Increase in Hypotonic Stress-Induced Endocytic Activity in Macrophages via CIC-3

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Extracellular hypotonic stress can affect cellular function. Whether and how hypotonicity affects immune cell function remains to be elucidated. Macrophages are immune cells that play key roles in adaptive and innate immune reactions. The purpose of this study was to investigate the role and underlying mechanism of hypotonic stress in the function of bone marrow-derived macrophages (BMDMs). Hypotonic stress increased endocytic activity in BMDMs, but there was no significant change in the expression of CD80, CD86, and MHC class II molecules, nor in the secretion of TNF-α or IL-10 by BMDMs. Furthermore, the enhanced endocytic activity of BMDMs triggered by hypotonic stress was significantly inhibited by chloride channel-3 (CIC-3) siRNA. Our findings suggest that hypotonic stress can induce endocytosis in BMDMs and that CIC-3 plays a central role in the endocytic process.

INTRODUCTION

Macrophages are multi-functional immune cells present in both peripheral blood and tissues (Cassetta et al., 2011). As innate immune cells, macrophages engulf infectious microorganisms, phagocytose tissue debris and apoptotic parenchymal cells, and repair wounded tissue (Shapiro et al., 2011). Macrophages are also involved in adaptive immunity by acting as antigen-presenting cells (APC) which initiate T cell-mediated immune responses (Medina-Contreras et al., 2011). Activated macrophages secrete a variety of cytokines which mediate immunity and inflammatory responses (Bozza et al., 2012; Liu et al., 2012). Macrophages are also associated with the occurrence and development of many diseases. For example, recent studies have shown a large influx of macrophages during myocardial infarction influence disease progression (Thorp, 2012).

In this study, we investigated the effect of hypotonic stress on macrophage function and report that a hypotonic environment upregulates endocytic activity in macrophages via CIC-3.

MATERIALS AND METHODS

Main reagents

The isotonic or hypotonic solution contained (mM): NaCl 95, KCl 4.5, CaCl₂ 1, MgCl₂ 1, Hepes 5, D-mannitol 110 or 0 (pH 7.4, adjusted with NaOH, 310 or 200 mOsm/kg H₂O)
(Abdullaev et al., 2003; Mohammad-Panah et al.). M-CSF was purchased from PeproTech (USA), and CD16/32 from Biolegend (USA). The fluorescent antibodies (Abs), including FITC-labeled anti-CD80, PE-labeled anti-CD86, PE-cys-labeled MHC class II, and APC-labeled F4/80 were bought from eBioscience (USA). Both TNF-α and IL-10 mouse ELISA kits were from Biolegend (USA), TRizol reagent was from Invitrogen (USA). PrimeScript 1st Strand cDNA Synthesis Kit was from TransGen Biotech (China). Rabbit anti-CIC-3 or rabbit anti-CIC-2 Abs were from Alomone Laboratories (Israel). Anti-GAPDH Ab was from BD Biosciences (USA). FITC-conjugated goat anti-rabbit secondary Ab was from eBioscience (USA).

Cell culture
Female C57BL/6 mice, 6-8 weeks old, were obtained from the Experimental Animal Center of Tongji Medical College, Hua-zhong University of Science and Technology (China). All mice were maintained under specific pathogen-free conditions and the studies were performed according to the guidelines of the Animal Care and Use Committee of Tongji Medical College, Hua-zhong University of Science and Technology (China). BMDMs were generated as previously described with minor modifications (Gong et al., 2012; Park and Bryers, 2012). In brief, bone marrow cells were obtained from the femurs and tibias of C57BL/6 mice at a density of 1 × 10^6/ml in DMEMs medium supplemented with 10% heat-inactivated FBS, penicillin (100 μg/ml), streptomycin (100 μg/ml), then seeded in 6-well plates (2 ml/well). Cultures were added with 10 ng/ml M-CSF. The cells were incubated for 7 days, and the medium were changed at the day 3 and 5 by aspirating 75% of the medium and adding back fresh medium containing 10 ng/ml M-CSF. BMDMs were harvested by trypsinization, and used for experiments. The cells were characterized as closely adherent mononuclear cells which expressed high levels of F4/80.

