Macrophage migration inhibitory factor contributes to hypoxic pulmonary vasoconstriction in rats

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A B S T R A C T

Background: Hypoxic pulmonary vasoconstriction may lead to pulmonary hypertension, but the underlying mechanisms of persistent vasoconstriction are still unclear. There is evidence that pulmonary inflammation contributes to the abnormalities of function in the pulmonary artery (PA) following chronic hypoxia exposure. Macrophage migration inhibitory factor (MIF) is an important pro-inflammatory cytokine, and we found that expression of MIF was increased in the smooth muscle of PA from hypoxic pulmonary hypertensive rats. Therefore, the aim of the study was to investigate the role of MIF in modulating vasoreactivity of isolated PA rings.

Methods: Sprague-Dawley rats were challenged by intermittent chronic hypoxia exposure for 4 weeks to establish hypoxic pulmonary hypertension models. Subsequently, immunohistochemistry and western blot assay were used to examine the MIF expression in pulmonary artery. Moreover, isometric force displacement was measured in isolated intrapulmonary artery.

Results: In the isolated PA, our results showed that MIF mediated the enhanced pulmonary arterial vasoconstriction under acute hypoxia. We also present the finding that MIF had no effect on force displacement on its own, but concentration-dependently potentiated constrictions pre-evoked by phenylephrine under normoxic condition. The potentiation was independent of the endothelium. MIF-induced potentiation of phenylephrine-evoked constriction was partially inhibited by PKC inhibitor chelerythrine, p38 inhibitor SB 203580, ERK1/2 inhibitor U0126, respectively.

Conclusions: Our results suggested that MIF enhanced vasoconstriction of pulmonary artery elicited by agonist through PKC, p38 and ERK1/2 signal pathways, which may contribute to hypoxic pulmonary vasoconstriction.

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Introduction

Hypoxic pulmonary vasoconstriction (HPV) is an important physiological mechanism that optimizes ventilation–perfusion matching. However, sustained HPV leads to detrimental increases in pulmonary artery (PA) pressure. Dysfunction of mechanisms regulating vascular tone and remodeling of the pulmonary vasculature contribute to the development of pulmonary hypertension (Ward and McMurtry, 2009), but they were not elucidated. Although it has been said that pulmonary hypertension is a disease of the distal lung circulation, it is increasingly appreciated that structural change in larger vessels may contribute directly to right ventricular work and failure and to changing flow dynamics (Stenmark et al., 2006). Therefore, the responses of larger vessels to local mediators need more work.

Several studies demonstrate that pulmonary inflammation contributes to the abnormalities of function in the PA following chronic hypoxic exposure (Frid et al., 2006; Perros et al., 2007; Savale et al., 2009; Stenmark et al., 2005). Hypoxic injury may activate pro-inflammatory signaling pathways, leading to production of lung inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and increased numbers of macrophages and neutrophils in the lungs (Madjdpour et al., 2003; Minamino et al., 2001; Savale et al., 2009). The increase in such inflammatory cytokines has significant effects on the local vascular wall cells, including changes vascular tone (De Salvatore et al., 2003; Liu et al., 1992).

Macrophage migration inhibitory factor (MIF) is an important pro-inflammatory cytokine, which is expressed by many cell types including vascular smooth muscle cells (Burger-Kentischer et al., 2002), and exhibits a broad range of immunostimulatory and inflammatory
activity (Calandra et al., 2000; Donnelly et al., 1997). It is known that MIF participates in the pathogenesis of acute inflammatory in the lung such as acute lung injury/acute respiratory distress syndrome (ALI/ARDS) (Donnelly et al., 1997; Gao et al., 2007). However, MIF has an undefined role in chronic inflammatory in the lung especially hypoxic pulmonary hypertension (HPH). In the study, we present evidence that MIF expression obviously increased in the smooth muscle of PA from HPH rats. We hypothesized that MIF might modulate pulmonary vasoconstriction like some inflammatory mediators. Therefore, we investigated the effect of MIF on the tension of PA rings and the underlying mechanisms.

