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ORIGINAL ARTICLE

Kinin B_2 receptor does not exert renoprotective effects on mice with glycerol-induced rhabdomyolysis

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Abstract

AIM: To investigate a potential protective role of the kinin B_2 receptor in a glycerol-induced rhabdomyolysis mouse model.

METHODS: We separated 28 C57Bl/6 male mice into 4 groups: untreated WT animals, untreated B_2 knockout mice, glycerol-treated WT and glycerol-treated B_2 knockout mice. Glycerol-treated animals received one intramuscular injections of glycerol solution (50% v/v, 7 mL/kg). After 48 h, urine and blood samples were collected to measure creatinine and urea levels. Additionally, kidney samples were extracted for histological evaluation, and the mRNA expression levels of kinin B1 and B2 receptors and inflammatory mediators were measured by real-time polymerase chain reaction.

RESULTS: Serum creatinine and urea levels showed differences between untreated wild-type and glycerol-treated wild-type mice (0.66 ± 0.04 *vs* 2.61 ± 0.53 mg/dL, $P < 0.01$; and 33.51 ± 2.08 *vs* 330.2 ± 77.7 mg/dL, $P < 0.005$), and between untreated B_2 knockout mice and glycerol-treated knockout mice (0.56 ± 0.03 *vs* 2.23 ± 0.87 mg/dL, $P < 0.05$; and 42.49 ± 3.2 *vs* 327.2 ± 58.4 mg/dL, $P < 0.01$), but there was no difference between the glycerol-treated wild-type and glycerol-treated knockout mice. Glycerol was able to induce a striking increase in kinin B_2 receptor expression (> 30 times, 31.34 ± 8.9) in kidney. Animals injected with glycerol had a higher degree of tubular injury than untreated animals. Wild-type and knockout mice treated with glycerol intramuscularly present kidney injury, with impairment in renal function. However, B_2 knockout mice treated with glycerol did not show a different phenotype regarding kidney injury markers, when compared to the wild-type glycerol-treated group.

CONCLUSION: We conclude that the kinin B_2 receptor

does not have a protective role in renal injury.

Key words: Kinins; acute kidney injury; Animal models; Rhabdomyolysis; Skeletal muscle

Core tip: In this work we are showing that glycerol-treated animals experienced impairment in renal function. Furthermore, we worked with kinin B_2 receptor knockout mice and our results suggest that kinin B_2 receptor does not exert renoprotective effects in this rhabdomyolysis model. In addition, we are presenting results of kidney expressions and we investigated several candidates that can participate in the kidney injury induced by glycerol.

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INTRODUCTION

Acute kidney injury (AKI) is a common life-threatening disease that places a heavy burden on the health system[1]. It was reported that the mortality rate for this disease ranges from 10% to 80%. Other studies suggest that AKI may be a step in the progression toward chronic kidney disease[2], in humans and animals[3].

The main factors that predispose patients to AKI include hemodynamic instability, hypovolemia, hypoxia, ischemia and reperfusion (I/R), and burns, among others[4]. There are several models used to study this disease; one of them is the glycerol-induced rhabdomyolysis model in which AKI occurs after muscle injury. In this nephrotoxic experimental model, the waste products from metabolism, such as enzymes and other molecules (*e.g.*, uric acid and myoglobin), cause kidney injury to the proximal and distal tubules[4]. Such injuries cause an abrupt (in a matter of hours or days) decline in renal function.

In renal inflammatory conditions such as AKI, the kallikrein-kinin system (KKS) plays an important role in glucose homeostasis[5,6]. This system is also involved in kidney inflammatory and vasodilation processes[7], which are directly involved in the inflammatory response mechanism of AKI. The AKI inflammatory response involves induction of local vascular ischemia, hypoxia and tubular injury[4]. KKS exerts its actions by activating two receptors: B_1 (B_1R) and B_2 (B_2R)[8]. The activation of B_1R is inducible and occurs under pathological conditions such as in ischemia[9], while B_2R is constitutively active under normal physiological circumstances[8]. Furthermore, some authors[10] have shown that there are fewer B_2R in rat nephrons affected by renal disease. It is well established that kinins are rapidly generated after tissue injury and that they have a central role in the development and maintenance of inflammatory processes, whether they are acute or chronic[1].

