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Neural control of Substance P-induced upregulation and release of macrophage migration inhibitory factor in the rat bladder

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Abstract

OBJECTIVE—Macrophage migration inhibitory factor (MIF) is increased in the intraluminal fluid after experimental inflammation and mediates pro-inflammatory effects on the bladder. We examined the contribution of nerve activity and of specific neurotransmitter systems on the mechanism of MIF release from the bladder during inflammation.

MATERIALS & METHODS—Male Sprague-Dawley rats were anesthetized, bladders were emptied and filled with saline. Rats received saline (s.c.; control; 0.1 ml/100 g bodyweight) or substance P (40 µg/kg in saline; s.c.; 0.1 ml/100 g bodyweight) and also received hexamethonium (50 mg/kg;i.p.; in saline; 0.1 ml/100 g body weight); intravesical lidocaine (2%; 0.3 ml), atropine (3 mg/kg in saline; i.v.; 0.1 ml/100 g body weight), propranolol (3 mg/kg in saline; i.v.; 0.1 ml/100 g body weight) or phentolamine (10 mg/kg in saline; i.v.; 0.1 ml/100 g body weight). After of 1 hour, the intravesical fluid was removed and the bladder was excised. MIF levels in the intraluminal fluid were measured by ELISA and Western-blotting. *MIF* expression in bladder homogenates was examined using RT-PCR.

RESULTS—Either intravesical lidocaine or ganglionic blockage with hexamethonium prevented Substance P-induced MIF release. In addition, pretreatment with atropine and phentolamine, but not propranolol, also prevented MIF release. *MIF* upregulation in the bladder, while increased with Substance P treatment, was only prevented by intravesical lidocaine.

CONCLUSION—Substance P-induced MIF release in the bladder is mediated through nerve activation. Post-ganglionic parasympathetic (via muscarinic receptors) and sympathetic (via alpha-adrenergic receptors) fibers mediate MIF release while activation of bladder afferent nerve terminals upregulate *MIF*.

Keywords

cytokine; cystitis; muscarinic; alpha-adrenergic; beta-adrenergic

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INTRODUCTION

Macrophage migration inhibitory factor (MIF) is a pluripotent cytokine considered to be an upstream regulator of other inflammatory cytokines¹. Our recent findings are consistent with an inflammatory role for MIF during bladder inflammation. MIF is constitutively expressed by urothelial cells which contain high concentrations of this cytokine. Under normal conditions urothelial cells exhibit basal release of MIF into the intraluminal space, a process that is increased by experimental bladder inflammation although the mechanism controlling MIF release in the bladder is not well-understood^{2, 3}. Similarly, we observed increased levels of MIF in the urine of patients with urinary tract infections compared to controls⁴. Our findings also showed *MIF* upregulation during bladder inflammation^{2, 3, 5}, upregulation of *CD74* (binding protein for MIF) and *CD44* (signaling component for MIF-CD74 complex)⁶ thus documenting that all the elements involved in signaling for MIF-mediated pro-inflammatory actions are present in the bladder during inflammation. In support of a pro-inflammatory role for intraluminal MIF, we reported that intravesical antibodies to MIF (to sequester released MIF) decreased experimental bladder inflammation⁵.

In the present study we examine further the mechanism of MIF release from the bladder during inflammation. We proposed earlier that noxious stimuli in the bladder lead to activation of C-fiber afferents that result in release of tachykinins and set up local and central (i.e. involving the spinal cord) reflexes to produce MIF release and induction of MIF-mediated pro-inflammatory effects in the bladder to maintain and/or augment bladder inflammation (see Fig 4⁵). In support of this hypothesis, we recently showed that intravesical capsaicin also elicited MIF release⁷ suggesting that activation of bladder sensory fibers are involved in this process.

