Inhibitory Effect of High Molecular Weight Water-Soluble Inflammatory Cytokine Production

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Chitosan is widely used to treat patients with hypoxia-induced diseases such as ischemia, neuronal death, cerebral stroke, and cerebral infarction. Using the ELISA method, we examined the effect of high molecular weight water-soluble chitosan (WSC) on inflammatory cytokine production in the desferrioxamine (DFX, known to mimic hypoxia)-stimulated human mast cell line HMC-1. DFX significantly increased interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α production compared with the control in a time-dependent manner (p < 0.05), but did not affect IL-1β production and mRNA expression. The increase in IL-6, IL-8, and TNF-α levels was significantly inhibited by WSC in a dose-dependent manner with IC50 values of 0.77, 0.88, and 2.5 μg/ml, respectively. The maximal inhibition rate of IL-6, IL-8, and TNF-α production by WSC was 64±9.7%, 80±4.9%, and 54±4.5%, respectively. In addition, WSC inhibited DFX-induced activation of nuclear factor (NF)-κB. In conclusion, these results suggest that WSC is an inhibitor of NF-κB under hypoxic conditions, which might explain its beneficial effect in the treatment of hypoxia-induced inflammatory diseases.

Key words: chitosan; inflammatory cytokine; nuclear factor kappa B

Hypoxia is an essential developmental and physiological stimulus that plays a key role in the pathophysiology of heart disease, cancer, neuronal death, cerebrovascular disease, and chronic lung disease, which are the most common causes of mortality in Western cultures.1–4 Hypoxia-induced diseases are mediated by inflammatory cytokines. For example, interleukin (IL)-1 and tumor necrosis factor (TNF)-α are involved in transient forebrain ischemia in the rat.2,3 IL-1, IL-6 and TNF-α are produced in the ischemic rat cortex.4 Hypoxia also results in the production of the chemotactic cytokine IL-8 in endothelial cells. IL-8 is a potent activator and chemotaxant of neutrophils and mediates their migration to local inflammatory sites.5 Although increased levels of several cytokines in hypoxia have been reported, the mechanism involved in secretion of cytokines from hypoxia-stimulated mast cells has not been examined.

Mast cells are widely distributed in the connective tissues of mammals and other vertebrates, where they are frequently located in close proximity to blood vessels.6 Activated mast cells can produce histamine as well as a wide variety of other inflammatory mediators such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines such as TNF-α, IL-1, IL-4, IL-6, IL-8, IL-13 and tumor growth factor (TGF)-β1.7–9 In chronically hypoxic rats, histamine-containing mast cells increase and contribute to a significant extent to acute hypoxia-induced injury.10,11 Nuclear factor (NF)-κB is a key transcription factor required for the expression of many inflammatory genes, including IL-1, IL-6, IL-8, and TNF-α. NF-κB is composed of homo- and heterodimeric complexes of members of the Rel protein family comprising Rel A (p65), p50, c-Rel, p52, and Rel B. NF-κB normally resides in the cytoplasm, where it’s retained by association with the IκB protein (α, β, γ), an endogenous inhibitor. However, when activated it translocates to the nucleus, binds to DNA, and activates genes.11–13 Chitosan is prepared by chemical N-deacetylation of chitin. Recent developments have enabled the use of these polymers in the medical and veterinary fields. Chitosan has been observed to accelerate wound-healing properties and cytokine production.14 Water-soluble chitosan (WSC) has higher reactivity than water-insoluble chitosan, but its pharmacological mechanisms have not yet been well defined. The iron chelator desferrioxamine (DFX) is capable of inducing NF-κB activation and hypoxia-dependent gene expression, probably by replacing or removing the central iron of the putative heme oxygen sensor.15–16 The purpose of this study was to determine whether the activation of human mast cells by DFX results in IL-1α, IL-6, IL-8, and TNF-α production and whether high-molecular-weight WSC can inhibit the production of these cytokines.

MATERIALS AND METHODS

Reagents Fetal bovine serum, Iscove’s modified Dulbecco’s medium (IMDM), ampicillin, and streptomycin were purchased from Gibco BRL (Bethesda, MD, U.S.A.). DFX and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Human recombinant (r) IL-1α, purified anti-human IL-1α, and biotin-conjugated anti-human IL-1α antibodies were purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.). Human rIL-6, IL-8, and TNF-α, purified anti-human IL-6, IL-8 and TNF-α, and biotin-conjugated anti-human IL-6, IL-8, and TNF-α antibodies were purchased from Pharmingen (San Diego, CA, U.S.A.). NF-κB antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture The mast cell line HMC-1 was used in this study. The cells were maintained in IMDM medium (Gibco BRL) with 10% fetal bovine serum (JRH Bioscience,
Rockville, MD, U.S.A.) at 37°C under 5% CO₂ in air. HMC-1 cell suspensions (3×10⁵ cells) were stimulated with DFX for 24 h. The cells were separated from the released cytokines by centrifugation at 400×g for 5 min at 4°C.

