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Protease activated-receptor 4 activation as a model of persistent bladder pain: Essential role of macrophage migration inhibitory factor and high mobility group box 1

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Abstract

Objectives: To develop a rodent model of persistent non-inflammatory bladder pain and to test macrophage migration inhibitory factor and high mobility group box 1 as mediators of bladder pain.

Methods: Female C57BL/6 mice received intravesical instillations of protease activated receptor 4 (100 μ mol/L, for 1 h) three times every other day and abdominal mechanical hypersensitivity (50% mechanical threshold) was tested on day 0 (baseline), and at days 1, 2, 3, 4, 7 and 9 after the first protease-activated receptor 4 injection. At the end of the experiment, micturition changes were measured and bladders were examined for histological changes. Macrophage migration inhibitory factor antagonist (MIF098; 40 mg/kg i.p. b.i.d.) or high mobility group box 1 inhibitor (glycyrrhizin; 50 mg/kg i.p. daily) was administered from day 2 until day 8.

Results: There was a significant and persistent decrease in abdominal mechanical threshold starting from day 3 in the protease-activated receptor 4-treated group that persisted until day 9 (5 days post-last instillation), but not in the control group. Glycyrrhizin fully reversed while MIF098 partially reversed abdominal mechanical hypersensitivity in protease-activated receptor 4-treated mice. The changes started on day 3 after the first protease-activated receptor 4 instillation, and analgesic effects lasted throughout the rest of the testing period. None of the groups had significant micturition changes or overt bladder histological changes.

Conclusions: Repeated intravesical protease activated receptor 4 instillations produce persistent bladder pain without inflammation. Macrophage migration inhibitory factor and high mobility group box 1 are possible effective target molecules for bladder pain alleviation.

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Conflict of interest

None declared.

Keywords

bladder pain; high mobility group box 1; interstitial cystitis/bladder pain syndrome; macrophage migration inhibitory factor; protease activated receptor

Introduction

IC/BPS is a debilitating chronic disease characterized by bladder-related pain or discomfort with some urinary symptoms.¹ Classic IC/BPS patients show bladder inflammation with Hunner's lesions.^{2,3} However, most IC/BPS patients do not show urinary infections, inflammation or other obvious bladder pathologies.⁴ Common current animal models of IC/BPS typically rely on direct bladder injury/insult to elicit bladder inflammation and thus mimic symptoms of IC/BPS.⁵ Bladder pain is usually secondary to bladder inflammation (or injury) in these animal models.

We recently reported that intravesical PAR4 instillation generated an acute (24 h) bladder pain model where mice showed bladder hypersensitivity without overt bladder inflammation, an important feature, as most IC/BPS patients do not show bladder inflammation.^{6,7} Furthermore, we examined the mechanisms responsible for the development of bladder hypersensitivity, and observed that PAR4 activation led to the release of urothelial MIF and HMGB1.^{7,8} Both MIF and HMGB1 are necessary for the development of bladder pain in this model,^{6,7} and these observations agree with reports showing a role for MIF⁹ and HMGB1 in nociception.¹⁰

Westropp and Buffington showed that the introduction of bladder inflammation and injury to urothelium might not result in a valid bladder pain model or a long-lasting model comparable with IC/BPS patients because of urothelial rapid regeneration after intravesical insult.¹¹ Recently, Dogishi reported a 14-day bladder pain model using intravesical H₂O₂ resulting in significant bladder inflammation.¹² We aimed to develop a rodent model where persistent bladder pain was devoid of obvious bladder injury and/or inflammation. In addition, we tested whether MIF and/or HMGB1 still mediated such an effect.

Methods

All animal experiments were approved by the Lexington Veterans Affairs Medical Center Institutional Animal Care and Use Committee (VER-11-016-HAF), and were carried out according to the guidelines of the National Institutes of Health.

Persistent bladder pain model: Repeated intravesical PAR4

Female C57BL/6 (SPF, 13–17-week-old; 20–25 g; Jackson Laboratory, Bar Harbor, ME, USA) were housed in ventilated animal cages with a 14/10 h light/dark cycle.