Continuing our investigation of MIF-mediated pro-inflammatory changes in the bladder and to test our hypothesis of reflex-mediated MIF release after noxious bladder stimuli, the aim of the present study was to investigate further the mechanism of Substance P(SP)-induced MIF release by first examining the contribution of nerve activity and more specifically, efferent nerves in the bladder. Thus, we compared the effects of intravesical lidocaine (to block bladder nerve activity) with the effects of ganglionic blockage (to stop efferent pathways to the bladder). Moreover, we also examined the specific contribution of parasympathetic pathways (by blocking muscarinic receptors) and/or sympathetic pathways (by blocking alpha and beta adrenergic receptors) to SP-induced MIF release. In this study we show that MIF release is mediated by activation of post-ganglionic fibers involving muscarinic and alpha-adrenergic receptors while *MIF* upregulation during bladder inflammation may only involve afferent stimulation.

METHODS

All experiments were approved by the local IACUC committee and conformed to National Institutes of Health guidelines. Male Sprague-Dawley rats (N=45; 300-350 gm; Harlan, IN) were anesthetized with sodium pentobarbital (60 mg/kg; i.p.) and placed on a heating pad. The bladders were exposed and ureters cut and allowed to drain. The bladder was emptied of urine (using a syringe and a 30 ga hypodermic needle) and 0.3 ml saline (or 2% lidocaine; see below) was placed in the bladder.

In experiment 1, we determined the contribution of nerve-activity vs. efferent nerves in Substance P-induced MIF release. The following groups (n= 5 rats/group) were examined: Control: Saline (s.c.); Substance P: Substance P (SP; 40 µg/kg; sc; Sigma, St. Louis, MO); Lidocaine: Lidocaine (2%; intravesically; 0.3 ml; Abbott, Chicago) + SP (s.c.); Hexamethonium: Hexamethonium (50 mg/kg; i.p.; Sigma) + SP (s.c.).

In experiment 2, we determined the contribution of parasympathetic or sympathetic pathways in Substance P-induced MIF release. The following groups (n= 5 rats/group) were examined: Control: Saline (s.c.); Substance P: Substance P (40 µg/kg; s.c.); Atropine: Atropine (3 mg/kg; i.v.; Sigma) + SP (s.c.); Propanolol: Propanolol (3 mg/kg; i.v.; Sigma) + SP (s.c.); Phentolamine: Phentolamine (10 mg/kg; i.v.; Sigma) + SP (s.c.).

All drugs were dissolved in saline, and systemic administration (s.c., i.p. or i.v.) of the agent was given in a volume of 0.1 ml of solution/100 g of body weight 5-10 minutes prior to SP (s.c.).

One hour after treatment, the intraluminal fluid was collected (using a syringe and a 30 Ga needle), the bladders excised and placed in Trizol (Invitrogen, Carlsbad, California) and the animals euthanized. MIF levels in the intraluminal fluid were determined by ELISA (Experiment 1) or Western-blotting (Experiment 2).

Rat MIF ELISA development and validation

We developed and validated an ELISA to detect rat MIF using commercially available antibodies. Briefly, white high binding ELISA plates (Microlite 2, ThermoScientific, Waltham, MA) were coated with 100 µl goat anti-MIF antibody (sc-16965, 2 µg/ml in phosphate-buffered saline; PBS, pH 7.4; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature overnight. Plates were blocked with 200 µl reagent diluent (1% BSA in PBS pH 7.4) for 1 h at room temperature. Recombinant rat MIF (Torrey Pines Biolabs, Houston, TX) was used to generate a standard curve from 2000 to 31.25 pg/ml in reagent diluent. Intraluminal fluid was diluted to the appropriate concentration in reagent diluent and applied in duplicate wells. Plates were covered with adhesive tape and incubated 2 h at room temperature. Individual wells were then washed three times with wash buffer (PBS containing 0.05% Tween-20, pH 7.4) using an automated plate washer (ThermoScientific). Detection antibody (biotinylated-goat anti-MIF, BAF289, R&D Systems, Minneapolis, MN) was added at a final concentration of 200 ng/ml, the plates recovered with adhesive and incubated 2 h at room temperature. The wells were washed as described above, 100 µl streptavidin-horseradish peroxidase (1:200 dilution in reagent diluent, DY998, R&D Systems) added to each well and the covered plate was incubated for 20 minutes at room temperature. The wells were washed as described above, 100 µl substrate (SuperSignal ELISA Pico Chemiluminescent Substrate, Pierce, Rockford, IL) was added and each well immediately read using a chemiluminescent microplate reader (GloRunner, Turner BioSystems, Sunnyvale, CA). A standard curve was created using linear regression and sample concentrations calculated by interpolation (PRISM v 4.02, GraphPad Software). This ELISA showed a linear relationship between chemiluminescence (arbitrary units) and MIF concentrations in the range of 31.25-2000 pg/ml (Figure 1). Repeated determinations using the recombinant MIF as standards showed an intraplate (n=10) and interplate (n=10) coefficient of variation of 7.3 and 12% respectively.

