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# Stimulation of phagocytosis by sulforaphane

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ABSTRACT

Sulforaphane, a major isothiocyanate derived from cruciferous vegetables, protects living systems against electrophile toxicity, oxidative stress, inflammation, and radiation. A major protective mechanism is the induction of a network of endogenous cytoprotective (phase 2) genes that are regulated by transcription factor Nrf2. To obtain a more detailed understanding of the anti-inflammatory and immunomodulatory effects of sulforaphane, we evaluated its effect on the phagocytosis activity of RAW 264.7 murine macrophage-like cells by measuring the uptake of 2-μm diameter polystyrene beads. Sulforaphane raised the phagocytosis activity of RAW 264.7 cells but only in the absence or presence of low concentrations (1%) of fetal bovine serum. Higher serum concentrations depressed phagocytosis and abolished its stimulation by sulforaphane. This stimulation did not depend on the induction of Nrf2-regulated genes since it occurred in peritoneal macrophages of *nrf2*$^{-/-}$ mice. Moreover, a potent triterpenoid inducer of Nrf2-dependent genes did not stimulate phagocytosis, whereas sulforaphane and another isothiocyanate (benzyl isothiocyanate) had comparable inducer potencies. It has been shown recently that sulforaphane is a potent and direct inactivator of macrophage migration inhibitory factor (MIF), an inflammatory cytokine. Moreover, the addition of recombinant MIF to RAW 264.7 cells attenuated phagocytosis, but sulforaphane-inactivated MIF did not affect phagocytosis. The inactivation of MIF may therefore be involved in the phagocytosis-enhancing activity of sulforaphane.

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## 1. Introduction

Phagocytosis is a major mechanism of cellular protection and manifestation of inflammatory and immunological responsiveness. The isothiocyanate sulforaphane (SF) is derived from its glucosinolate precursor (glucoraphanin) which is abundant in cruciferous plants such as broccoli [1,2]. There has been intense interest in the chemoprotective capabilities of SF in defending against carcinogenesis initiated by DNA-damaging electrophiles, reactive oxygen intermediates, inflammation, and radiation (UV and ionizing) damage [3–5]. These protective effects, which have been largely attributed to the upregulation of cytoprotective (phase 2) genes, have been demonstrated in systems ranging in complexity from cells in culture to humans. The potential implications of these findings for devising strategies to reduce the risk of chronic disease are widely appreciated, and SF has been identified as a potentially promising new chemoprotective agent.

Inflammation plays a key and critical role in the pathogenesis of many chronic diseases, and SF has now been shown to dampen many inflammatory responses in a variety of experimental systems. Overexpression or abnormal accumulation of inflammatory agents such as chemokines, cytokines, nitric oxide (NO), and prostaglandins (PGs) are at the root of many age-related and chronic diseases. SF has been reported to attenuate the lipopolysaccharide-induced production of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), $PGE_2$, and NO in murine peritoneal macrophages and murine macrophage-like RAW 264.7 cells [6,7]. These inflammatory agents also play critical roles in $T_H2$ (humoral) immune allergic responses such as in atopic diseases and allergic bronchitis. SF also showed protective effects against age-related decrease in $T_H1$ (cellular) immunity [8]. The respiratory system is one of the front lines of both humoral and cellular immunities, and it has been reported that SF protects against disorders induced by xenobiotics including cigarette smoke [9] and diesel exhaust particles [10]. Phagocytic cells such as macrophages act to eliminate foreign materials that accompany inflammation. We therefore asked whether in such systems, in some of which SF had already been shown to suppress inflammation, this isothiocyanate also regulated phagocytosis. For these purposes, we used the established mouse RAW 264.7 macrophage-like cell line, as well as mouse peritoneal macrophages produced in response to an inflammatory stimulus. In both types of cells SF raised phagocytosis activity when fetal bovine serum

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concentrations were low or absent, probably by inactivation of macrophage migration inhibitory factor (MIF) [11] which under some conditions depresses phagocytosis of these cells [12].

## 2. Materials and methods

### 2.1. Materials

Triterpenoid 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (TP-225) [13] was a gift from M.B. Sporn (Department of Pharmacology and Toxicology, Dartmouth Medical School). Endotoxin-free recombinant MIF was from R&D Systems. L-Dopa methyl ester was from Sigma. *RS*-Sulforaphane was obtained from LKT Laboratories.

### 2.2. Cell cultures

RAW 264.7 cells, a macrophage-like, Abelson leukemia virus-transformed cell line from BALB/c mice, were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### 2.3. Peritoneal macrophages

Experimental animal procedures were in compliance with the National Institutes of Health Guidelines and approved by the Johns Hopkins University Animal Care and Use Committee. C57BL/6 background- *nrf2*$^{-/-}$ mice ($n = 4$, female, 17 weeks old) initially established by Itoh et al. [14] and *nrf2*$^{-/-}$ hairless SKH1 mice crossed with the *nrf2*$^{-/-}$ mice in the C57BL/6 background and SKH1 mice ($n = 2$, female, 52 weeks old) were used. The mice were challenged by intraperitoneal injection of 4% thioglycollate solution (3 ml/mouse). After 4 days, the animals were euthanized by cervical dislocation, and the peritoneal cavity was lavaged with two 10-ml injections of cold sterile PBS. After centrifugation of the recovered fluid (5 min at 100*g*), the cell pellet was suspended in RPMI 1640 culture medium (Invitrogen). About $10^7$ cells were obtained from each mouse.