There is a renal KKS[11] that can respond in a more specific way to AKI. Another system that could be involved in the mechanism of AKI, as observed in the glycerol-induced rhabdomyolysis model, is the renin-angiotensin system (RAS). A drop in blood pressure, typically observed during a hypovolemic state, will activate this system and induce renal perfusion[12]. It is important to note that, the renal KKS can activate RAS[13]. In a recent study from our laboratory[14], we showed that there is a relationship between the genetic polymorphisms of ACE and B_2R, where the former can modulate the kinins in transplanted kidney patients. In other animal models of renal injury, it has been shown that B_2 has a possible protective role[15], while others have shown that it has a deleterious role[16].

It is described in the literature that the B_2R are in all portions of the nephron, except in podocytes of human kidney[17]. Moreover, it is described that the NO levels fall in rhabdomyolysis[4], and that the B_2R activation is related with NO production[18]. Thus, the purpose of this study is to investigate the role of B_2R in the kidney in an animal model of glycerol-induced rhabdomyolysis.

MATERIALS AND METHODS

Experimental design

This study began only with approval of the ethics committee of the Federal University of Sao Paulo (UNIFESP, nº 0300/11), and experiments were performed in accordance with the guidelines established by the Brazilian College for Animal Experimentation. The animals were provided by the Center for Development of Experimental Medicine and Biology (CEDEME) at UNIFESP. Twenty-eight male 3-month-old C57Bl/6 mice were used. Animals were randomly divided into 4 groups: WT (n = 5), glycerol WT (GWT, n = 9), B_2KO (n = 6), and glycerol B_2KO (GB$_2$KO, n = 8). All animals were placed in individual cages (Alesco, Brazil) in an environment with controlled temperature (21 ℃), a light/dark cycle of 12 h for one week and water and standard chow *ad libitum*.

The WT and B_2KO groups were control groups and received no treatment. The animals in the treated groups were deprived of water for 18 h to better evidence of AKI. Animals of both treatment groups (GWT and GB$_2$KO) were then slightly sedated with an intraperitoneal injection of ketamine-xylazine solution (150 μL, Vetnil, Brazil; at 10 μL/g, Vetbrands Brazil, Brazil), and received an i.m. injection of glycerol solution (50% v/v, Merk, Brazil, 7 mL/kg), with half a dose in each gastrocnemius muscle. Then, animals were placed back in their cages in a heated environment until recovery.

The urine samples were placed in 2 mL tubes and stored at -20 ℃ until analysis. Then, 48 h after the injec-

tions, the animals were anesthetized with *i.p.* ketamine-xylazine solution (300 μL at 10 μL/g), and blood samples were collected by intracardiac puncture into non-heparinized 1.5 mL tubes. These samples were incubated for approximately 20 min at room temperature and were then centrifuged at 4 ℃ at 4000 rpm for 10 min. The serum was collected and stored in a 1.5 mL tube at -20 ℃ until analysis. After blood sample collection, the animals were sacrificed by cervical dislocation, and each kidney was removed, weighed and transversally cut. One piece was placed in one 2 mL tube and immediately immersed in liquid nitrogen and then stored at -80 ℃ until analysis, while the other half was placed in a 2 mL tube with 10% buffered formaldehyde solution for 24 h, then placed into a new tube with a 70% ethanol solution and stored until sectioning.