The BMDMs were stimulated by isotonic or hypotonic solution for 15 min, then cultured in normal environment (37°C, 5% CO_2) for the following experiments.

Apoptosis detection
Apoptosis was determined by annexin V-FITC/PI staining using flow cytometry. BMDMs were pretreated with hypotonic solution or isotonic solution for 15 min respectively. Thereafter, cells at a density of 1 × 10^6 were collected, centrifuged and washed with PBS for two times. Binding buffer was then added to each tube and cells were re-suspended. The re-suspended cells were incubated with 5 μl annexin V-FITC and 10 μl of PI for 15 min at room temperature in the dark. Then, the percentage of apoptotic cells was determined by using flow cytometry. BMDMs which pretreated with isotonic served as control group.

Pinocytosis and phagocytosis assay
To measure the pinocytotic activity of BMDMs, a previously reported method was used with little modifications (Tamura et al., 2009). Briefly, BMDMs (1 × 10^6/ml) were pre-incubated with or without a hypotonic solution for 15 min and incubated with FITC-dextran (1,000 μg/ml) for 3 h at 37°C in normal environment. Cells were washed twice with PBS. Cells were collected and analyzed on BD LSR-II flow cytometer. The mean fluorescence intensity (MFI) of cells incubated with FITC-dextran at 0°C was set as fluorescence background. BMDMs which pre-treated with isotonic solution served as control group.

To examine the phagocytosis, cells were treated in the same way with pinocytosis. After hypotonic stimulation, cells were cocultured with IgG-coated latex particle in 1:10 ratio for 5 min at 37°C, and their phagocytosis was compared with the control group. The reaction was stopped by the addition of 2 ml ice-cold PBS, and non-engulfed beads were removed with Accu-tase. Cells were washed four times with cold PBS and fixed in formaldehyde. Fifteen fields with phagocytosis were randomly chosen under light microscopy, and ingested beads of at least 200 macrophages were quantitated. Phagocytosis index (Link et al., 2010) was calculated by a formula: Phagocytosis index = (Σ n Pn)/1000, where n is the number of engulfed particles by a macrophage, and Pn is the percent of cells that phagocytosed n particles.

Expression of CD80, CD86, and MHC II Ag
To check up the influence of hypotonicity on the antigen presenting capability of BMDMs, the expression of CD80, CD86 and MHC class II was measured by using flow cytometry. BMDMs (1 × 10^6/ml) were pre-incubated with or without the hypotonic solution for 15 min, and cultured in normal environment for 24 h. Cells (1 × 10^5) were stained with fluorescent mAbs diluted in PBS containing 1% BSA (FACS buffer). Before staining, cells were incubated with purified anti-mouse CD16/32 for 10 min on ice to block FcγR. The following Abs were used: FITC-labeled anti-CD80, PE-labeled anti-CD86, PE-cys-labeled MHC class II, and APC-labeled F4/80. The cells were incubated for 30 min at room temperature, washed twice with FACS buffer and analyzed by LSR II flow cytometer (BD Bioscience, USA). The results were shown as mean fluorescence intensity (MFI). BMDMs which pretreated with isotonic solution served as control group.

Cytokine production
The effect of hypotonicity on IL-10 and TNF-α production was determined by using ELISA.

Briefly, BMDMs (2 × 10^6/ml) were pre-incubated in the presence or absence of the hypotonic solution for 15 min, then in isotonic solution for 6 h. Supernatants were harvested for analyzing the levels of TNF-α and IL-10 by mouse ELISA kits. 100 ng/ml LPS (sigma, USA) was used as a positive control. BMDMs which pretreated with isotonic solution served as control group.