MIF Western-blotting

Western-blotting of intraluminal fluid was performed under non-reducing conditions following the manufacturer's protocols (NuPAGE Bis-Tris gels, Invitrogen) as described previously^{7, 8}. Briefly, 10 µl of intraluminal fluid were loaded onto NuPAGE Bis-Tris gels (4-12%; Invitrogen). After electrophoresis, separated proteins were transferred to a polyvinylidene fluoride membrane. MIF protein bands were detected using a polyclonal antibody to MIF (R&D Systems; biotinylated; BAF289) and chemiluminescent substrate (Pierce). MIF Western-blotting under denaturing (non-reducing) conditions results in multiple bands reflected MIF association with $\alpha 1$ -inhibitor 3⁷. Band intensities were quantified using Kodak Image Station (Kodak, Rochester, NY), added and expressed as a ratio of the saline group.

MIF RT-PCR

The bladders were processed for *MIF* RT-PCR as previously described⁸. Briefly, Bladder total RNA was isolated using TriZol reagent (Invitrogen). 1 µg total RNA was reverse transcribed using random hexamers (Promega, Madison, WI) and amplified for *MIF* by endpoint PCR (ReadyTaq, Sigma). Rat specific *MIF* PCR primers used were sense: 5' CTCTCCGAGCTCACCCAGCAG 3' and antisense: 5' CGCGTTCATGTCGTAATAGTT 3'. Relative gene expression was determined by measuring net intensity of ethidium bromide staining DNA bands using Kodak Image Station (Kodak). Gene specific PCR product intensities were normalized to an 18S rRNA internal standard (Classic, Ambion, Austin, TX) by determining a ratio (net-intensity of gene-specific band divided by net-intensity of 18S rRNA band).

Statistical Analyses

Data are reported as mean+S.E.M. Data were analyzed using ANOVA followed by Dunnett's test (using Saline group as control) if the overall ANOVA was significant ($p < 0.05$). Statistical analyses were carried out using R⁹, and using the multcomp package for post-hoc comparisons¹⁰.

RESULTS

We first examined whether blocking nerve activity using intravesical lidocaine or selectively blocking only post-ganglionic (efferent) activity using hexamethonium would be effective in reducing SP-induced MIF release, measured by ELISA. An analysis of variance showed that the effect of treatment was significant ($F(3,16) = 4.02$, $p = 0.026$). Subsequent post-hoc analyses using Dunnett's post-hoc t-test (using saline group as control) indicated that SP treatment significantly increased intraluminal fluid MIF (79.55 ± 28.91 µg MIF/ml) compared to saline treatment (10.89 ± 1.04 µg MIF/ml; Dunnett's test, $p = 0.016$; Figure 2) similar to our previous findings^{3, 7}. Pretreatment with either hexamethonium (50 mg/kg; i.p.) or intravesical lidocaine prevented MIF release and in these groups, intraluminal MIF amounts were not significantly different from saline treated animals (18.32 ± 6.07 µg MIF/ml, Dunnett's test, $p = 0.62$; 33.05 ± 8.38 µg MIF/ml, $p = 0.97$, respectively).