Methods

Animals, drugs and chemicals

Male Sprague–Dawley rats (body weight 250–300 g) were used. All of the experimental procedures were approved by the Animal Use and Care Committee for Research and Education of the Fourth Military Medical University (Xi’an, P.R. China). MIF antibodies against rats MIF was obtained from Abcam (Cambridge, UK). Antibodies against phospho-ERK1/2 (extracellular signal-regulated kinase 1/2) and phospho-p38 were from Cell Signaling (Beverly, MA, USA). Phospho-antibody to MLC20 (20 kDa regulatory light chain of myosin II) was obtained from Santa Cruz Biotechnology (New York, NY, USA). Recombinant mouse MIF was purchased from R&D Systems (Minneapolis, MN, USA). ISO-1 was obtained from Calbiochem (San Diego, CA, USA). Phenylephrine, acetylcholine and SB 203580 were from Sigma (St. Louis, MO, USA). U0126 was from Promega (Madison, WI, USA).

Assessment of pulmonary hypertension

According to the previous reports, rats were housed in a chamber containing 10% oxygen and taken hypobaric hypoxia challenge of 8 h/d for 4 weeks (Wang et al., 2010; Xu et al., 2010). Control rats were housed continuously in room air. At the end of hypoxia exposure, the rats were anesthetized with 20% ethylurethan (4 ml/kg i.p.), and a soft silicagel catheter connected to a pressure transducer (AD Instruments, Colorado Springs, CO, AUSTRALIA) was inserted into the right ventricle through jugular vein to record right ventricular systolic pressure (RVSP). The ratio of right ventricle/left ventricle + septum (RV/[LV+S]) weight was determined. Increases in RVSP, RV/[LV+S] and thickened tunica media in small distal pulmonary arteries were taken as indicators of pulmonary hypertension.

Right lung sagittal sections were placed in 4% paraformaldehyde and processed for paraffin embedding. Sections (5 μm) were cut and mounted on the glass slides, and hematoxylin and eosin staining of lung tissues were done. The stained lung sections were processed in parallel. The sections of the right lung were incubated overnight at 4 °C against phospho-ERK1/2, phospho-p38, phospho-MLC20. The levels of proteins were detected with enhanced chemiluminescent substrate (Pierce, Rockford, IL, USA). The relative gray levels were evaluated using software.

Tension measurements

Isolated PA ring preparation

Normoxic and HPH rats were anesthetized with pentobarbital (150 mg/kg ip). After median sternotomy was performed, the heart and lungs were removed and placed in ice-cold oxygenated Krebs-Henseleit (KH) solution containing (in mM) NaCl 127, KCl 4.7, NaHCO3 17, MgSO4 1.17, KH2PO4 1.18, CaCl2 2.5, d-glucose 5.5. Under a dissecting microscope, the third-division intrapulmonary arteries were isolated carefully and cut into 2- to 3-mm-long rings. Each pulmonary ring was suspended on steel hooks connected to force transducers for isometric force recording in the chamber containing KH solution sparged continuously with 95% O2/5% CO2 and maintained at 37 °C. Force displacement was recorded using a PowerLab (AD Instruments) eight-channel data acquisition system.

Studies on the effect of MIF

Suspended pulmonary artery rings from normoxic and HPH rats were studied in parallel. The rings were stretched to a predetermined optimal passive tension of 750 mg and equilibrated for 60 min with washouts at 20-minute intervals. Then reproducibility of contractile responses to 1 μM phenylephrine (~300 mg) was established. Endothelial integrity of each artery ring was confirmed by demonstrating whether acetylcholine (1 μM)–induced relaxation of intact pulmonary vessel was adequate (~80% of phenylephrine-induced tone) or not (~20% of phenylephrine-induced tone). After washout of acetylcholine, PA rings were constricted with phenylephrine. In another group, MIF antagonist ISO-1 (50 μM) was added to the organ baths 30 min before the onset of phenylephrine.

In separate experiments, after phenylephrine was added to establish a stable contractile tone of pulmonary artery ring from normal rat, hypoxia was induced by changing the gas to 95% N2/5% CO2. Experiments were terminated after 60 min of hypoxia. This protocol was performed in the presence of the ISO-1 (50 μM). ISO-1 was added to the organ baths 30 min before the onset of phenylephrine.