The experimental design using repeated intravesical PAR4 instillation to develop a persistent bladder pain model is shown in Figure 1. Isoflurane-anesthetized mice were transurethrally catheterized (PE10, 11-mm length) and the bladder drained of urine. PAR4-AP or PAR4 scrambled peptide control (100 µmol/L in PBS; 150 µL; Peptides International, Louisville,

KY, USA) were instilled into the bladder lumen and held for 1 h, and then the mouse was allowed to recover from anesthesia and returned to its cage.⁸ Intravesical instillation was repeated twice every 48 h on day 0, and days 2 and 4 (Fig. 1) for a total of three intravesical instillations. The abdominal mechanical threshold (see below) was evaluated before intravesical instillation (day 0; Fig. 1) and repeated several times as shown in Figure 1 up to day 9. On the last day, micturition parameters were tested (see below) and bladders collected for histology (see below).

In some experimental groups, mice were treated with HMGB1 inhibitor, glycyrrhizin (50 mg/kg, with 100 mmol/L NH₄OH in PBS i.p. daily; Calbiochem, Burlington, MA, USA),⁶ or a MIF receptor CD74 coupling inhibitor, MIF098 (40 mg/kg, with 40% HP-P-CD and 10% PEG400 in H₂O i.p. b.i.d.¹³). Treatment was administered daily starting from day 2 until day 8 (Fig. 1).

Abdominal mechanical hypersensitivity test

Abdominal mechanical hypersensitivity was tested in mice by recording the 50% mechanical threshold, as previously described.^{8,14} Briefly, the mechanical threshold was measured with eight VF fibers (0.008, 0.02, 0.07, 0.16, 0.4, 1.0, 2.0 and 6.0 g). Whenever a positive response to a stimulus occurred, the next smaller VF filament was applied. Otherwise, the next higher filament was applied to detect the mechanical threshold. A positive response was defined as any one of the three following behaviors: (i) licking the abdomen; (ii) flinching/jumping; or (iii) abdomen withdrawal. The 50% mechanical threshold was tested at baseline (day 0 before PAR4-AP or scrambled peptide instillation), and days 1, 2, 3, 4, 7 and 9 after the first PAR4 instillation (Fig. 1; VF).

VSOP

Micturition volume and frequency were measured in awake mice at the end of the experiment (day 9; Fig. 1), as previously described.^{15,16} Micturition tests were carried out in the same enclosures and in the same room used for VF testing to ensure adaptation to the testing environment. All measurements were carried out between 9:00 am to 12:00 pm. Briefly, mice were gavaged with water (50 μ L/g bodyweight) to induce diuresis, and then placed in a plastic enclosure. Mice were free to move and filter paper was placed under the animal to collect urine during a 2-h observation period. Micturition volumes were determined by linear regression using a set of known volumes. Micturition frequency was defined as the total number of micturitions in 2 h. At the end of the experiment, the animal was deeply anesthetized with isoflurane, the bladder excised and placed in 4% paraformaldehyde for histology (see below) and the animal was euthanized.

Histology and immunohistochemistry

Bladder paraffin sections (5 μ m) were processed for routine HE staining. HE stained sections were evaluated by a pathologist blinded to the experimental treatment, and scored for edema and inflammation according to the following scale: 0, no edema and no infiltrating cells; 1, mild submucosal edema and no inflammatory cells; 2, moderate edema and several inflammatory cells; and 3, frank edema, vascular congestion and many inflammatory cells.

For immunohistochemistry, batch-stained paraffin sections ($n = 6$) were blocked (5% goat serum, 0.2% Triton X-100 in PBS, 30 min at room temperature), then incubated overnight at 4°C with goat polyclonal anti-MIF antibody (1:100; AF-289-PB; R&D, Minneapolis, MN, USA) or rabbit polyclonal anti-HMGB1 antibody (1:100; ab18256; Abcam, Cambridge, MA, USA). Omission of primary antibody and non-specific (goat or rabbit) IgG were used as controls. Immunoreactivity was detected with donkey anti-goat TRITC or donkey anti-rabbit FITC-labeled secondary antibody (1:100 in PBS with 1% goat serum, 0.2% Triton X-100; 1 h at room temperature; Jackson ImmunoResearch, West Grove, PA, USA) before coverslipping (Vectashield; Vector Laboratories, Burlingame, CA, USA). Computer-assisted densitometry of HMGB1 immunostaining intensity was carried out on images captured using a Leica DMI4000B microscope equipped with the LAS V4 program and ImageJ (NIH, Bethesda, MD, USA).