RT-PCR
BMDMs were generated as previously described. After treated with hypotonicity, cells were used for the following test. Total RNA was isolated from BMDMs using TRizol reagent according to the manufacturer’s instructions. cDNA synthesis was performed using a PrimeScript first Strand cDNA Synthesis Kit. PCR amplifications of cDNA were performed by standard methods. The heart tissue obtained from the same mouse with BMDMs, and act as a positive control. The following specific mouse primers (forward and reverse) were used:

- ClC-2 (Enz et al., 1999) 5′-CAG TTT CCT CTC CCT CTT G-3′; 5′-GAA CTG TCC AAA GGC AGG G-3′; CIC-3 (Okamoto et al., 2008) 5′-TGG GTT GTC TCT GGT GGT TAT TG-3′; 5′-GAA AGA GAT GGA GTA TGC TG-3′; GAPDH, 5′-AAT CTT CCA AGT ATG ATG AC-3′; 5′-TAC CAG GAA ATG AGC TTG AC-3′; GAPDH was used for normalization. PCR products were analyzed by 2% agarose gel electrophoresis.

Western blot
After treated with hypotonicity, BMDMs were harvested as previously described and were used for the following test. BMDMs were lysed in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl containing 1% Nonidet P-40, 0.02% NaN3, 0.5 mM PMFS, 1
Fig. 1. Hypotonic environment promotes endocytosis in BMDMs. (A) The purity of BMDMs analyzed by flow cytometry. IgG was used as an isotype control. (B) Apoptotic fraction of cells detected by annexin V staining (x-axis)/propidium iodide staining (y-axis) after treatment with hypotonic solution. (C, D) Gray fill represents hypotonic group. (C) Pinocytotic activity of BMDMs in hypotonic environment shown by mean fluorescence intensity (MFI) of cells incubated with FITC-dextran. (E, F) Uptake of IgG-coated latex beads analyzed by light microscopy and calculated as phagocytosis index. Photos taken from a representative experiment. The data represent mean ± SD from five independent experiments (n = 5 for each group). ***P < 0.001 for group comparisons.

Fig. 2. Expression of CD80, CD86, and MHC class II molecules in BMDMs was not affected by hypotonic stress. (A) Histograms from representative experiments. Gray fill represents hypotonic group. (B) MFI values of CD80, CD86, and MHC class II analyzed by flow cytometry (n = 5 for each group).

mg/ml aprotinin, and 1 mg/ml leupeptin). The whole-cell lysate was mixed with 5× SDS loading buffer and boiled for 10 min. The proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% (w/v) nonfat milk in TBST for 2 h at 37°C and then incubated with rabbit
anti-CIC-3 or rabbit anti-CIC-2 Abs, as well as mouse anti-
GAPDH antibody (serving as a loading control) diluted in 5% 
(w/v) nonfat milk in 0.1% TBST overnight at 4°C. After washing
three times (10 min each) in 0.1% TBST, the membranes were
incubated with peroxidase-conjugated goat anti-rabbit second-
ary antibody for 2 h at 37°C. The membrane was washed three
times in 0.1% TBST, and the proteins were detected using Peroxide Solution with a Kodak Image Station 4000MM.

For negative controls, the specificity of anti-CIC-3 antibody (CICn3) was confirmed by incubating the membrane fraction with the antigen pre-absorbed antibody. The antigen of CIC-3 with a molecular weight of 35 kDa was provided by Alomone labs.

**Immunocytochemistry**
The immunofluorescence was used to determine the localization of CIC-3 in BMDMs. First, BMDMs were cultured on glass coverslips, washed with PBS once, fixed with 4% paraformaldehyde in PBS for 20 min, washed three times for 5 min in PBS, and then blocked with 3% BSA for 2 h at 37°C. BMDMs were incubated with rabbit anti-CIC-3 primary Abs diluted in 3% BSA overnight at 4°C. After washing three times in PBS, cells were incubated with the FITC-conjugated goat anti-rabbit secondary antibody diluted in 3% BSA for 1 h at room temperature. After extensive washing, coverslips were mounted on glass slides with 50% glycerol. Staining was visualized with an Olympus FV500 confocal microscope.

