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Involvement of Src tyrosine kinase and protein kinase C in the expression of macrophage migration inhibitory factor induced by H_2O_2 in HL-1 mouse cardiac muscle cells

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Abstract

Macrophage migration inhibitory factor (MIF), a pleiotropic cytokine, plays an important role in the pathogenesis of atrial fibrillation; however, the upstream regulation of MIF in atrial myocytes remains unclear. In the present study, we investigated whether and how MIF is regulated in response to the renin-angiotensin system and oxidative stress in atrium myocytes (HL-1 cells). MIF protein and mRNA levels in HL-1 cells were assayed using immunofluorescence, real-time PCR, and Western blot. The result indicated that MIF was expressed in the cytoplasm of HL-1 cells. Hydrogen peroxide (H_2O_2), but not angiotensin II, stimulated MIF expression in HL-1 cells. H_2O_2-induced MIF protein and gene levels increased in a dose-dependent manner and were completely abolished in the presence of catalase. H_2O_2-induced MIF production was completely inhibited by tyrosine kinase inhibitors genistein and PP1, as well as by protein kinase C (PKC) inhibitor GF109203X, suggesting that redox-sensitive MIF production is mediated through tyrosine kinase and PKC-dependent mechanisms in HL-1 cells. These results suggest that MIF is upregulated by HL-1 cells in response to redox stress, probably by the activation of Src and PKC.

Key words: Macrophage migration inhibitory factor; HL-1 cells; Hydrogen peroxide; Atrial fibrillation; Protein kinases

Introduction

Recent evidence indicates that inflammation plays an important role in the pathology of atrial fibrillation (AF). In atrial tissue, inflammation is associated with activation of a variety of cells including lymphocytes, monocytes/macrophages, fibroblasts, and cardiac myocytes, which express and secrete proinflammatory cytokines such as interleukin 6, tumor necrosis factor α, and C-reactive protein. These cytokines can modulate atrial functions and atrial remodeling, leading to development of AF (1-7).

Macrophage migration inhibitory factor (MIF), a proinflammatory cytokine, also functions as a regulator of the inflammatory response. It has been associated with inflammatory cardiovascular diseases including coronary heart disease and myocarditis, pulmonary hypertension, and cardiac dysfunction following burn injury and sepsis (8-11). In previous research, we found that reduced calcium channel current amplitude in AF is associated with increased MIF levels (12,13). However, the regulation of MIF production in atrial myocytes remains unclear.

Experimental and clinical data suggest that there are interactions among angiotensin (Ang) II-related signaling, inflammation, and oxidative stress in the pathogenesis of AF-promoting structural or electrical remodeling (14,15). Reactive oxygen species (ROS) and Ang II could regulate the expression of MIF in cardiac myocytes and tubular epithelial cells (16,17). Therefore,

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in the present study, we investigated whether and how MIF is regulated in response to the renin-angiotensin system (RAS) and oxidative stress in an atrial-derived cell line (HL-1).

Material and Methods

HL-1 cardiomyocytes

HL-1 cells, a mouse cardiac cell line derived from a transplantable mouse cardiomyocyte lineage (AT-1), were obtained from the laboratory of Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA, USA). Cells were cultured in Claycomb medium (JRH Biosciences, USA) supplemented with 10% fetal bovine serum (JRH Biosciences), 2 mM L-glutamine (Gibco, USA), 100 µM norepinephrine (Sigma, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco) in flasks precoated with fibronectin and gelatin (Sigma), then incubated at 37°C in 5% CO_2-95% air. The medium was changed every 24-48 h.

Immunohistochemistry

For the immunohistochemical analyses, cultured cells were fixed in paraformaldehyde and incubated overnight with polyclonal antibodies against MIF (Santa Cruz Biotech, USA) or nonimmune immunoglobulin G (IgG) at dilutions of 1:50. The slides were then washed three times with Tris-buffered saline (TBS) for 5 min and incubated with peroxidase-labeled rabbit anti-goat antibody (Calbiochem, Germany) at a dilution of 1:2000 for 45 min. After they were washed three times for 5 min in phosphate-buffered saline (PBS), the sections were incubated in diaminobenzidine tetrahydrochloride (DAKO, Denmark) in PBS with 0.01% H_2O_2 for 15 min. The reaction was stopped by washing the sections in distilled water. The slides were mounted and observed under a light microscope.