Cytokine Assay  A modified ELISA described previously was used to measure cytokines. The ELISA was performed by coating 96-well plates with 6.25 ng/well of human monoclonal antibody with specificity for IL-1α, IL-6, IL-8, and TNF-α. Before use and between subsequent steps in the assay, the coated plates were washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween-20 and twice with PBS alone. All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. The standard curve was generated from a known concentration of cytokine provided by the manufacturer. After exposure to the medium, the assay plates were washed sequentially with the biotin-conjugated secondary antibody (IL-1α, IL-6, IL-8, and TNF-α), avidin peroxidase. Optical density readings were made within 10 min of the addition of the ABTS substrate solution (containing 30% H₂O₂ on the ELISA reader (molecular device) with a 405 nm filter. Appropriate specificity controls were included. All samples were run in duplicate.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis  Total RNA was isolated from HMC-1 cells according to the manufacturer’s specifications using the Easy-BLUE™ RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final eluate was determined by spectrophotometry. Total RNA (2.5 μg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using a cDNA synthesis kit (AmershamPharmacia, Piscataway, NJ, U.S.A.). PCR was performed with the following primers for IL-1α (5’CAC TCC ATG AAG GCT GCA TGG 3’; 5’GGT GTG TCG TTC TGA TGA CCC A 3’), GAPDH (5’CAA AAG GGT CAT CAT CTC TG 3’; 5’CCT GCT TCA CCA CCT TCT TG 3’) was used to verify that equal amounts of RNA were used for reverse transcription and PCR amplification under different experimental conditions. The annealing temperature was 65°C for IL-1α and 60°C for GAPDH. Amplified fragment sizes for IL-1α and GAPDH were 631 bp and 446 bp, respectively. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

MTT Assay  The MTT colorimetric assay of cell survival was performed using the method of Ben Trivedi et al., with minor modifications. Cell aliquots (3×10⁵) were seeded in microplate wells and incubated with 20 μl of MTT solution (5 mg/ml) for 4 h at 37°C under 5% CO₂ and 95% air. Consecutively, 250 μl of DMSO was added to extract the MTT formazan. An automatic microplate reader read the absorbance of each well at 540 nm.

Nuclear Protein Extraction  Preparation of crude nuclear extract was basically as described previously. Briefly, after cell activation for the times indicated, 1×10⁵ cells were washed in 1 ml of ice-cold PBS, centrifuged at 1000×g for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (HEPES/KOH 10 mM, MgCl₂ 2 mM, EDTA 0.1 mM, KCl 10 mM, DTT 1 mM, PMSF 0.5 mM, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15000×g for 30 s. Pelleted nuclei were gently resuspended in 50 μl of ice-cold saline buffer (HEPES/KOH 50 mM, KCl 50 mM, NaCl 300 mM, EDTA 0.1 mM, 10% glycerol, DTT 1 mM, PMSF 0.5 mM, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15000×g for 5 min at 4°C. Aliquots of the supernatant which contained nuclear proteins were frozen in liquid nitrogen and stored at −70°C. Protein was determined using a Biochrominic acid protein assay method (Sigma Chemical).

Western Blot Analysis of NF-κB  Samples were heated at 95°C for 5 min and briefly cooled on ice. Following centrifugation at 15000×g for 5 min, 50 μl aliquots were resolved by 12% SDS-PAGE. Resolved proteins were electro-transferred overnight to nitrocellulose membranes in Tris 25 mM, pH 8.5, glycerin 0.2 m, and 20% methanol at 25 V. Blots were blocked for at least 2 h with 1×TBST containing 10% nonfat dry milk and then incubated with anti-NF-κB antibodies (1 : 500 dilution in blocking solution). After washing in PBS-Tween-20 three times, the blots were incubated with secondary antibodies (1 : 1000 dilution in blocking solution) for 30 min and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp., Newark, NJ, U.S.A.).