**siRNA-mediated knockdown of CIC-3**
The 21-nucleotide siRNA (Okamoto et al., 2008) duplexes with two overlapping tRNA nucleotides at the 3′-end targeted to the mouse CIC-3 channel were designed and synthesized by Invi- trogen (USA). The following specific primers (forward and re-
verse) were used: CIC-3, siRNA 5′-CGA GAG AAG UGU AAG 
GAC ATT-3′, 5′-GUU CCU ACU ACA UCU CUC GTT-3′; con-
trol siRNA, 5′-AUC CGC GCC AUA GUA CGU ATT-3′, 5′-UAC 
GUU CUA UCG CGA GGA UTT-3′. siRNAs were transfected with nuclear transfer according to the manufacturer’s instruction.

BMDMs were harvested and transfected with FAM (carboxyfluorescin)-conjugated CIC-3 siRNA. 6 or 72 h after transfection, BMDMs were collected to test transfection efficiency by flow cytomtery and interference efficiency by Western blot. After transfection, BMDMs were stimulated with hypotonic and the endoscopy activity was measured. BMDMs without transfection served as normal control group, and transfected with control siRNA served as control siRNA group.

**Statistical analysis**
Data are presented as mean ± SD. The Student’s t-test with paired comparisons was used to evaluate the differences. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Purity of BMDMs**
To insure sufficient purity of BMDMs, the cells were tested by flow cytomtery and achieved a purity of 92.1% (Fig. 1A).

**Effect of hypotonic solution on apoptosis of BMDMs**
To determine if a hypotonic solution can affect the function of BMDMs and induce apoptosis, the Annexin V-FITC/PI double staining assay was used to detect apoptotic cells. The distribution of early apoptotic cells did not appreciably differ between BMDMs treated with an isotonic v. hypotonic solution (1.71% v. 1.74%, respectively; Fig. 1B).

**Hypotonic environment promotes endocytosis by BMDMs**
Endocytosis is an important function of macrophages. To investig-
tigate the effect of a hypotonic environment on endocytic func-
tion in BMDMs, we examined pinocytosis of FITC-dextran and phagocytosis of IgG-coated latex beads. Both FITC-dextran pinocytosis (Figs. 1C and 1D) and IgG-coated latex phagocyto-
sis (Figs. 1E and 1F) were significantly increased (\( P < 0.001 \)) after stimulation of BMDMs with a hypotonic solution compared to BMDMs in a control isotonic environment (arrows show in-
gested particles/beads).

**Effect of hypotonic solution on expression of CD80, CD86, and MHC class II molecules in BMDMs**
Macrophages act as APCs to trigger adaptive immunity. To fur-
ther examine the effect of a hypotonic environment on the APC function of macrophages, expression of CD80, CD86 and MHC class II molecules in BMDMs was examined by flow cytomtery. As shown in Figs. 2A and 2B, there were no obvious differences between the hypotonic and control groups.

**Effect of hypotonic solution on secretion of IL-10 and TNF-α by BMDMs**
IL-10 and TNF-α are anti- and pro-inflammatory cytokines, respectively, released by activated BMDMs. To examine the effect of a hypotonic environment on secretion of these cyto-
kines, IL-10 and TNF-α were quantified by ELISA. LPS was 
used as positive control. As shown in Figs. 3A and 3B, following stimulation of BMDMs by LPS, levels of both IL-10 and TNF-α were significantly increased in BMDM culture supernatants (\( P < 0.001 \)), whereas levels of either cytokine were unchanged when BMDMs were placed in a hypotonic environment.

**Expression of CIC-3 in BMDMs**
It has been previously reported that CIC-2 and CIC-3, ex-
pressed in diverse tissues including heart (Dick et al., 1999), 
mediate changes in cellular function in a hypotonic environment.
The expression of ClC-3 and ClC-2 mRNA in BMDMs was examined using RT-PCR. The sizes of the expected PCR products for ClC-3 and ClC-2 were 456 and 499 bp, respectively. We found that only ClC-3 mRNA was expressed in BMDMs, whereas both transcripts were expressed in heart (Fig. 4A). The expression of ClC-3 and ClC-2 proteins in BMDMs was detected using Western blot and immunocytochemistry. As shown in Fig. 4B, ClC-3 but not ClC-2 was detected in BMDMs. ClC-3 resolved as two bands between 72 and 90 kDa on Western blot, which may reflect glycosylated and non-glycosylated isoforms, and was predominantly expressed in the cytoplasm of BMDMs determined by immunocytochemistry (Fig. 4C). Stimulation of BMDMs by a hypotonic solution did not alter ClC-3 expression, but distribution of the protein changed to accumulate in clusters around the cytomembrane (shown by arrows, Fig. 4F right panel).