Renal function analysis

For renal function analysis, we measured serum creatinine (S_{Cr}) levels and urine creatinine levels (U_{Cr}) according to the method described by Jaffé with slight modifications[19]. Briefly, we deproteinized the samples by adding 100 μL of each serum sample to a 1.5 mL tube containing 200 μL of 1.84 % H_2SO_4 and 300 μL of sodium tungstate and thoroughly mixed the resulting solution. The tubes were maintained at room temperature for 15 min and then centrifuged for 10 min at 4000 rpm at room temperature. The supernatant was then collected and placed in another 1.5 mL tube. Then, 5.28 mL of picric acid was mixed with 1.32 mL of a 10% NaOH solution in a 14 mL falcon tube. Next, 100 μL of picric acid (Labtest Diagnostica SA, Brazil) was added to 63 wells of a 96-well flat-bottomed ELISA microplate (Cral plast, Brazil), followed by pipetting and mixing of 200 μL of Milliq H_2O (Millipore, United States) in triplicate for blank samples, 200 μL of standard protein at 5 mg/mL in triplicate, and 200 μL of each deproteinized sample in duplicate. The plate was incubated for 20 min and then read in an EPOCH spectrophotometer (Biotek Instruments Inc., United States) at 450 nm. To measure U_{Cr} levels, we used the same method, but we did not deproteinize the samples. Instead, we diluted the samples (1:125) and multiplied the results by 25. The blood urea nitrogen (BUN) level were measured by colorimetric reaction kit (Labtest diagnostica, Brazil) in spectrophotometer at 600 nm, following the manufacturer's instructions.

Urine collection

Urine was collected by direct punction in bladder, for measure of creatinine and urea levels. The urine was collected in 1.5 mL tubes.

We also quantified the total urine protein (mg/dL) by colorimetric assay at 660 nm using the Pierce Protein Assay Kit (Thermo Scientific, United States).

Gene expression quantification

Total RNA was extracted using the TRIzol Reagent method (Invitrogen, United States) from tissues and stored at -80 ℃. The samples were run on 1% agarose gels to evaluate the integrity of the samples, and the samples were then quantified using a nanoDrop (NanoDrop Technologies, Inc., United States). Single-stranded cDNA was synthesized for each sample, using MML-V reverse transcriptase (Promega, United States). The qRT-PCR was carried out using Taqman (Applied Byosystem, United States) probes for GAPDH, B_1R and B_2R gene expression, and SYBR Green Real-time polymerase chain reaction (PCR) (Applied Byosystem, United States) probes for β-actin (sense - 5' CTG GCC TCA CTG TCC ACC TT 3' , antisense - 5' CGG ACT CAT CGT ACT CCT GCT T 3'), TGF-β1 (sense - 5' TTA GGA AGG ACC TGG GTT GG 3' , antisense - 5' AAG TTG GCA TGG TAG CCC TT 3') and IL-1β (sense - 5' AGG AGA ACC AAG CAA CGA CA 3' , antisense - 5' CGT TTT TCC ATC TTC TTC TTT 3') gene expression. The plates were placed in a 7500 real-time PCR system (Applied Biosystems, United States) for reading. The fold change was calculated by taking the $2^{-\Delta CT}$ of each sample and dividing it by the wild type (WT) group average.

Histological analysis

Slices 5 μm thick were cut from the samples stored in 70% ethanol solution and stained with HE. The samples were analyzed using an optical microscope at 200 × magnification. The criteria used to analyze the tubular injury in the samples included epithelium desquamation, cellular debris in the lumen, flattening of epithelium, the presence of cylinders and dilation of the lumen. Based on these criteria, an expert classified and scored the injuries: grade Ⅰ (< 10%), grade Ⅱ (10%-25%), grade Ⅲ (25-50%), and grade Ⅳ (> 50%).

Statistical analysis

The values are expressed as the mean ± SE. Statistical analyses were carried out by one-way ANOVA (analysis of variance) followed by Tukey's *post-hoc* test, and associations were made by the Spearman correlation test. P values < 0.05 were considered statistically significant. The statistical software used for graphs and analysis was GraphPad Prism 5.