Statistical Analysis  The experiments shown are a summary of the data from at least three experiments and are presented as mean±S.E.M. Statistical evaluation of the results was performed using one-way ANOVA with the Tukey post-hoc test. The results were considered significant at a value of p<0.05.

RESULTS

Production of Cytokines in DFX-Treated HMC-1 Cells  HMC-1 cells were treated with DFX 0.1 mM for various time intervals. The constitutive and inducible production of IL-1α, IL-6, IL-8, and TNF-α was demonstrated by the ELISA method. DFX significantly enhanced IL-6 (0.315±0.015 ng/ml), IL-8 (0.686±0.1 ng/ml) and TNF-α (0.226±0.01 ng/ml) production compared with the media control (p<0.05), but did not affect IL-1α production and mRNA expression at various times (Figs. 1A, E). Production of IL-6 appeared 4 h following stimulation, and maximal levels were achieved at 8 h and then slowly declined thereafter (Fig. 1B). IL-8 production increased in a time-dependent manner (Fig. 1C). The production of TNF-α was rapid, reaching a peak at 4 h, revealing a 2.1-fold increase over the control level after DFX treatment, and declining thereafter from 8 to 24 h (Fig. 1D). We used 8 h incubation for optimal cell stimulation in subsequent experiments.

WSC Inhibits DFX-Induced Cytokine Production  To determine whether WSC can modulate DFX-induced IL-6, IL-8, and TNF-α production, the cells were pretreated with various concentrations of WSC for 1 h prior to DFX stimulation. Culture supernatants were assayed for IL-6, IL-8, and TNF-α protein levels using the ELISA method. WSC did not affect IL-6 (unstimulated cells, 0.05±0.007 ng/ml; WSC, 0.049±0.005 ng/ml), IL-8 (unstimulated cells, 0.08 ng/ml; WSC, 0.081±0.001 ng/ml) and TNF-α (unstimulated cells, 0.1 ng/ml; WSC, 0.12±0.03 ng/ml) production in the absence of DFX. However, in DFX-stimulated cells, IL-6, IL-8, and TNF-α production decreased with WSC treatment (about 64±9.7% for IL-6, 80±9.4% for IL-8, and 54±4.5%
for TNF-α). WSC inhibited DFX-induced IL-6 and TNF-α production in a dose-dependent manner with IC50 values of 0.77 and 2.5 μg/ml, respectively. The inhibition rate of IL-8 production tended to increase at low concentrations of WSC, but the inhibitory effect of WSC of IL-8 production became weaker when the concentrations were high. The maximum effective concentration of WSC was 10 μg/ml for IL-6 and TNF-α and 0.1 μg/ml for IL-8 (Fig. 2). Cell cytotoxicity of WSC was not observed.

**WSC Inhibits DFX-Induced NF-κB Activation**

Expression of inflammatory cytokines was regulated by the transcription factor NF-κB/Rel A. The expression level of NF-κB/Rel A in the nucleus was examined by Western blot analysis. In DFX-stimulated cells, the expression level of NF-κB/Rel A increased. However the expression level of NF-κB/Rel A in the nucleus decreased with WSC treatment (Fig. 3A). Rel A protein levels were quantitated by densitometry (Fig. 3B). β-Actin expression level did not change with any treatment in the nuclear extract (data not shown).

**DISCUSSION**

In the present study, we demonstrated that DFX induced proinflammatory cytokine (IL-6, IL-8, and TNF-α) production in HMC-1 cells but did not affect IL-1α production. We also reported that WSC inhibited the DFX-induced proinflammatory cytokine production via blockade of NF-κB activation in HMC-1 cells.
Hypoxia induces myocardial ischemia and cerebral infarction.11 Numerous inflammatory cytokines have been implicated in the development of hypoxia-induced diseases including the IL-1 family (IL-1α, IL-1β, IL-1 receptors, and IL-1 receptor antagonist), IL-6, IL-8, and TNF-α.20,21 First, IL-1 production is increased by hypoxia. Increased IL-1α induces the binding of the heterodimer hypoxia-inducible factor (HIF)-1 to the HIF consensus sequence.22 Liu et al. reported that IL-1α was released by chemical hypoxia (KCN) in PC12 cells.23 Benyo et al. also reported that incubation of human placental explants under reduced oxygen conditions results in increased environmental hypoxia in the dorsocaudal brainstem of rats, which might contribute to the treatment of hypoxia-induced diseases. We also found that WSC effectively inhibits the production of proinflammatory cytokines in DFX-stimulated HMC-1 cells. Therefore we speculate that the in-...