function of this chemical inhibitor is advantageous for minimizing AKI during CS (**Figure 6**).

5. Conclusion

Based on the present data, we conclude that TNF-*α*-TNFR1 axis is one of the most key targets to inhibit progression of AKI after the onset of rhabdomyolysis. TNFR1 directly induces apoptotic cell death in renal tubules. Furthermore, TNFR1-mediated NF-κB activation leads to renal inflammation and destruction, in part, via induction of hyper-IL-6-nemia. In these pathogenic spirals, R-7050 suppresses apoptotic events, possibly, via inhibiting binding of TRADD (or RIP3) to TNFR1 intracellular domains. The pharmacological approach to arrest TNFR1 intracellular cascade would open up an avenue to treat AKI-associated pathological events, including hyperkalemia.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (16590312 and 15K08402 to S.M.) from the Japanese Society for the Promotion of Science.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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