RESULTS

Renal function evaluation

The S_{Cr} levels were different between the control wild-type and glycerol, and knockout control group and glycerol, with glycerol groups greater about 4 times than control groups (Figure 1A). There were no significant differences in the U_{cr} levels, despite the wild-type showed about 11 times higher levels than glycerol wild-type group (Figure 1B). The BUN levels were different about 10 times between the control wild-type and glycerol, and about 8 times between control knockout mice and glycerol (Figure 1C). Urinary urea content differed between the control and glycerol groups, about 24 times for wild-type groups and about 33 times for knockout groups

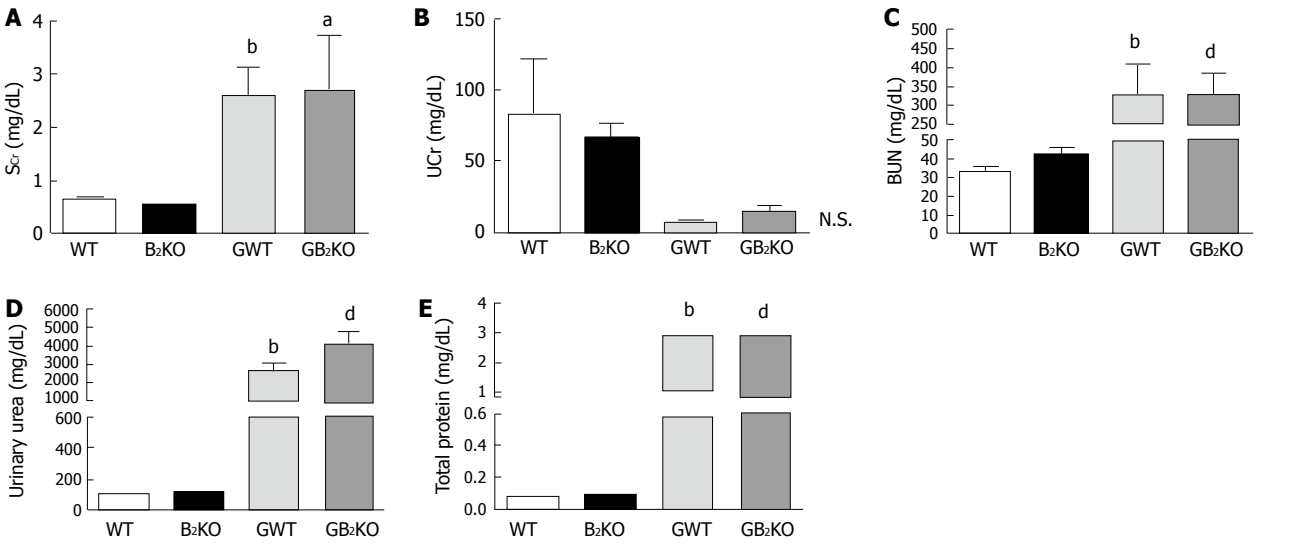

Figure 1 Serum and urinary parameters. A: S_{Cr} levels ($^aP < 0.05$ *vs* B_2KO, $^bP < 0.01$ *vs* WT); B: U_{Cr} levels; C: BUN levels ($^bP < 0.005$ *vs* WT, $^dP < 0.01$ *vs* B_2KO); D: Urinary urea ($^bP < 0.01$ *vs* WT, $^dP < 0.005$ *vs* B_2KO); E: Total urine proteins ($^bP < 0.005$ *vs* WT, $^dP < 0.005$ *vs* B_2KO). WT: Wild type; B_2KO: Kinin B2 receptor knockout mice; GWT: Glycerol wild type; B_2KO: Glycerol kinin B2 receptor knockout mice; BUN: Blood urea nitrogen.