In other pulmonary artery rings from normal rats, when a stable constriction evoked by phenylephrine was attained, concentration–response curve was constructed by cumulative addition of MIF (0.1–1000 ng/ml) at 10-minute interval on the rings. We also removed endothelium by gently rubbing the lumen in some rings to test whether the effect of MIF on the rings is endothelium-dependent. 100 ng/ml MIF was administrated after the constriction induced by phenylephrine reached a plateau level. We also used several inhibitors to determine the intracellular signal pathways in the action of MIF. These protocols were performed in the presence of the protein kinase C (PKC) inhibitors chelerythrine (10 μM) or ERK1/2 inhibitor U0126 (10 μM) and p38 inhibitor SB

203580 (10 μM). All inhibitors were incubated for 20 min after the constriction induced by phenylephrine reached a plateau level.

Statistical analysis

Hemodynamic values were presented as mean±S.D. Force displacement was expressed as percentage change from the amount of phenylephrine preconstriction and shown as mean±S.E.M. Paired Student’s t-test and analysis of variance (ANOVA) were used to compare the differences between treatments. A p value less than 0.05 was considered statistically significant.

Results

Hemodynamic variables and histological remodeling

Compared with the control, a significant increase in RVSP was observed in rats exposed to intermittent hypoxia for 28 d (Table 1). A significant increase in the ratios of RV/[LV+S] was also observed in these rats, indicating that right ventricular hypertrophy had developed (Table 1). The pulmonary arterial wall thickness, destruction of alveolar structure and inflammatory cell infiltration were markedly increased in HPH rats compared with that in control rats (Fig. 1A). Also, the medial wall thickness (MT)% (Fig. 1B) and the medial wall area (MA)% (Fig. 1C) of the arteries was significantly elevated after chronic hypoxia exposure.

Protein expression of MIF

Positive immunoreactivity for MIF was observed in the smooth muscle of intrapulmonary arteries (Fig. 2A). MIF expression in PA of HPH rats was more than that in normal rats (n=6). Western blot also showed that MIF protein expression was increased obviously in the pulmonary artery of HPH rats compared with that in normal rats (n=6, P<0.05) (Fig. 2B), which was consistent with the immunohistochemical results. In Fig. 2B(b), bar graph shows MIF signal intensity obtained from quantitative densitometry analysis of 6 independent experiments. The expression of the MIF is normalized to the levels of β-actin.

Role of MIF on pulmonary vasoconstriction

We measured the effect of MIF on pulmonary arterial rings when exposed to chronic and acute hypoxia respectively. Chronic hypoxia enhanced the pulmonary vascular response to phenylephrine (n=6, P<0.01), but the vasoreactivity was attenuated by pretreatment with ISO-1 (Fig. 3). We also gassed phenylephrine-precontracted normal PA with 95% N2/5% CO2, with or without ISO-1

Table 1

Hemodynamic variables.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>RVSP (mm Hg)</th>
<th>RV/[LV+S]</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>22±0.6</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>10</td>
<td>49±2.5*</td>
<td>0.41±0.03*</td>
</tr>
</tbody>
</table>

Values are means ± S.D. RVSP = right ventricular systolic pressure. RV = right ventricle. [LV+S] = [left ventricle + septum].

* P<0.05 compared with corresponding value in control rats.

Fig. 1. Effects of hypoxia on pulmonary artery morphology. A: The lung sections were stained with HE staining; B: Medial wall thickness (MT%) of pulmonary arteries; C: Medial wall area (MA%) of pulmonary arteries. n=50. **P<0.01 vs. control.

pretreatment. As shown in Fig. 4A, the response to hypoxia in isolated PA is biphasic: a rapid, transient vasoconstriction lasting about 10–15 min and a slowly developing vasoconstriction. Maximum constrictions were measured as the differences between the highest and lowest force displacements during hypoxia and expressed as a percentage of the precontractile force. The maximum early constriction of PA was 38.1±5.7%, and the maximum delayed constriction was 72.2±8.7%. MIF inhibition significantly attenuated delayed hypoxic constriction, but not early (Fig. 4B). The maximum early constriction was 30.1±3.1%, whereas the maximum delayed constriction was 41.2±6.9% (Fig. 4C) (n=8, \( P<0.01 \)).