Western blot analysis

Cells were lysed in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.15 M sodium chloride, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% nonidet P (NP-40), and a Protease Inhibitor Cocktail Set (Calbiochem). Cell lysates were centrifuged at 12,000 *g* for 15 min at 4°C. Protein concentrations were determined. Samples were diluted with 4X loading buffer (Invitrogen, USA) and heated at 95°C for 5 min. The proteins (30 µg) were fractionated on 12% (for MIF) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham, USA) according to standard protocols. Membranes were blocked with dried skimmed milk powder in TBS Tween (TBST) for 2 h at room temperature before overnight incubation at 4°C with the primary antibodies (rabbit polyclonal to MIF, 1:500). The signals were normalized to the protein levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000; Zymed, USA). After washing in TBST, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (KPL, USA) in blocking solution. Protein bands were visualized by electrochemiluminescence reagents (Pierce, USA), and films were evaluated densitometrically with the Gel-Pro Analyzer 4.0 software (http://gel-pro-analyzer.software.informer.com).

RNA extraction and reverse transcription polymerase chain reaction (PCR)

Total RNA extracted from cultured HL-1 cells was treated with DNase I to remove genomic DNA contamination. First-strand cDNA was synthesized from 1 µg total RNA using a reverse transcription system (Promega, USA). Real-time quantitative PCRs were run in an MJ Research DNA Engine Opticon® 2 continuous fluorescence detection system (MJ Research, Inc., USA). cDNA (2 µL) in a final volume of 25 µL was amplified using SYBR Premix Ex Taq™ (TaKaRa Biotechnology, China). For mouse MIF, the forward primer was 5′-GTG CCA GAG GGG TTT CTG T-3′ and the reverse was 5′-AGG CCA CAC AGC TTA CT-3′; for β-actin, the forward primer was 5′-TGT CCC TGT ATG CCT CTG GT-3′ and the reverse was 5′-GAT GTC ACG CAC GAT TTC C-3′. Relative expression levels were calculated as $2^{-\Delta\Delta CT}$. Fold-changes were calculated according to Livak and Schmittgen (18).

Confocal microscopy

Cells were grown on glass coverslips precoated with fibronectin and gelatin, fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.25% Triton X-100 in PBS containing 1% BSA for 15 min. They were incubated overnight at 4°C with a 1:100 dilution of primary antibody (mouse anti-MIF) and for 1 h at room temperature with a 1:200 dilution of secondary antibody. The coverslips were mounted onto microscope slides in Vectashield mounting medium containing DAPI for fluorescence microscopy (Vector Laboratories, USA). Fluorescent images were visualized and captured using a Leica DMI 6000 CS upright fluorescent microscope and a Leica TCS SP5 laser scanning confocal microscope (Leica, Germany).

Drugs

Genistein, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1), PD098059, SB203580, calphostin C, and GF109203X were purchased from Sigma, and U0126 was obtained from Calbiochem. These kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO, Calbiochem). The concentration of DMSO in the working solutions did not exceed 1.5%.

Data analysis

All data are reported as means ± SE. One-way ANOVA was used for multiple comparisons. Values of $P<0.05$ were considered to indicate statistical significance.

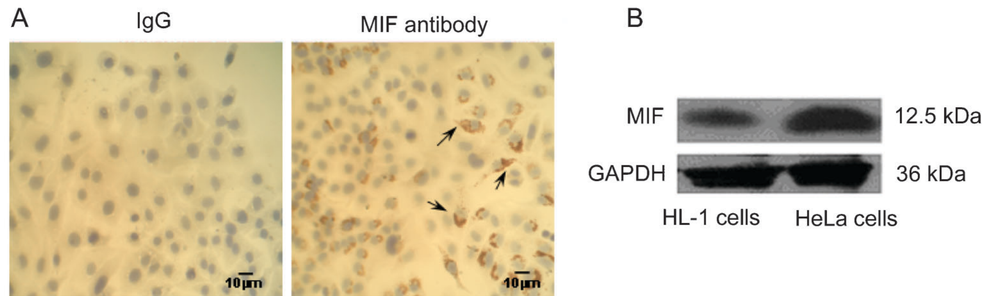

Figure 1. Expression of macrophage migration inhibitory factor (MIF) in HL-1 cells. *A*, Representative examples of immunohistochemical analysis of HL-1 cells. MIF expression in HL-1 cells with positive cytoplasmic staining (arrows). The staining specificity was tested by substituting IgG for the primary antibody. *B*, Western blot analysis of MIF performed on cell lysates showing a single band of 12.5 kDa.