Increased endocytosis in BMDMs under hypotonic environment via ClC-3
To investigate if ClC-3 is required for the increased endocytic activity of BMDMs under hypotonic conditions, siRNA was used to suppress the expression of ClC-3. Flow cytometric analysis showed that the siRNA transfection efficiency of control and ClC-3 siRNAs tracked by FAM was 42.6% and 46.4%, respectively (Fig. 5A), while the interference efficiency of ClC-3 siRNA was approximately 50% (Figs. 5B and 5C). Uptake of both FITC-dextran (Fig. 5D) and IgG-coated latex beads (Figs. 5E and 5F) in hypotonia-exposed BMDMs was significantly decreased in the group transfected with ClC-3 siRNA compared to the normal control and control siRNA groups (P < 0.001).

DISCUSSION
Macrophages play an important role in innate immunity and adaptive immune responses, including destruction of pathogens, endocytosis of antigens, and possible mediation of tumor development (Torr et al., 2012). Endocytosis is an important macrophage function. Studies to date have shown that endocytosis in macrophages is regulated by many factors, including cytokines (Parveen et al., 2013; Roger et al., 2013), medicines (Li and Liu, 2005) and extracellular acidosis (Kong et al., 2013). Utilizing endocytosis, macrophages can eliminate invading micro-organisms and remove necrotic tissue and cells. Our results suggest that hypotonic stress may enhance the endocytic activity of macrophages.

In the present study, we examined the impact of extracellular hypotonicity on BMDMs and investigated the mechanism by which hypotonic stress regulates their function. First, we
examined the effect of hypotonic stress on endocytosis in BMDMs. Recent work has shown that during ischemia-reperfusion episodes, the local tissue microenvironment becomes hypotonic, producing additional tissue damage and cell death (Okada et al., 2004; 2009). Macrophages are known to play a role in the immunopathology of ischemia-reperfusion injury (Zhang et al., 2013), but the extent to which hypotonic stress affects macrophage function is unknown. In our experiments, FITC-dextran was used to detect pinocytosis, while IgG-coated latex beads were used to monitor receptor-mediated endocytosis in BMDMs. Our results showed that extracellular hypotonic stress increased both pinocytosis and receptor-mediated phagocytosis in BMDMs.

CD80, CD86 and MHC-II molecules are known markers of macrophage maturation. To examine the effect of hypotonic stress on BMDM maturation, we quantified expression of these molecules during hypotonic stress in BMDMs. Our results showed that extracellular hypotonic stress increased both pinocytosis and receptor-mediated phagocytosis in BMDMs. CD80, CD86 and MHC-II molecules are known markers of macrophage maturation. To examine the effect of hypotonic stress on BMDM maturation, we quantified expression of these molecules during hypotonic stress. After the exposure of BMDMs to hypotonicity, levels of CD80, CD86 and MHC-II secreted into BMDM cell culture supernatants were similar in the hypotonic and control groups. Activated macrophages also secrete different cytokines, two of which, IL-10 and TNF-α, are inhibitory and stimulatory cytokines, respectively. Our results showed that after exposure to hypotonic stress, IL-10 and TNF-α levels in BMDM culture supernatants were unchanged.

The mechanism by which BMDMs sense extracellular hypotonic stress is unclear. Recent work has focused on the role of ion conduction pathways in lymphocyte function and immunity. Our group has shown that acid sensing ion channels are crucially involved in the modulation of BMDMs and contribute to the effect acidosis imposes on dendritic cells (Kong et al., 2013; Tong et al., 2011).