Table 1 Serum and urinary creatinine

Variable	Groups			
	WT	B_2KO	GWT	GB_2KO
S_{Cr}	0.6621 ± 0.041	0.5584 ± 0.027	2.613 ± 0.536[b]	2.233 ± 0.867[a]
U_{Cr}	50.31 ± 29.24	32.80 ± 15.44	4.540 ± 2.137	9.084 ± 3.931

$^aP < 0.05$ *vs* B_2KO; $^bP < 0.01$ *vs* WT. B2KO: Kinin B2 receptor knockout mice; WT: Wild type; GB_2KO: Glycerol kinin B2 receptor knockout mice.

(Figure 1D). The levels of total urine proteins were different between the wild-type and glycerol group (around 42 times), while the difference between knockout and glycerol group was approximately 36 times. There were no difference between wild-type glycerol and knockout glycerol group (Figure 1E).

Gene expression evaluation

The fold changes in B_1R, B_2R, β-actin, IL-1β and TGF-β1 renal expression are shown in Figure 2. Also shown are the associations between these expression levels. The fold change of the B_2R was different between the control and glycerol groups about 30 times (Figure 2A). The B_1R fold change was different between the control and glycerol groups about 14 times, and between glycerol groups almost 2 times (Figure 2B). The associations were not different between the B_1R and B_2R (Figure 2C), but showed difference between B_2R and IL-1β, where greater expression of B_2R results in lower expression of IL-1β (Figure 2D), and between B_1R and TGF-β1 groups, with greater expression of B_1R when the expression of TGF-β1 is lower (Figure 2E).

Histological evaluation and histomorphometry

The histological evaluation and histomorphometry shown in Figure 3 demonstrate that both sets of animals (WT and B_2KO) had a high degree of tubular injury when injected with glycerol (Figure 3F and H), but WT had a higher degree IV compared with B_2KO.

DISCUSSION

It is described that the NO levels fall in rhabdomyolysis[4], and that the B_2R activation is related with NO production[18] we considered investigate the role of kinin B2 receptor in glycerol-induced rhabdomyolysis model.

Our findings demonstrate that the renal expression of B_2R in animals that received glycerol is 30 times greater than that of the controls. Another study showed a 5-fold increase of B_2R expression[15]. These results suggest that B_2R may be involved in the kidney inflammatory process. Interestingly, the peak renal expression of B_2R coincided with a significant reduction in renal function, which could suggest that the renal upregulation of these receptors occurs in response to renal injury.

Renal function was evaluated by measuring serum creatinine and BUN levels. In mice, particularly male C57Bl/6 mice, the normal serum creatinine level (S_{Cr}) is approximately 0.8 ± 0.1 mg/dL[20], which is similar to the values found in the control groups (Table 1). In a recent study[21], the authors showed using the glycerol model in rats, that the peak of renal function impairment occurs between 48 and 72 h, as evidenced by S_{Cr} levels. In our study, we investigated the peak of injury.

Kidney injury in the glycerol model is due to the release of nephrotoxic molecules and proteins (*e.g.*, myoglobin) into the bloodstream, causing damage upon reaching the kidney[4]. The literature describes, using rat and cell culture models[22-27], the deleterious role of this protein, specifically the heme prosthetic group, in the tubular endothelium. This role is associated with the production of reactive oxygen species (ROS) and free radicals in the mitochondria, which initiate lipid peroxidation reactions.