Next we examined whether blocking parasympathetic pathways (through blockade of muscarinic receptors) or sympathetic pathways (through blockade of alpha or beta adrenergic receptors) was able to prevent SP-induced MIF release. Using Western-blot analysis we examined the effects of SP and pre-treatment with atropine, propranolol and phentolamine on the levels of MIF in the intraluminal fluid (Figure 3). Figure 3A shows MIF Western blotting of intraluminal fluid for 3 different animals in each of the treatment groups, and high molecular weight bands (approximate molecular weights 170 and 130 kDa) as well as a 12 kDa band were detected. High-molecular weight bands are MIF- $\alpha 1$ -inhibitor 3 complexes as recently shown by us⁷, while monomeric MIF is 12 kDa. As can be seen in Figure 3A, SP increased the intensity of the high molecular weight bands compared to saline treated rats. Atropine and phentolamine prevented the increase in intensity while propranolol had no effect. Figure 3B shows the densitometric analysis of the Western-blotting MIF bands (170, 130 and 12 kDa) normalized to the saline group. ANOVA showed the effect of treatment was significant ($F(4,10) = 8.41$, $p = 0.003$), while post-hoc tests indicated that SP produced a greater than four-fold increase (compared to saline) in the amounts of MIF detected by Western blotting (Dunnett's test, $p = 0.0029$), particularly the 170 and 130 kDa bands that are associated with $\alpha 1$ -inhibitor 3⁷ (Fig 3A). Pretreatment with atropine (3 mg/kg; i.v.) prevented the increase in SP-mediated MIF release and this group was not significantly different from saline (Dunnett's test, $p = 0.979$). Similarly, pretreatment with phentolamine prevented SP-induced MIF release and this group was not significantly different from saline (Dunnett's test, $p = 0.848$). However,

pretreatment with propranolol did not prevent SP-induced effects and intraluminal MIF levels increased four-fold when compared to saline (Figure 3A,3B; Dunnett's test, $p=0.024$).

We also examined the effect of the different treatments on upregulation of bladder *MIF* using RT-PCR. Figure 4 shows that SP treatment alone upregulated *MIF* mRNA in the bladder as shown by us earlier^{3, 5}. Similar findings were observed for all the other treatments, except for intravesical lidocaine pre-treatment which abolished *MIF* upregulation after SP treatment (Figure 4).

DISCUSSION

SP elicits MIF release into the intraluminal fluid of the rat as we have shown in this and previous studies^{3, 5, 7}. In this study we show that blocking nerve activity in the bladder with intravesical lidocaine or blocking ganglionic transmission (with hexamethonium) prevents SP-induced MIF release indicating that nerve activity is necessary for SP-induced MIF release and that such release is mediated via activation of post-ganglionic pathways to the bladder.

Intravesical lidocaine likely blocked both afferent and efferent nerve activity at the bladder since intravesical lidocaine has also been reported to abolish micturition contractions and produce overflow incontinence during cystometry in rats¹¹. In the clinical literature, on the other hand, poor penetration by lidocaine (and other local anesthetics) has been reported, necessitating increasing the pH of the lidocaine solution to maximize absorbance¹².

SP-induced MIF release was prevented by either blocking nerve fibers (intravesical lidocaine) or a ganglionic blocker (hexamethonium), suggesting that SP activated bladder afferent fibers (probably C-fibers, since capsaicin also elicited MIF release⁷) to activate reflex pathways in the spinal cord leading to activation of post-ganglionic nerves to the bladder and MIF release into the intraluminal fluid.

Then, we selectively blocked the contribution of the transmitters (cholinergic or adrenergic) in efferent post-ganglionic pathways to the bladder^{13, 14}. Administration of atropine to block muscarinic receptors or phentolamine to block alpha-adrenergic receptors at the bladder, both prevented SP-induced MIF release. Administration of propranolol to block beta-adrenergic receptors, on the other hand, was not effective. Therefore, our data suggest that SP-induced MIF release may be due to afferent activation of reflex post-ganglionic pathways to the bladder, involving both parasympathetic (acting via muscarinic) and sympathetic (acting via alpha-adrenergic) innervation. As a first step, we used broad receptor blockers at concentrations reported effective for blocking transmission in the rat bladder^{13, 14}, the receptor subtypes involved remain to be elucidated and future experiments will investigate this question. It should be noted, that cytokine release modulated by the parasympathetic and/or sympathetic nervous systems has been reported in other organs¹⁵⁻¹⁷ and suggests that neural modulation of the immune system is likely to play a pivotal role in health and disease.