Results

MIF expression and distribution in HL-1 cells

To investigate whether MIF was expressed in HL-1 cells, we used specific antibody to probe MIF in HL-1 cells. HeLa cells were used as a positive control. Immunohistochemistry assay showed that MIF was predominantly expressed in the cytoplasm of HL-1 cells, which was further confirmed by Western blot (Figure 1).

MIF production in HL-1 cells in response to redox stress

To determine whether MIF was regulated by redox stress in atrial myocytes, HL-1 cells were incubated in medium containing 100 or 300 µM H_2O_2 for 6 h. H_2O_2 induced HL-1 cells to produce significantly increased levels of MIF in a concentration-dependent manner as shown by real-time PCR and Western blot (1.0 *vs* 2.6±0.1, $P<0.05$, and 3.2±0.8, $P<0.01$, gene expression for β-actin;

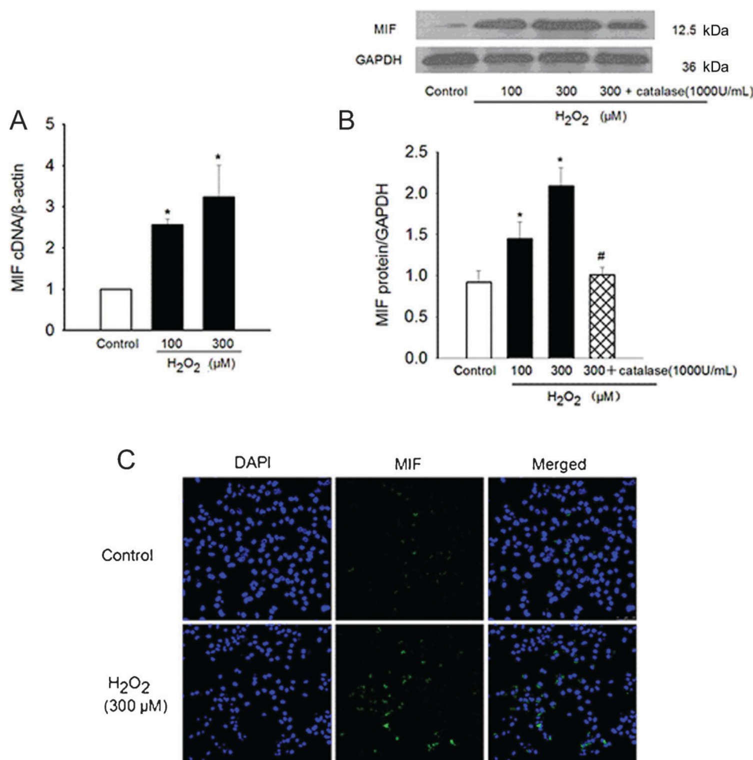

Figure 2. H_2O_2-stimulated migration inhibitory factor (MIF) production in HL-1 cells. *A*,*B*, HL-1 cells were stimulated by H_2O_2 (100 or 300 µM) for 6 h or by H_2O_2 (300 µM) for 6 h in the presence of catalase (1000 U/mL). Levels of MIF mRNA and protein were analyzed using real-time PCR or Western blotting with β-actin or GAPDH as internal controls, respectively. Mean values for each group were determined from 3 separate experiments each performed in duplicate. *$P<0.05$ *vs* control. #$P<0.01$ *vs* 300 µM H_2O_2 group (one-way ANOVA). *C*, Immunostaining results of MIF stimulated by 300 µM H_2O_2 detected by confocal microscope. HL-1 cells were co-stained with anti-MIF antibody, and DAPI. Merged images of MIF (green), and DAPI (blue) staining are shown.

0.9 ± 0.1 *vs* 1.5 ± 0.2, $P<0.05$, and 2.1 ± 0.2, $P<0.01$, protein expression for GAPDH; Figure 2A and B). This H_2O_2-induced MIF production was completely abolished in the presence of catalase (1000 U/mL; from 2.1 ± 0.2 to 1.0 ± 0.1, $P<0.05$; Figure 2B). Immunofluorescence revealed similar results (Figure 2C). Morphological changes of HL-1 cells in response to H_2O_2 treatment were observed under an inverted microscope. Compared to controls, treated cells exhibited nuclear condensation, plasma membrane shrinkage, and a decreased beating rate, and few cells detached at 6 h after treatment; the morphological changes were H_2O_2-dose dependent.