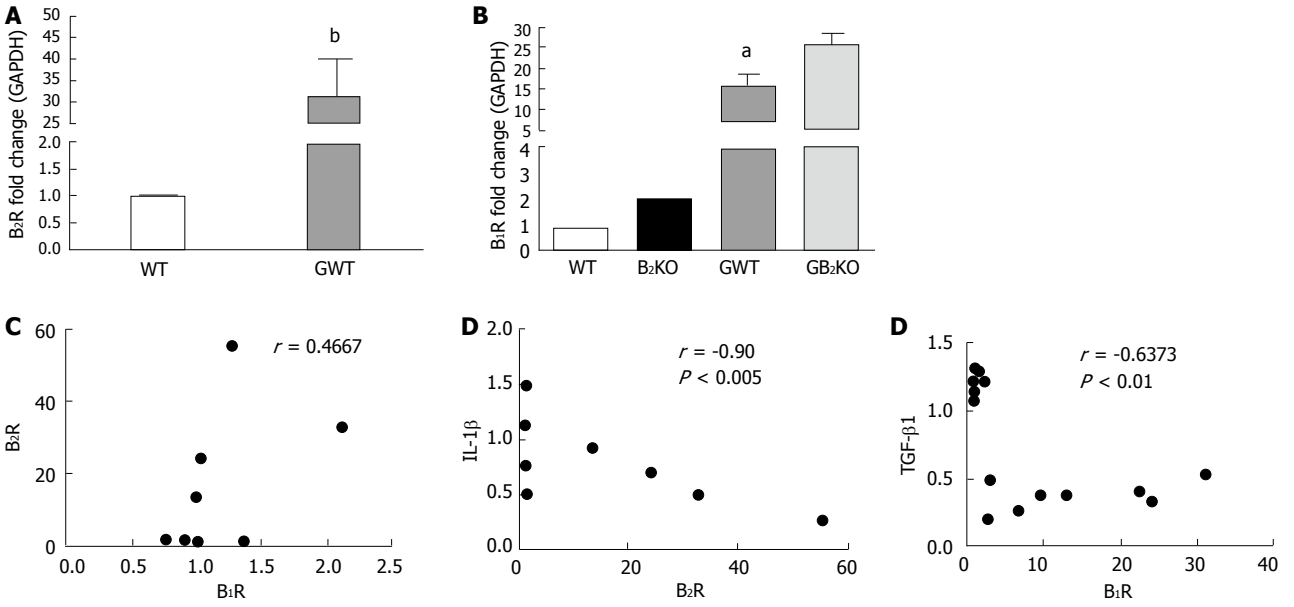

Figure 2 Renal gene expression and association between genes. A: B_2R fold change ($^bP < 0.01$ *vs* WT); B: B_1R fold change ($^aP < 0.05$ *vs* B_2KO); C: Association between B_2R and B_1R; D: Association between IL-1β and B_1R; E: Association between TGF-β1 and B_1R. WT: Wild type; B_2KO: Kinin B2 receptor knockout mice; GWT: Glycerol wild type; GB_2KO: Glycerol kinin B2 receptor knockout mice; BUN: Blood urea nitrogen.