Although either ganglionic blockade or selective blockade (of either muscarinic or alpha-adrenergic receptors) prevented SP-induced MIF release, these treatments had no effect on *MIF* upregulation in the bladder produced by SP. Thus, activation of efferent pathways to the bladder does not appear to be involved in *MIF* upregulation in the bladder after SP treatment. It is possible that SP is acting directly on bladder epithelia or muscle to upregulate *MIF*. However, intravesical lidocaine prevented *MIF* upregulation after SP. Thus, it appears that nerve activity is necessary for *MIF* upregulation after SP and since blockade of post-ganglionic efferent pathways to the bladder had no effect, it suggests that afferent nerve activity alone is sufficient to elicit *MIF* upregulation. Consistent with this possibility, intravesical capsaicin alone was observed to upregulate *MIF* in the bladder (unpublished observations), indicating that activation of capsaicin-sensitive afferent fibers mediate *MIF* upregulation.

Our observations suggests that neurogenic inflammation produced by the release of neuropeptides at the bladder and proposed as one of several factors involved in chronic pelvic pain conditions (including interstitial cystitis)¹⁸, may be capable of upregulating bladder *MIF* production and result in MIF release into the intraluminal fluid which, in turn, can augment or maintain inflammatory processes in the bladder (Figure 5). Increased MIF release alone, however, is not likely to be sufficient to produce pro-inflammatory effects without the presence of CD74 (recognized MIF-binding protein) and CD44 (MIF-CD74 signaling component), which are both upregulated during experimental bladder inflammation⁶. We also recently presented preliminary evidence of upregulation of MIF and CD74 in the urothelium of IC patients¹⁹. Since IC patients have increased levels of urinary SP²⁰, it is possible that neurogenic inflammation in these patients may be eliciting similar results (in terms of *MIF/CD74* upregulation) as we have observed in an animal model of experimental cystitis.

CONCLUSIONS

SP-induced MIF release in the bladder is nerve-mediated and involves activation of post-ganglionic fibers (via muscarinic and alpha-adrenergic receptors) indicating that activation of both parasympathetic and sympathetic pathways to the bladder elicit MIF release. Activation of bladder afferent fibers mediates *MIF* upregulation in the bladder. These results show that neurogenic inflammation modulates MIF production and release in the bladder through activation of different nerve terminals.

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ABBREVIATIONS

SP

Substance P

MIF

Macrophage migration inhibitory factor

RT-PCR

reverse transcriptase polymerase chain reaction

ANOVA

analysis of variance

Figure 1.

Validation of rat MIF ELISA. Representative regression showing high correlation (adjusted $r^2=0.9979$) between MIF concentration (pg/ml) and chemiluminescence (arbitrary units).

Repeated determinations using recombinant MIF as standards were carried out to determine intraplate (n=10) and interplate (n=10) variability. Results showed coefficient of variation of 7.3 and 12% respectively.

Figure 2.

Changes in MIF levels in the intraluminal fluid increased after different treatments. The overall ANOVA showed a significant difference ($p=0.026$) across treatment groups. Substance P ($n=5$) increased the intraluminal levels of MIF (as measured by ELISA) compared to saline treatment ($n=5$) and this difference was statistically significant (Dunnett's test, $p=0.0162$). Pretreatment with hexamethonium (Hex; 50 mg/kg; i.p; $n=5$) or intravesical lidocaine (Lid; 0.3 ml; 2%; $n=5$) prevented Substance P-induced increase in intraluminal MIF so that the level of MIF in those groups were not significantly different from saline (Dunnett's test, $p=0.621$; $p=0.973$, respectively). Sal= Saline; SP=Substance P (s.c.); Hex+SP: Pretreatment with hexamethonium followed by Substance P; Lid = Intravesical Lidocaine followed by Substance P; $*=p<0.05$.