Real-time PCR

Total RNA was extracted from wild-type and MIF knockout mouse bladder tissue through Trizol (15596026; Thermo-Fisher Scientific, Grand Island, NY, USA), DNA removed by DNase and reversed transcribed to cDNA (A3500; Promega, Madison, WI, USA). PCR products were detected using SYBR green (4472903; ThermoFisher Scientific) binding with primers (MIF, PPM02985H; HMGB1, PPM05059F; GAPDH, PPM02946E; Qiagen, Germantown, MD, USA) to quantify the level of mRNA in bladder tissue from wild-type and MIF knockout mice. The detection of GAPDH PCR product was used as the internal control.

Statistical analysis

All parameters are reported as mean $\pm$ SE. Changes in the 50% mechanical threshold to VF stimulation were determined by comparing the AUC from day 3 to 9 and analyzed using Tukey tests. Changes in micturition or histology were analyzed using one-way ANOVA. All statistical analyses were carried out using R software (CRAN, Vienna, Austria).¹⁷

Results

Persistent bladder pain model: Repeated PAR4 instillation

The 50% mechanical threshold was tested at baseline and at several time-points after PAR4 instillation (Fig. 1). A total of 24 h after the first PAR4 instillation, the 50% mechanical threshold was markedly decreased compared with the PAR4 scrambled peptide group (Fig. 2a; day 1), in agreement with our earlier reports.^{6,8} The mechanical threshold was increased at day 2 before the second intravesical treatment (Fig. 2a; day 2). After the second intravesical treatment, the PAR4-treated mice showed a markedly decreased mechanical threshold that persisted throughout the rest of the experiment (Fig. 2a; PAR4). In contrast, mice treated with intravesical scrambled peptide (controls) showed no change in abdominal mechanical threshold through the 10 days of testing (Fig. 2a; Scrambled). The AUC (days 3–9) analyses showed a significant decrease in the intravesical PAR4 group compared with the PAR4 scrambled peptide group ($P < 0.05$; Fig. 2b).

Effect of MIF antagonism on abdominal mechanical hypersensitivity

We treated persistent abdominal mechanical hypersensitivity with a systemic MIF antagonist (MIF098; 40 mg/kg i.p. b.i.d.) starting from day 2 after the first intravesical PAR4 instillation until day 8. On day 3, the abdominal mechanical threshold was increased compared with the intravesical PAR4 group. The 50% mechanical threshold was kept at a similar level as the mechanical threshold on day 3 until day 9 (Fig. 2a). AUC measurements showed that treatment with MIF antagonist (MIF098) significantly increased from PAR4 alone (no treatment; $P < 0.05$), whereas there was still a significant difference compared with PAR4 scrambled peptide ($P < 0.05$; Fig. 2b).

Effect of HMGB1 antagonism (glycyrrhizin) on abdominal mechanical hypersensitivity

We also treated persistent abdominal hypersensitivity with a systemic HMGB1 antagonist (glycyrrhizin; 50 mg/kg i.p. daily) starting from day 2 after the first intravesical PAR4 instillation until day 8. Similar to the effects observed with MIF antagonist, the abdominal mechanical threshold was increased compared with the intravesical PAR4 group on day 3, 1 day after treated started. The 50% mechanical threshold continuously increased toward baseline thereafter until day 9 (Fig. 2a). AUC analysis showed that glycyrrhizin treatment significantly improved PAR4-induced abdominal hypersensitivity when compared with PAR4 alone, and there was no significant difference compared with PAR4 scrambled peptide ($P > 0.05$; Fig. 2b).

Micturition changes after repeat intravesical PAR4 or treatments

Micturition parameters were measured day 9 after first intravesical PAR4 instillation. Repeat PAR4 did not result in statistically significant changes in urine volume (in μL) or frequency when compared with scrambled peptide (volume: 300.96 ± 12.90 vs 329.76 ± 24.99 in controls; frequency: 2.33 ± 0.27 vs 2.33 ± 0.27 in controls). Furthermore, treatment with either MIF098 (volume: 285.51 ± 11.43 ; frequency: 3.50 ± 0.25) or glycyrrhizin (volume: 292.90 ± 22.41 ; frequency: 3.00 ± 0.24) also did not produce statistically significant changes on micturition volume or frequency when compared with PAR4-only treatment (Table 1; Fig. 3).