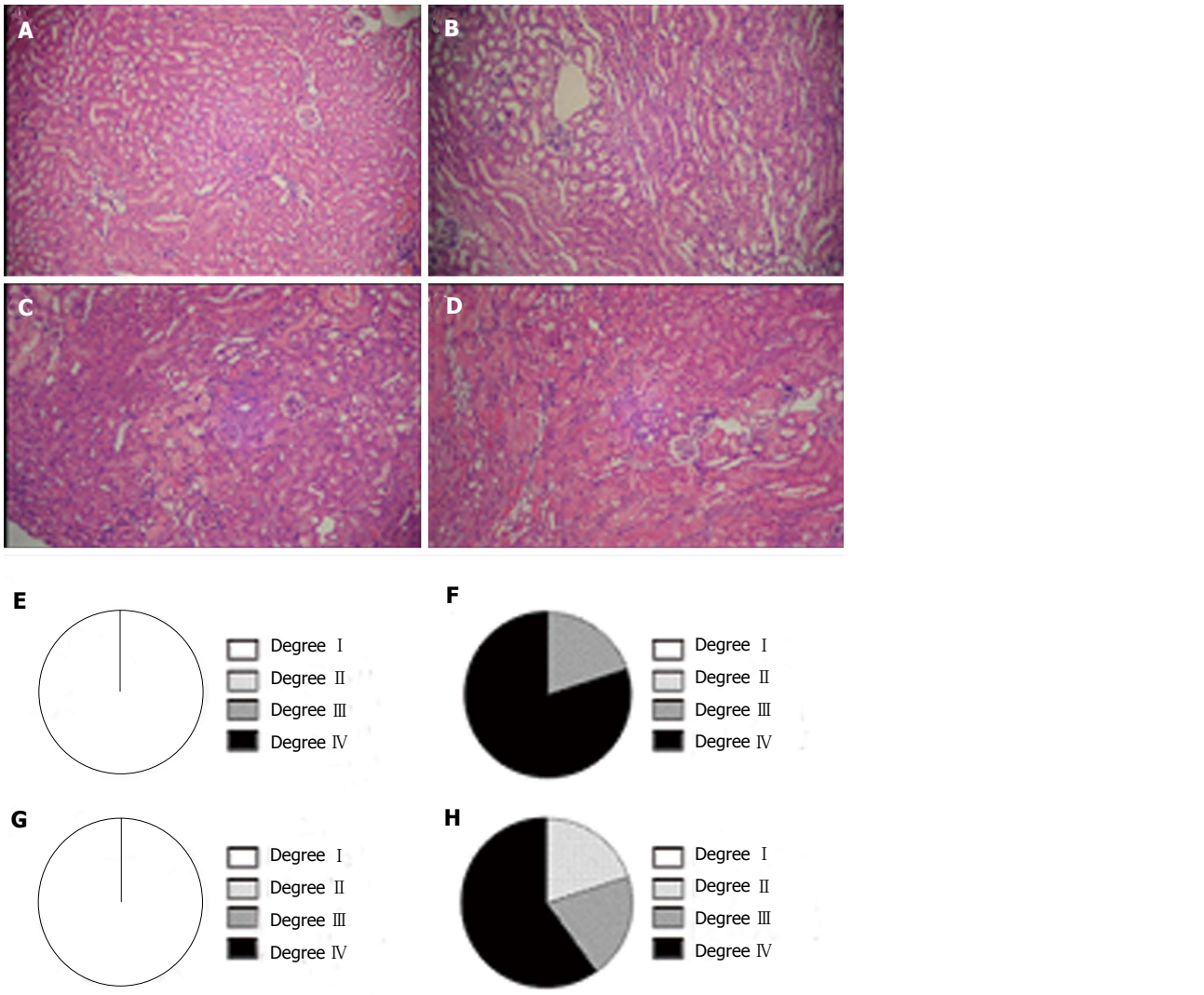

Figure 3 Histological evaluation and Graphs show the degrees of renal injury. A: Degree Ⅰ; B: Degree Ⅱ; C: Degree Ⅲ; D: Degree Ⅳ; E: WT; F: GWT; G: B_2KO; H: GB_2KO. WT: Wild type; B_2KO: Kinin B2 receptor knockout mice; GWT: Glycerol wild type; GB_2KO: Glycerol kinin B2 receptor knockout mice;

It has been shown that human skeletal muscle can survive for up to 3 or 4 h during circulatory ischemia[28]. In other studies[24], the authors showed that in the rat glycerol-induced rhabdomyolysis model, the renal mitochondria were already markedly degenerated 3 h after the glycerol application. Twenty-four hours after treatment, there were clear signs of tubular necrosis (proximal and distal). The authors attributed this to the formation of intra-tubular aggregates. This conclusion suggests that disruption of skeletal muscle can occur approximately 3 h after the glycerol administration.

Recent studies[28-31] show that deletion of B_1R and B_2R exacerbates the renal phenotype in diabetic mouse models, suggesting that both receptors have a protective effect on diabetic nephropathy by suppressing oxidative stress *via* NO and prostaglandins. However, because the absence of one of the receptors causes increased expression of the other, it is difficult to determine the precise function of each receptor[28]. In other models, such as the I/R model[15] the receptors' role was also studied. Blocking B_1R showed an antifibrotic effect, which therefore has a protective effect. Some authors[16] have demonstrated that renal injury by ischemia and reperfusion is significantly increased by B_2R activation and that this activation is related to increased production of ROS, suggesting that B_2R activation is deleterious.