**Effect of MIF on agonist-induced PA vasoconstriction**

The experiments were performed in endothelium-intact PA rings. MIF had no detectable effect on the rest tone of the rings on its own (Fig. 5B). However, MIF concentration-dependently caused a substantial developing potentiation of the constriction elicited by phenylephrine (1 \( \mu \)M) under normoxic condition (Fig. 5A), with maximum effect of 26.8±1.6% at 100 ng/ml (n = 10, \( P<0.01 \)). Fig. 5B(c) depicts a typical trace of the rat PA rings to 100 ng/ml MIF.

As Fig. 6 showed, that MIF-induced potentiation of phenylephrine-evoked constriction was not dependent on an intact endothelium, since similar level of constriction was achieved in PA rings with or without functionally intact endothelium (n = 7).

**Effects of inhibitors on the potentiation induced by MIF**

We tested the effect of inhibitors of PKC, ERK1/2 and p38 on the potentiation induced by MIF under normoxic condition respectively. As shown in Fig. 7, PA were incubated with chelerythrine or U0126, SB 203580 for 20 min, MIF-induced potentiation of phenylephrine-elicted constriction was significantly reduced by 86%, 68% and 83% respectively (n = 8, \( P<0.01 \)).

Next, we detected whether MIF could activate phosphorylation of ERK1/2, p38 and MLC20 in vitro. We found that 100 ng/ml MIF-induced ERK1/2, p38 and MLC20 activation was increased at both 30 min and 60 min (Fig. 8). Treatment with chelerythrine (10 μM), MIF-induced such protein activation was attenuated markedly.

Discussion

Sustained hypoxic pulmonary vasoconstriction may lead to vascular remodeling and pulmonary hypertension, but the underlying mechanisms are not clearly elucidated. We prepared the rat model of HPH as the previous papers reported (Wang et al., 2010; Xu et al., 2010). It was observed that intermittent hypoxia exposure caused remarkable changes in the functional properties of pulmonary vascular smooth muscle. The hypoxic regime did eventually lead to marked right ventricular hypertrophy and pulmonary arterial elevations. Moreover, we found that MIF, an important pro-inflammatory mediator, was increased obviously in smooth muscle of PA rings from HPH rats compared with that of normal rats. Besides, MIF modulated pulmonary vasoconstriction through enhancing vasoconstriction elicited by agonist, which PKC, p38 and ERK1/2 signal pathways may be involved in it.

Changes in pulmonary vasoconstriction are important in the development of HPH. Firstly, we investigated the role of MIF in pulmonary vasoconstriction when exposed to hypoxia. In our study, we found that chronic hypoxia enhanced the pulmonary vascular response to phenylephrine, but the vasoreactivity was attenuated by pretreatment with MIF antagonist ISO-1. It is reported that ISO-1 inhibition of this MIF catalytic site suppresses the cytokine pro-inflammatory activities and significantly increases the survival rate in severe sepsis induced by endotoxin (Al-Abed et al., 2005). In isolated pulmonary arteries, we know that acute hypoxia causes PA vasoconstriction pre-evoked by agonist which is typically biphasic, characterized by a large but transient first phase and a sustained and often gradually developing second phase [18]. Our results showed that MIF inhibition significantly attenuated delayed

pulmonary vasoconstriction, but not early, when exposed to acute hypoxia. The above finding indicated that MIF may play a role in hypoxic pulmonary vasoconstriction and contribute to pulmonary hypertension.