Histology after repeat intravesical PAR4 or treatments

HE stained bladder sections from mice that received repeat intravesical PAR4 or treatments of glycyrrhizin or MIF098 were examined by a pathologist blinded to the treatments, and scored for inflammation and edema changes. Neither PAR4 scrambled peptide ($n = 6$, edema: 0.00 ± 0.00 ; inflammation: 0.00 ± 0.00) nor repeat PAR4 ($n = 6$, edema: 0.17 ± 0.15 ; inflammation: 0.00 ± 0.00) produced edema or inflammation. MIF098 ($n = 4$, edema: 1.00 ± 0.35 ; inflammation: 0.25 ± 0.22) and glycyrrhizin ($n = 6$, edema: 0.17 ± 0.15 ; inflammation: 0.00 ± 0.00) did not induce any bladder inflammation in PAR4 instilled mice (Table 2). HE staining showed that neither the catheterization procedure with PAR4 scrambled peptide (Fig. 4a) nor repeat PAR4 caused bladder inflammation (Fig. 4b).

MIF and HMGB1 mRNA and protein levels in the bladder after treatments

Bladder mRNA and protein levels of MIF and HMGB1 were examined in all the groups with different treatments at the end of the study. The real-time PCR result showed no difference between PAR4 scrambled peptide control (MIF: 1.00 ± 0.21 , $n = 5$; HMGB1: 1.00 ± 0.15 , $n = 5$), PAR4 (MIF: 0.81 ± 0.15 , $n = 6$; HMGB1: 0.79 ± 0.01 , $n = 6$), PAR4-MIF098 (MIF: 1.43 ± 0.24 , $n = 5$; HMGB1: 0.74 ± 0.05 , $n = 5$) and PAR4-glycyrrhizin groups (MIF: 1.45 ± 0.10 , $n = 6$; HMGB1: 0.79 ± 0.02 , $n = 6$) in both MIF and HMGB1 mRNA when normalized to GAPDH (Table 3; Fig. 3). Control slides incubated with non-specific goat IgG (Fig. 5a) or non-specific rabbit IgG (Fig. 5d) showed no immunofluorescence. MIF and HMGB1 immunofluorescence showed similar levels of fluorescence in bladder urothelium of PAR4 scrambled peptide (Fig. 5b, MIF; Fig. 5e, HMGB1) and PAR4 groups (Fig. 5c, MIF; Fig. 5f, HMGB1), and densitometry analysis did not detect any differences (data not shown).

Discussion

We reported that intravesical PAR4 activation is a useful acute (24 h) model of bladder pain without histological signs of bladder inflammation.^{6,7} In the current study, we expanded our results by describing a persistent mouse bladder pain model lasting 9 days. In the present model, repeated (every other day) intravesical PAR4 instillations (total of 3) resulted in extended abdominal mechanical hypersensitivity that persisted for 7 consecutive days (from day 3 to the end of the experiment) and persisted even 5 days after the last intravesical instillation. Remarkably, no overt histological evidence of bladder inflammation was found. Likewise, micturition was not affected in this persistent model.

Pelvic pain and urinary frequency were identified as the two key components of animal models of IC/BPS.¹⁸ The pain component of IC/BPS models is most commonly evaluated using VF testing of abdominal mechanical hypersensitivity in awake animals.^{19,20} Micturition changes can be evaluated in awake and/or anesthetized animals.²¹ The persistent model described in this experiment showed bladder nociception (as determined by VF testing, as we tested behavior daily) over several days while showing no changes in awake micturition testing (urinary frequency/volume) or bladder histology. These features make this model suitable for studying the mechanisms of bladder pain in the absence of micturition changes caused by bladder injury.