Kinin receptors in renal tissue were studied in the I/R model, where it was shown that both receptors have a protective role in this type of injury[30]. However, a different study[15] found that during I/R in mice, the double knockout showed an extremely high S_{Cr} along with a proinflammatory profile. The renal B_2R expression alternated, beginning with low expression after 4 h, high expression after 24 h, and 48 h after the reperfusion onset, its expression level was similar to that observed at 4 h and then increased to values similar to those observed at 24 h post-reperfusion.

An important difference between the previous two studies[15,24] and ours is that in the other studies, ischemia and reperfusion were induced exclusively in the kidneys, whereas in our work, nephrotoxic kidney injury occurs after induction of primary skeletal muscle injury. Furthermore, in the model we use, there is no time for reperfusion to occur because the peak of injury would be 48 h later, allowing the kidneys to therefore remain in an ischemic state.

Regarding the release of proinflammatory cytokines (*e.g.*, IL-1β) and growth factors with a known profibrotic effect (TGF-β1), some authors[21] have shown that in the rat glycerol model, the mRNA expression levels of IL-1β peak 48 h after treatment, while in our work, the same expression levels were observed in GB_2KO animals, whereas GWT animals showed expression levels similar to those found 24 h post-treatment by these authors. In our study, we found a strong negative association between the expression of B_2R and IL-1β, which may indicate that B_2R does not induce inflammation in the kidneys.

Previous studies show[21] that renal TGF-β1 expression 48 h after *im* glycerol injection in mice is 1.5 times greater than our results indicate. It is important to note that the lineage of the animals used was different and that the endogenous gene studied was also different. Furthermore, in our work, we did not find any association between renal expression of B_2R and TGF-β1 (data not shown). Meanwhile, B_1R and TGF- β1 had a strong negative association.

Another important issue regarding analysis of TGF-β1 expression is the methodological difference between the studies. Although both studies use rodents, rats and mice have differences in several genes, including TGF-β1; the rat version is on chromosome 1, while the murine version is located on chromosome 7.

In conclusion, our results suggest that B_2R does not have a renoprotective role in mice with glycerol-induced rhabdomyolysis.

COMMENTS

Background

Acute kidney injury (AKI) is a common life-threatening disease that places a heavy burden on the health system and may be a step in the progression toward chronic kidney disease. It was reported that the mortality rate for this disease ranges from 10% to 80%, depending on the population studied. According to some authors, prevention is the key to avoiding the morbidity and mortality associated with AKI.

Research frontiers

Kinins is related with several models of inflammatory process in different organs as well in kidney. However, there is a lack of acknowledgments about kinins and rhabdomyolysis. The kinins are related with pathologies as well in physiology process. However, the absence of B2 receptor can induces the expression of the kinin B1 receptor and it difficult the determination of the precise function of each receptor. In this study, the authors demonstrated that the overexpression of kinin B_2 receptor could be involved with kidney injury once that the peak renal expression of kinin B_2 receptor coincided with a significant reduction in renal function.

Innovations and breakthroughs

This is the first study reporting that kinin B_2 receptor is over-expressed in kidney in rhabdomyolysis model. Furthermore, these results suggest that the overexpression may be one of the causes of kidney injury in this animal model.

Applications

Kinin B2 receptor antagonist could be tested in future to avoid kidney injury in rhabdomyolysis process.

Terminology

Rhabdomyolysis is a skeletal muscle disease where the muscle tends to break, that commonly result in acute kidney injury. Bradykinin is a nonapeptide messenger that is enzymatically produced from kallidin and that act via activation of two membrane receptors: kinin B_1 receptor and kinin B_2 receptor. Kinin B_1 receptor is a bradykinin receptor that is induced in response to inflammation, it may play a role in chronic inflammation. Kinin B_2 receptor is a bradykinin receptor that is constitutively expressed and may play a role in the acute phase of inflammation.

Peer review

Gattai *et al* reported that the kinin B_2 receptor knockout. The authors provided detailed data on this and the manuscript is well written.

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