In our study, we examined ion channels ClC-2 and ClC-3, members of the chloride channel superfamily. These channels play important roles in the regulation of cellular excitability, cell volume regulation, and acidification of intracellular organelles (Tang and Chen, 2011). Previous studies have reported that ClC-2 and ClC-3 mediate functional changes induced by hypotonicity. For example, ClC-2 was shown to control the functional response of trabecular meshwork cells to a hypotonic environment (Comes et al., 2005). A more recent study using an induc-
ible heart-specific ClC-3 knockout mouse found that inactivation of ClC-3 gene produced myocardial hypertrophy and heart failure (Xiong et al., 2010). In our study, we confirmed the expression of ClC-3 in BMDMs by RT-PCR, Western blot and immunocytochemistry. We used the Cl channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (data not shown) and ClC-3 siRNA to investigate the role of ClC-3 in mediating the effects of extracellular hypotonicity on the biological behavior of BMDMs. We found that ClC-3 siRNA abrogated the increase in endocytosis induced by hypotonic stress. These data provide strong evidence that ClC-3 is crucially involved in stimulating endocytic activity in BMDMs by hypotonic stress.

Our results also showed that stimulation of BMDMs by a hypotonic solution had no significant impact on ClC-3 expression but rather altered distribution of the protein into clusters of accumulation around the cytomembrane. Two explanations may account for this phenomenon. On the one hand, our results were similar to findings in neurons and mouse osteoclasts where ClC-3 expression predominantly localized to endosomal compartments in the cytoplasm (Stobrawa et al., 2001). We propose that in BMDMs ClC-3 may be located to lysosomes where it functions in lysosome acidification. The exchanger may produce a Cl- current into a subcellular vesicle, reducing the potential difference caused by electrogenic H+ transport. On the other hand, ClC-3 is an important component of volume-sensitive outwardly rectifying chloride channels (VSORs) (Hermoso et al., 2002). VSORs are ubiquitously expressed and involved in cell volume regulation after osmotic swelling, called regulatory volume decrease, in various cell types (Inoue et al., 2010; Min et al., 2011). The hypotonicity-induced relocation of ClC-3 we observed in BMDMs may be related to endocytosis. We hypothesize that intracellular ClC-3 may act as a cell volume sensor and translocate to the cell membrane in response to cellular swelling induced by hypotonic stress. Consequently, VSORs become activated and redundant materials are discharged from the BMDMs in order to increase macrophage endocytic capacity and efficiency.

To exclude the possibility that apoptosis in BMDMs induced by hypotonic stress resulted in increased endocytic activity, we carried out flow cytometric apoptosis detection. Our results showed that apoptosis was nearly undetectable in BMDMs treated with the hypotonic solution used in our study. The endocytic activity of BMDMs induced by hypotonic stress thus appears to have no relationship to apoptosis.

Recently, studies have shown that ClC-3 is necessary for activation of smooth muscle cells by TNF-α. Deficiency in ClC-3 markedly reduced neointimal hyperplasia following vascular injury (Chu et al., 2011). Our investigation showed that ClC-3 is also involved in macrophage function. Control of ClC-3 may represent a novel therapeutic target to prevent clinical blood vessel damage. Furthermore, intraperitoneal chemotherapy with hypotonic perfusion is an important technique for the prevention of postoperative retroperitoneal tumor relapse. The mechanism underlying this therapy is thought to involve increased permeability of the tumor cell membrane in response to hypotonicity, rendering tumor cells more sensitive to chemotherapy and thus more easily killed. Our study suggests a new pharmacodynamic mechanism in which the endocytic ability of macrophages is enhanced by a hypotonic environment, allowing macrophages to engulf tumor cells and debris more efficiently.

In summary, the increased endocytic ability of BMDMs in a hypotonic environment appears to require ClC-3, which may depend on VSORs and organellar acidification.

ACKNOWLEDGMENTS
This work was supported by grants from the National Natural Science Foundation of China (No. 81273221 and Major State Basic Research Development Program of China (973 Program) (No. 2013CB530505).

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