Figure 3.

Effect of different receptor blockade on Substance P-induced MIF release. A) Western-blotting under denaturing (non-reducing) conditions showed multiple MIF bands. High molecular weight bands (170,130 kDa, indicated on the right side of the figure) correspond to MIF- α -1 inhibitor 3 complexes as reported earlier⁷, whereas a band at 12 kDa corresponds to monomeric MIF. Each lane represents intraluminal fluid from one animal. Substance P (SP) increased the intensity of the MIF Western-blotting bands at 170 and 130 kDa (previously shown to be associated with α 1-inhibitor 3⁷). MIF bands at 170, 130 and 12 kDa (monomeric MIF) are indicated by markers on the left hand side of the figure. Atropine (3 mg/kg; i.v.) or phentolamine (10 mg/kg; i.v.) pretreatment blocked the effect of Substance P. Propanolol (3 mg/kg; i.v.) had

no effect. Although 5 animals were used in each group, we randomly selected 3 to allow side-by-side comparisons during Western-blotting. This procedure was repeated twice include all animals and to insure reproducibility of results. B) Densitometric analysis of the MIF Western-blot bands (170,130, 12) in panel A. The overall ANOVA showed a significant difference across treatment groups ($F=8,41$, $p=0.0036$). A significant increase in levels of intraluminal MIF was observed after Substance P when compared to saline treatment (Dunnett's test, $p<0.003$). This increase was prevented by pre-treatment with atropine and phentolamine, and in these groups the intraluminal MIF levels were not significantly different from saline treated animals (Dunnett's test, $p=0.980$; $p=0.848$, respectively). The group treated with propranolol still showed increased levels of MIF in the intraluminal fluid when compared to saline (Dunnett's test, $p=0.024$). Abbreviations: Sal=Saline; SP=Substance P; ATR+SP: Atropine pretreatment followed by Substance P; PRO+SP: Propranolol pretreatment followed by Substance P; PHE+SP: Phentolamine pretreatment followed by Substance P. $*$ = $p<0.05$; $**$ = $p<0.01$.

Figure 4.

Effect of different treatments on *MIF* expression in the bladder. The overall ANOVA showed a significant difference across treatment groups ($F=12.29$, $p=0.00002$). When compared to saline treated rats ($n=3$), Substance P treatment ($n=3$) significantly increased *MIF* expression in the bladder (Dunnett's test, $p=0.039$) and only intravesical lidocaine was able to prevent this effect (Dunnett's test, $p=0.999$; compared to saline). The remaining treatments still showed significantly increased *MIF* upregulation as a result of Substance P treatment. (Dunnett's test, atropine, $p=0.001$; propranolol, $p=0.001$; phentolamine, $p=0.033$). $*$ = $p<0.05$; $**$ = $p<0.01$; $***$ = $p<0.001$.

Figure 5.

Proposed mechanism by which MIF maintains and/or enhances bladder inflammation (modified from Siegler & Vera⁵ to incorporate new evidence). Noxious stimulation activate C-fiber afferents in the bladder to produce local release of tachykinins (e.g. neurogenic inflammation) and also set up reflexes involving the central nervous system (CNS). C-fiber activation (as shown with intravesical capsaicin⁷) of CNS reflexes results in release of MIF mediated via activation of muscarinic and alpha-adrenergic receptors (presumably through activation of efferent activity to the major pelvic ganglia (MPG) and sympathetic ganglia (SG) innervating the bladder) and upregulation of MIF production in the bladder. Released MIF can then bind to the CD74/CD44 receptor-signaling complex that is also upregulated in the urothelium during inflammation⁶ and induce production of pro-inflammatory mediators⁵. These factors can then further activate local afferent fibers thus setting up a positive feedback

loop that maintains inflammation. Continued release of MIF and other pro-inflammatory mediators, if left unchecked, can then set up conditions of chronic inflammation and further activation of central nervous system reflexes including viscerovisceral interactions.