One advantage of the persistent model presented here is that it provides a better opportunity for a treatment (rather than prevention) window to alter bladder pain. Our previous results showed that antagonism of MIF or MIF receptors prevented acute bladder pain.^{7,22} We now show that established bladder pain treatment with a MIF receptor inhibitor partially reversed bladder pain. In our acute PAR4 bladder pain model, MIF was released after urothelial PAR4 activation followed by urothelial HMGB1 release, and blocking either of these substances prevented abdominal mechanical hypersensitivity.^{7,8} MIF interacts with several receptors to mediate its effect including CD74 (cognate receptor), CXCR4 and CXCR2.⁷ We showed that urothelial CXCR4 and CD74 mRNA expression were significantly increased after single PAR4 instillation. Furthermore, a MIF antagonist (ISO-1) fully blocked acute bladder pain, whereas a CXCR4 antagonist AMD3100 partially blocked acute bladder pain.^{7,22} Further

evidence for a central role of MIF in acute bladder pain as a result of intravesical PAR4 stimulation is the fact that bladder pain was not observed in MIF-deficient mice treated with PAR4.¹⁶ Therefore, we tested the effect of blocking the binding of MIF with CD74 (MIF098²³) on persistent bladder pain. The present results show that MIF acts on multiple receptors to mediate bladder pain. Similarly, in the present study we also tested the effectiveness of using a HMGB1 antagonist (glycyrrhizin) to treat persistent bladder pain. The present results show that treatment with a HMGB1 inhibitor completely reversed persistent bladder pain, extending our previous observation of HMGB1 inhibition's preventive effect on acute bladder pain.^{6,8}

Our previous results in our acute bladder pain model clearly showed that early (1 h) changes in the bladder; for example, urothelial MIF and HMGB1 release, play an integral role in developing acute bladder pain.^{6,7} In the current study, there were no changes in bladder expression of MIF or HMGB1 at the end of the experiment period (day 9). Mean-while, targeting MIF and HMGB1 at an early stage of the persistent bladder pain model successfully alleviated or diminished persistent bladder pain induced by repeat intravesical PAR4. It remains to be tested whether MIF and/or HMGB1 are mediating bladder pain through a central mechanism of action. Evidence shows that MIF protein in the spinal cord was increased, which makes a major contribution to pain induced by nerve injury.⁹ MIF elevation in the spinal cord was reported in a rat bladder pain model induced by intravesical injection of lipopolysaccharide.²⁴ In addition, spinal HMGB1 modulated pain in collagen antibody-induced arthritis.¹⁰ Therefore, our systemic treatments with MIF or HMGB1 antagonists might be modulating bladder pain at multiple levels (organ/central nervous system), and strategies targeting MIF and HMGB1 need to be optimized according to persistent bladder pain phases as well as levels (bladder vs spinal). These questions require further elucidation.

In summary, the present results show that repeated intravesical PAR4 produces a mouse persistent bladder pain model without bladder inflammation, which provides a better chance for a treatment window. Furthermore, MIF and HMGB1 mediate persistent bladder pain. Glycyrrhizin is widely available as a nutritional supplement,²⁵ whereas MIF inhibitors are currently undergoing clinical testing for several inflammatory conditions.²⁶ Our recent report of urine MIF elevation in IC patients with Hunner's lesions further supports a potential role for MIF in IC/BPS and warrants further investigation.²⁷ Therefore, MIF and HMGB1 represent novel targets for treating bladder pain and might provide a therapeutic avenue for the treatment of IC/BPS.

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Abbreviations & Acronyms

AUC	area under the curve
CYP	cyclophosphamide

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HE	hematoxylin–eosin
HMGB1	high mobility group box 1
IC/BPS	interstitial cystitis/bladder pain syndrome
IgG	immunoglobulin G
MIF	macrophage migration inhibitory factor
PAR4	protease-activated receptor 4
PAR4-AP	protease-activated receptor 4-activating peptide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
VF	von Frey
VSOP	voided stain on paper

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Fig. 1.

Study design and time line for developing and treating persistent bladder pain. The 50% mechanical threshold tested by VF on days 0, 1, 2, 3, 4, 7 and 9. PAR4-AP was instilled on days 0, 2 and 4 after the VF test. Mice were treated by glycyrrhizin or MIF098 from days 2 to 8. On day 9, VSOP was measured after the VF test and the bladder was collected at the end of the experiment.

Fig. 2.