Next, we examined how MIF modulated vasoconstriction of PA. Interestingly, MIF could not induce constriction of rings on its own, but concentration-dependently potentiate constrictions pre-evoked by phenylephrine in normoxic condition. Many factors like vasoconstrictors, such as catecholamine, contribute to the pulmonary vasoconstriction during chronic hypoxia. MIF-induced potentiation of constriction evoked by phenylephrine may be due to augmentation of PA sensitization in response to vasoconstrictors under hypoxic condition, which may be one of the mechanisms of MIF modulating hypoxic pulmonary vasoconstriction. MIF can directly or indirectly induce expression of inflammatory cytokine such as TNF-α, IL-6 (Calandra and Roger, 2003; Morand, 2005). It is reported TNF-α, IL-6 induced the constriction of the arterial segment (De Salvatore et al., 2003; Liu et al., 1992). Additionally, IL-6 modulates arterial vascular tone via endothelium-dependent mechanisms. Therefore, we tested whether MIF-induced potentiation of tension is dependent on the endothelium. In our experiments, removal of endothelium failed to affect the vasoreactivity of MIF, implying that other endothelial-derived mediator might not contribute to the MIF-induced potentiation of constriction, and the effect of MIF on the non-small intrapulmonary arteries was not dependent on an endothelium.

Several investigators have demonstrated that PKC may have a central role in modulating hypoxic pulmonary vasoconstriction (Goirand et al., 2003; Tsai et al., 2004; Weissmann et al., 1999). PKC is a regulatory enzyme activated by numerous effectors, growth factors, hormones, and neurotransmitters. Studies showed that MIF activates PKC in the migration of inflammatory cells and upregulation of matrix metalloproteinases 2 in synovial fibroblast (Onodera et al., 2004; Pakozdi et al., 2006). Harnett and Biancani (2003) reported that agonist-activated contractile pathway of circular muscle from the esophageal body is mediated by activation of the PKC. This contractile pathway depends on activation of the ERK1/2 and p38 MAP kinase, resulting in MLC20 phosphorylation and contraction. In the present study, we found that MIF-induced potentiation of constriction in PA...
was significantly inhibited when the rings were incubated with the inhibitor of PKC chelerythrine, which suggested that PKC activation contributes to the MIF enhanced vasoconstriction. MIF signaling is known to activate ERK1/2 (Mitchell et al., 1999). Stojanović et al. (2009) found that the effect of MIF on interleukin (IL)-17 production in lymph node cells was dependent on p38 and ERK. Our results showed that MIF-induced potentiation of constriction was significantly reduced when the rings were incubated with ERK1/2 inhibitor U0126 or p38 inhibitor SB 203580. In vitro, MIF could activate the phosphorylation of p38, ERK1/2 and MLC20, which was inhibited by PKC inhibitor, confirming that MIF could elicit PKC pathway in smooth muscle cells. Considering the above, we speculate that PKC, p38 and ERK1/2 pathway may be involved in the potentiation of PA vasoactivity evoked by MIF.

Study limitations

1. Although our study showed that chronic hypoxia lead to increased expression of MIF in the pulmonary artery wall, MIF may be involved in hypoxia-induced constriction of PA and contribute to hypoxic pulmonary vasoconstriction, it was not enough to represent MIF-mediated PA remodeling in vivo. (2) MIF possess various biological properties, in which many signal pathways are implicated, including tyrosine kinases, MAPKs, PKC, phosphoinositid 3-kinase (PI3K) and Rho kinases. Whether other signal pathways contribute to MIF modulating PA constriction needs to be further investigated.

Conclusions

In summary, we report the finding that MIF expression is increased in PA of HPH rats, and it is also proved to have a role in modulating hypoxic pulmonary vasoconstriction through enhancing vasoconstriction elicited by agonist in an endothelium independent manner. PKC, p38 and ERK1/2 signal pathways may be involved in the vasoreactivity of MIF. MIF may be considered a new player in the PA vasoconstriction underlying the development of HPH. However, the full characterization of the MIF in pulmonary vasoconstriction and its role in HPH requires future investigation.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Zhi-Chao Li designed the study and performed analyses. Bo Zhang performed the experiment, interpreted the data, and wrote the manuscript. Ying Luo and Man-Ling Liu performed experimental measurements and helped to write and revise the manuscript. Jing Wang and Dun-Quan Xu helped with the animal experiment and experimental measurements. Ming-Qing Dong, Yi Liu and Min Xu carried out immunohistochemistry and western blot assay. Hai-Ying Dong, Peng-Tao Zhao performed experimental measurements and helped to write the manuscript. Yu-Qi Gao reviewed the manuscript. All authors read and approved the final manuscript.

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References