MIF production in HL-1 cells in response to Ang II

We also investigated the role of Ang II in the production of MIF. In contrast to H_2O_2, there was no significant change in MIF mRNA in HL-1 cells treated with three concentrations of Ang II (1, 10, and 100 μM) for 3, 6, and 12 h, respectively. Nevertheless, the level of MIF mRNA was significantly repressed by Ang II for the group treated with Ang II for a longer time (24 h) (1 *vs* 0.63 ± 0.03, 0.66 ± 0.06, and 0.66 ± 0.07, $P<0.01$; Figure 3A). However, protein levels of MIF were not altered by treatment of Ang II as detected by immunoblotting (Figure 3B). Perhaps a longer treatment with Ang II would lead to the inhibitory effect of Ang II on MIF protein levels.

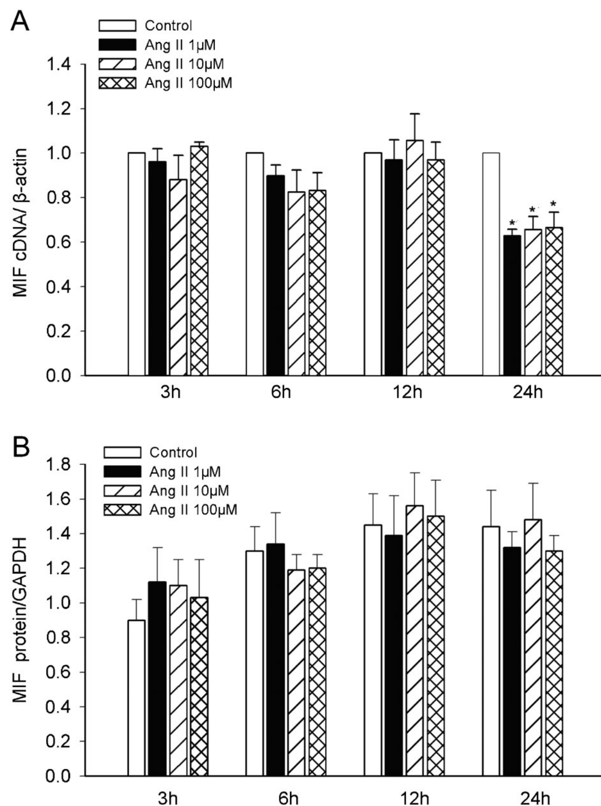

Figure 3. Effects of angiotensin II (Ang II) on the migration inhibitory factor (MIF) expression of HL-1 cells. HL-1 cells were stimulated by Ang II (1, 10, 100 μM) for the indicated periods. Levels of MIF mRNA and protein were analyzed using Western blotting or real-time PCR with β-actin (*A*) or GAPDH (*B*) as internal controls, respectively. Data are reported as means ± SE of MIF expression for 4 independent experiments in duplicate. *$P<0.05$ *vs* control (one-way ANOVA).

Effects of various kinase inhibitors on MIF production

We then examined whether protein kinases were involved in H_2O_2-induced MIF production in HL-1 cells using tyrosine kinase inhibitors (genistein, a nonspecific PTK inhibitor and PP1, a specific Src antagonist), mitogen-activated protein (MAP) kinase inhibitors (PD098059 and U0126 for extracellular-regulated kinase 1/2 and SB203580 for p38-MAP kinase), and protein kinase C (PKC) inhibitors (GF109203X and calphostin C). Genistein, PP1, and GF109203X could all inhibit H_2O_2-induced MIF production, whereas PD098059, U0126, SB203580, and calphostin C had no significant effect (Figure 4). These results suggest that H_2O_2 induces MIF production through PKC and tyrosine kinase-dependent mechanisms in HL-1 cells.