Abdominal mechanical threshold changes after treatments in persistent bladder pain. (a) Repeated intravesical injections of PAR4 scrambled peptide (e; control) did not change the mechanical threshold when compared with its own baseline. PAR4-AP (▲) significantly decreased the mechanical threshold 1 day after the first injection (day 1), second injection (day 3), and then the mechanical hypersensitivity lasted until day 9. Systemic MIF098 (■; MIF inhibitor) partially reversed abdominal mechanical hypersensitivity when daily injections started from day 2. The analgesic effect lasted from day 3 until day 9, but mechanical hypersensitivity did not diminish. Glycyrrhizin (●; HMGB1 inhibitor) increased the mechanical threshold starting from 1 day after treatment (day 3) and completely reversed mechanical hypersensitivity by day 9. (b) The AUC showed that PAR4 is significantly decreased compared with PAR4 scrambled peptide. MIF098 is significantly decreased compared with PAR4 scrambled peptide and significantly increased compared with PAR4 group. Glycyrrhizin did not show any significant difference compared with PAR4 scrambled peptide. * $P < 0.05$ compared with PAR4 scrambled peptide; # $P < 0.05$ compared with PAR4. The arrows at the bottom in (a) show total three PAR4-AP instillations every other day. The bar across the top in (a) shows the duration of MIF098 or glycyrrhizin treatment (days 2–8).

Fig. 3.

Histograms showing micturition and mRNA fold change in all groups. Repeated intravesical injection of PAR4 with and without treatments did not change (a) micturition volume and (b) micturition frequency when compared with the scramble group. There are no significant fold changes in both (c) MIF and (d) HMGB1 mRNA in all groups.

Fig. 4. Bladder histology after repeated instillations. HE staining of bladder sections showed normal histology after repeat instillation of (a) PAR4 scrambled peptide and (b) PAR4-AP.

Fig. 5.

Bladder immunostaining of MIF and HMGB1 after repeated instillations. (a) Bladder sections incubated with non-specific goat IgG showed no immunofluorescence. Arrowhead marks luminal edge of urothelium, while arrow marks lamina propria. (b,c) MIF immunostaining of bladder sections showed a pattern similar to that described before,²⁸ where umbrella cells had little or no MIF immunofluorescence (arrowhead marks edge of urothelium) while basal and intermediate cells showed moderate immunofluorescence (arrow). Similar MIF immunofluorescent intensity was observed in urothelial cytoplasm (arrow) of (b) PAR4 scrambled peptide (c) PAR4-AP measured by image J (data not shown). (d) Bladder sections incubated with non-specific rabbit IgG showed no immunofluorescence in urothelial nuclei (arrowhead marked luminal edge; arrow marks lamina propria). HMGB1 immunofluorescence of bladder sections showed nuclear immunofluorescence as described.⁶ Similar fluorescent intensity in urothelial nuclei (arrow) of (e) PAR4 scrambled peptide and (f) PAR4-AP. Weak or no HMGB1 immunostaining was detected in umbrella cells (e; arrowhead).

Table 1

Micturition volume and frequency in all groups

	Scrambled	PAR4	PAR4-MIF098	PAR4-glycrrrhizin
Volume (μL)	329.76 ± 24.99	300.96 ± 12.90	285.51 ± 11.43	292.90 ± 22.41
Frequency	2.33 ± 0.27	2.33 ± 0.27	3.50 ± 0.25	3.00 ± 0.24

Values presented as mean ± SE. PAR4, PAR4-AP; Scrambled, PAR4 scrambled peptide.

Table 2:

Histological changes in all groups

	Scrambled	PAR4	PAR4-MIF098	PAR4-glycyrrhizin
Edema	0.00 ± 0.00	0.17 ± 0.15	1.00 ± 0.35	0.17 ± 0.15
Inflammation	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.22	0.00 ± 0.00

Values presented as mean ± SE. PAR4, PAR4-AP; Scrambled, PAR4 scrambled peptide

Table 3:

mRNA fold changes in all groups

	Scrambled	PAR4	PAR4-MIF098	PAR4-glycyrrhizin
MIF	1.00 ± 0.21	0.81 ± 0.15	1.43 ± 0.24	1.45 ± 0.10
HMGB1	1.00 ± 0.15	0.79 ± 0.01	0.74 ± 0.05	0.79 ± 0.02

Values presented as mean ± SE. PAR4, PAR4-AP; Scrambled, PAR4 scrambled peptide.