### 2.4. Phagocytosis assay

The method of Link et al. [15] was modified and used to assess the phagocytosis activity. RAW 264.7 cells were plated in 8-well glass slides ($6 \times 10^4$ cells/well; Nalge Nunc International) and incubated for 24 h (37 °C, 5% $CO_2$). Cytoprotective (phase 2) inducers were added in acetonitrile (final concentration 0.1% by volume), and incubation was continued for a further 24 h, unless otherwise stated. The medium was replaced by DMEM containing polystyrene beads (2 μm diameter; Bangs Laboratories) which had been treated for 1 h at 37 °C with mouse IgG (5 mg/ml; Equitech Bio). The ratio of polystyrene beads to cells was 8:1. The chamber slide was incubated at 37 °C for 10 min, nonadherent beads were removed by washing with cold PBS, and cells were fixed with 3.7% formaldehyde for 12 min. Ten to fifteen randomly-selected fields were photographed in each well with a dark-field microscope (TMS; Nikon) and a digital camera (D40; Nikon). The numbers of cells and beads were confirmed with ImageJ (ver.1.43, NIH), a software program for photo-analysis. Phagocytosis activity was expressed as a Phagocytosis Index (PI), which is the number of beads phagocytosed by 100 cells under these conditions. The agreement between the PI measurements by two independent observers who counted the same 21 randomly-selected fields was 99.7 ± 4.0%.

### 2.5. NQO1 assay

NAD(P)H quinone oxidoreductase 1 (NQO1) activity in RAW 264.7 cells was measured by the Prochaska microtiter plate bioassay [16]. Cells were plated into 96-well microtiter plates ($5 \times 10^4$ cells/well). After 3 h at 37 °C, the medium was replaced by DMEM containing serial dilutions of phase 2 inducers in acetonitrile (final concentration 0.1% by volume) and incubated for 24 h. The cells were lysed by digitonin (0.08% in 2 mM EDTA, pH 7.8; 75 μl/well) and aliquots (20 μl/well) were transferred to newly prepared 96-well microtiter plates for measuring cellular proteins by the bicinchoninic acid assay [17]. NQO1 activity was obtained from the initial velocity of formation of the reduced tetrazolium dye in cell lysates with an optical microtiter plate scanner (SpectraMax Plus 384; Molecular Devices). The specific enzyme activity was expressed as the ratio of reaction velocity to protein concentration, and the CD values (Concentration required to Double the NQO1 specific activity) were used to compare inducer potencies.

### 2.6. mRNA determination by quantitative real-time PCR

Quantitative real-time PCR analysis was performed to determine the expression of the *nqo1* gene (7000 Sequence Detection System, Applied Biosystems). Total RNA was isolated from RAW 264.7 cells by a column-based RNA extraction kit (RNeasy Mini kit, Qiagen) and reverse transcribed into cDNA (iScript, BioRad). The cDNA was diluted 50-fold to measure the 18s ribosomal RNA for accurate normalization. The cDNA was subjected to quantitative real-time PCR by using the TaqMan Gene Expression Assay primer probe sets for NQO1 and 18s ribosomal RNA (assay ID Mm00500821_m1 and Hs03928990_g1, Applied Biosystems). Relative expression of the NQO1 message was normalized to the 18s RNA as an endogenous control and the expression rate was calculated using the comparative ΔΔCT method. Average relative mRNA expression and confidence intervals are indicated (number of dishes, $n = 3$).

### 2.7. MIF tautomerase activity assay

RAW 264.7 cells were suspended in DMEM (with 0 or 10% FBS) and plated in 96-well microtiter well plates ($10^5$ cells/well). The plates were prepared in duplicate for measurement of MIF tautomerase activity and cell proliferation. After incubation (37 °C, 5% $CO_2$) for 24 h, the media were recovered and the cells were washed with PBS. Lysis buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, pH 7.4, 1% CHAPS) was added to each well (30 μl/well) and incubated at room temperature for 10 min. Recovered medium and cell lysates (40 μl of medium; 20 μl of cell lysate) were transferred to freshly prepared 96-well microtiter plates for measurement of MIF enzymatic activity, by a modification of the method of Rosengren et al. [18]. L-Dopachrome methyl ester was prepared just before use by mixing L-dopa methyl ester solution (13 mM, 500 μl) and sodium periodate (24 mM, 500 μl) in 19 ml of the reaction buffer (50 mM Bis-Tris, 1 mM EDTA, pH 6.2). Then 200 μl of the L-dopachrome methyl ester solution were added to each well, and the decrease in absorbance at 475 nm was monitored for 10 min after addition of the solution to be assayed. The tautomerase activity was calculated as the maximum velocity of the initial rate of decrease in absorbance, corrected for the nonenzymatic rate (usually about 0.001 per min). A molar absorption coefficient of $3000\ M^{-1}\ cm^{-1}$ at 475 nm was assumed. The specific enzyme activities were normalized by dividing the tautomerase velocity by a measure of cells obtained from MTT reduction assays. Briefly, MTT was added to each well (50 μg/well) of the 96-well plate and incubated at 37 °C for 2 h. Formazan products were solubilized with DMSO (150 μl/well) and diluted 10-fold. The absorbance of

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