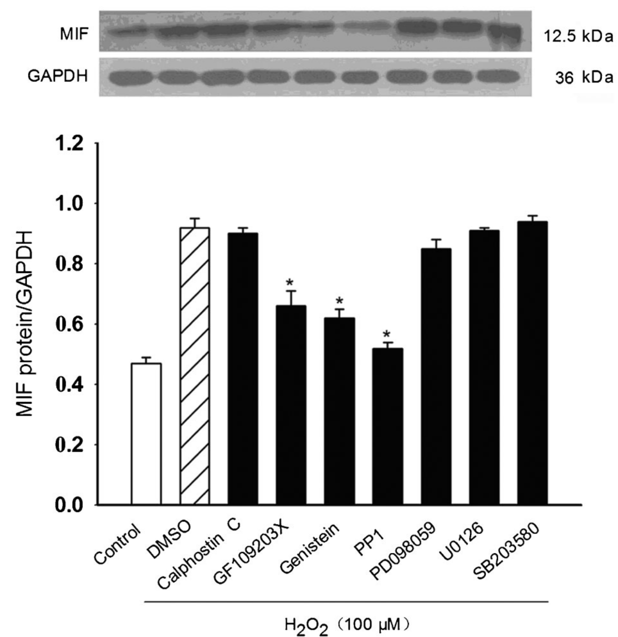

Figure 4. Effects of various inhibitors on H_2O_2-induced migration inhibitory factor (MIF) production in HL-1 cells. After HL-1 cells were pretreated for 1 h with 0.1% DMSO (control), calphostin C (100 nM), GF109203X (5 μM), genistein (10 μM), PP1 (10 μM), PD098059 (10 μM), U0126 (20 μM) or SB203580 (10 μM), cells were stimulated with H_2O_2 (100 μM) for 6 h. The MIF concentration was determined by Western blot analysis as described in Material and Methods. Data are reported as means ± SE of 4 independent experiments. *$P<0.01$ *vs* DMSO + H_2O_2 group (one-way ANOVA).

Discussion

We demonstrated that 1) MIF was expressed in the cytoplasm of HL-1 cells; 2) levels of MIF expression were increased significantly by H_2O_2, but not Ang II, in a dose-dependent manner; 3) this MIF expression was completely inhibited by treatment with the tyrosine kinase inhibitors genistein and PP1, as well as the specific PKC inhibitor GF109203X.

Recent findings demonstrated the involvement of oxidative stress and inflammation in atrial tissue during AF, suggesting a potential role in the remodeling phenomenon (1,14,15). MIF, an important cytokine, is known to play a role in the pathoetiology of inflammatory cardiovascular diseases including coronary heart disease, myocarditis, pulmonary hypertension, and cardiac dysfunction after burn injury and sepsis (8-10). In our previous studies (12,13), increased MIF expression was found in atrial tissue from patients with AF and contributed to the development of electrical remodeling in AF.

However, the upstream regulatory pathway of MIF in atrial myocytes is still not clear. Recent evidence has indicated that ROS could regulate the expression of MIF in cardiac myocytes (16). Ang II has been shown to upregulate MIF mRNA production and MIF protein secretion by renal tubular epithelial cells (17). These results suggest that ROS and RAS might promote oxidative stress and inflammation via induction of MIF synthesis and secretion. In the present study, we therefore examined the concentration of MIF under the stimulation of H_2O_2 and Ang II in HL-1 cells. We found that H_2O_2 was able to stimulate MIF production in HL-1 cells in a dose-dependent manner, whereas Ang II had no effect. These findings suggest that MIF may function in the myocardium as a redox-sensitive cytokine. In contrast to H_2O_2, after a longer, 24-h treatment, Ang II had an inhibitory effect on MIF mRNA levels, but not on the protein levels of MIF. Perhaps a longer treatment with Ang II would help explore the exact role of Ang II on MIF protein expression.

We also investigated the pathways of redox-sensitive intracellular signaling and found that MIF production induced by H_2O_2 was completely inhibited by genistein, PP1, and GF109203X, suggesting that redox-sensitive MIF production is mediated through tyrosine kinase and PKC-dependent mechanisms in HL-1 cells. However, calphostin C, another PKC inhibitor, had no effect on the MIF production induced by H_2O_2. This difference might result from the different mechanisms of action of GF109203X and calphostin C. GF109203X inhibits PKC by competitive inhibition of the ATP-binding site, whereas calphostin C inhibits PKC by interaction with the protein's regulatory domain, which is the binding site of diacylglycerol and phorbol esters. A study by Takahashi et al. (16) reported that H_2O_2-induced MIF production is mediated through phorbol ester-insensitive PKC in cardiac myocytes, which could explain the different effects seen in our study. From the results of the present study, we propose that MIF is expressed by HL-1 cells in response to redox stress, probably by the activation of Src and PKC.

Acknowledgments

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