Hypoxia-Induced HMGB1 Secretion Is Crucial in Airway Inflammation

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INTRODUCTION

Nuclear high-mobility group box 1 (HMGB1) functions as a DNA chaperone.1 In the extracellular space, HMGB1 binds to transmembrane receptors, including Toll-like receptor (TLR) 2 and TLR4 and the receptor for advanced glycation end products (RAGE).2 Binding of HMGB1 to receptors triggers activation of proinflammatory signaling pathways, and induces pleiotropic effects depending on the cell type.3 Recently, not only extracellular HMGB1 but also cytoplasmic HMGB1 has been found to function as an autophagy regulator and a chaperone-like molecule. And the translocation of HMGB1 from nucleus to the extracellular area was dependent on the post-translational modifications such as, phosphorylation, acetylation and oxidation. In our previous study, we proved that ROS was important in translocation of HMGB1. HMGB1 may play a role in upper airway inflammatory diseases that originate in a hypoxic tissue environment, however, no studies have investigated the role of HMGB1 in the upper airway under hypoxic conditions. Thus, the current study was undertaken to investigate the effects of hypoxia on the expression and translocation of HMGB1 protein in upper airway epithelium and to determine the underlying mechanism behind this expression and translocation following exposure to hypoxic conditions in upper airway epithelium. Furthermore, we sought to unveil the role of HMGB1 protein in hypoxia-associated upper airway inflammatory diseases.

Materials and Methods

Human sino-nasal mucosa and nasal secretions were obtained from patients who underwent endoscopic sinus surgery (ESS). Total 35 nasal secretions and sino-nasal mucosa samples were collected before surgery. All patients gave informed consent before surgery. Patients with a history of atopy/allergic rhinitis and who had taken corticosteroid in the previous 4 weeks were excluded from our study.

RESULTS

Fig 1. HMGB1 is secreted into primary upper airway epithelial cells (a) NHNE cells were incubated under hypoxic conditions and real time PCR was performed to evaluate relative amount of HMGB1 mRNA. (b) NHNE cells were incubated under hypoxic conditions and Western blot assay was performed to evaluate the relative amount of HMGB1 protein. (c) Relative band intensities of HMGB1 protein in whole cell lysates were calculated and compared between control and hypoxic conditions (N=3). (d) NHNE cells were incubated under hypoxic conditions and then immunofluorescence assay was performed for HMGB1 (red) and with DAPI (blue) for DNA. Arrows indicate cytoplasmatic HMGB1 protein. (e) Apical culture supernatants of NHNE cells were harvested and concentrated. A Western blot assay was performed to compare the HMGB1 protein level secreted into the extracellular space. (f) Relative band intensities of HMGB1 protein in culture supernatants were calculated and compared between control and hypoxic conditions (N=3). *, P<0.05.

Fig 2. HMGB1 is secreted into extracellular area through hypoxia induced ROS (a) NHNE cells that were incubated under hypoxic conditions in the presence or absence of NAC treatment were stained with DCFDA for 20 min at 37°C, and stained cells were visualized by confocal microscopy. (b) Mean fluorescence was quantified using Image J software. Results are shown as mean ± SEM (N=3). *, P<0.05. (c) NHNE cells were incubated under hypoxic conditions in the presence or absence of NAC pretreatment, and an immunofluorescence assay was performed for HMGB1 (green) and DAPI staining (blue) for DNA. (d) Culture supernatants from NHNE cells were harvested and concentrated. A western blot assay was performed to compare the levels of HMGB1 protein secreted into the extracellular space after hypoxia with/without NAC pretreatment. (e) ELISA assay using apical NHNE culture supernatant was performed to compare the levels of HMGB1 protein secreted into the extracellular space after hypoxia with/without NAC pretreatment. (N=3). *, P<0.05.

Fig 3. DUOX2 plays a significant role in hypoxia-induced HMGB1 secretion (a) Gene expression of DUOX1 and DUOX2 relative to GAPDH expression in NHNE cells after transfection with shCont, shDUOX1, and shDUOX2. (b) NHNE cells transfected with shCont, shDUOX1, or shDUOX2 were incubated under hypoxic conditions for 8 h and stained with DCFDA for 20 min at 37°C to visualize ROS. (c) Mean fluorescence of the images in (b) was quantified using Image J software. Results are presented as mean ± SEM (N=3). (d) HMGB1 ELISA assay was performed to compare the levels of HMGB1 protein secreted into the extracellular space after hypoxia with/without DUOX1 or DUOX2 knockdown. (N=3). *, P<0.05; **, P<0.01.

Fig 4. HMGB1 increases IL-8 secretion in NHNE cells after hypoxic stimulation. (a) NHNE cells were incubated under hypoxic condition with/without NAC pretreatment. The amount of IL-8 secreted into apical culture medium was quantified by ELISA. (b) NHNE cells were incubated with mammalian HMGB1 protein (4 μg/ml) for 24 h. Amounts of IL-8 in the apical supernatants produced by NHNE cells were determined by ELISA. Flagellin (100 ng/ml) was used as a positive inducer of IL-8. (c) NHNE cells were incubated under hypoxic conditions for 8 h, and then the apical culture medium was harvested. HMGB1 protein that had been secreted into the apical culture medium was precipitated and depleted using anti-HMGB1 antibody. HMGB1-precipitated culture supernatants were separated, and a western blot assay was performed to confirm the precipitation of secreted HMGB1 protein. (d) NHNE cells were incubated under hypoxic conditions for 8 h, and apical culture medium, which might contain secreted HMGB1 protein, was harvested. This culture supernatant was precleared using protein G-Sepharose or serially precleared and precipitated using anti-HMGB1 antibody to remove secreted HMGB1 protein. Supernatants that were only precleared or that had had HMGB1 removed were applied into NHNE cells for 24 h. Then, IL-8 produced in the apical supernatants by NHNE cells was determined by ELISA. (e) NHNE cells were incubated under hypoxic conditions with or without anti-HMGB1 blocking antibody for 24 h. Apical culture medium was harvested, and IL-8 produced by NHNE cells was determined using ELISA. For all panels, results are shown as mean ± SEM. (N=3). *, P<0.05.

Fig 5. HMGB1 is secreted into extracellular space in hypoxic human sino-nasal mucosa (a) Hypoxic sino-nasal mucosa was obtained from patients whose nasal ostium was totally obstructed in a preoperative CT scan and compared to control mucosa from patients whose natural ostium was not obstructed. Immunohistochemistry was performed to compare expression and location of HMGB1 protein in sino-nasal mucosa. (b) Percentage of nasal epithelial cells that show positivity for cytoplasmatic HMGB1 were calculated. (c) Nasal lavage fluids were collected from a total of 35 patients. Of these, 16 patients had a completely obstructed maxillary sinus opening, and 19 patients had a patent maxillary sinus opening. HMGB1 ELISA was performed to compare the HMGB1 protein secreted into the extracellular space. (d) An ELISA for IL-8 was performed to compare the IL-8 in hypoxic and normoxic nasal lavage fluid samples. For all panels, data are shown as mean ± SEM, and * represents P<0.05.

CONCLUSIONS

HMGB1 is secreted from nasal epithelium under hypoxic conditions both in vitro and in vivo. Furthermore, extracellular HMGB1 induced upregulation of IL-8 via an autocrine or paracrine pathway. These findings suggest that HMGB1 plays a crucial role in the pathogenesis of hypoxia-mediated upper airway inflammatory diseases.

REFERENCES