EXTENDED ABSTRACT

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Protandim® Treatment Causes Reversible Nuclear Translocation of Nrf2 and Activation of the Antioxidant Response Element

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Abstract

Protandim® is a natural supplement which activates antioxidant protective pathways through induction of the Nrf2 transcription factor. We set out to elucidate the mechanism and kinetics by which this occurs through a series of cell-based assays. AREc32 cells, containing an Nrf2-responsive reporter gene encoding luciferase, were treated with Protandim. A time dependent evaluation of Protandim treatment showed an increase in ARE-controlled reporter gene expression starting at 12 hours, and peaking at 24 hours post-treatment. To better understand the kinetics of Nrf2 subcellular localization, immunofluorescence and ELISA protein quantification in normal intestinal epithelial cells were measured. The cells were treated with 24 µg/mL Protandim. Nrf2 protein was translocated to the nucleus within 1 hour of treatment, and reached a maximum level at approximately 10 hours. This inducible translocation was reversible: from 12 hours to 24 hours post-treatment, Nrf2 signal gradually returned to baseline pre-treatment levels. To determine whether Nrf-2 nuclear localization would stimulate the growth of cancer cells, we treated HCT-116 and CaCo-2 cancer cells with varying doses of Protandim. At no point in time or dose was the activation of Nrf2 by Protandim associated with increased tumor cell viability. These data support the inducible mechanism of action of Protandim.

Introduction

Oxidative damage is generally accepted as a causative agent in the development and maintenance of many pathological states, including inflammation, cancer, and the manifestation of aging-related health issues [1]. Evidence has come to light in recent years, pointing to an increase in cancer risk as a result of overuse of antioxidant supplements [2] Protandim is a supplement made of 5 herbal extracts (Milk thistle, Bacopa monieri, Ashwagandha, Green tea and Turmeric) that is geared to protect against oxidative stress through prophylactic activation of the body's endogenous antioxidant defenses, rather than through antioxidant supplementation.[3-7] Protandim targets a central regulator of the antioxidant response pathway: the Nrf2 transcription factor. [1]

Nrf2 regulates the expression of several antioxidant-related genes (those of the glutathione system, NQO1, and superoxide dismutase) in response to oxidative stress.[8] This response is controlled through the actions of KEAP-1, a protein suppressor which binds to Nrf2 in the

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cytoplasm and targets it for ubiquitination and subsequent destruction. This interaction is broken in the presence of reactive oxygen species (ROS) or electrophiles, freeing Nrf2 to enter the nucleus and effect transcription of antioxidant response genes through binding to the Antioxidant Response Element (ARE) in their promoters. In this paper, we showed that Protandim promotes the nuclear translocation of Nrf2 and the activation of Nrf2 pathway as seen by induction of luciferase.

Materials and methods

Cell Culture

Human primary small intestinal epithelial cells were sourced from Cell Biologics. HCT-116 and CaCo-2 cells were purchased from the American Type Culture Collection. AREc32 is a stable human MCF7-derived reported cell line, which contains a luciferase gene construct controlled by eight copies of the antioxidant response element (ARE).

Nuclear/cytoplasmic protein extraction

Nuclear protein extracts were obtained using the NE-PER system (ThermoFisher).

ELISA

Nuclear protein levels for Nrf2 were evaluated using an ELISA assay from Active Motif. (Catalog #50296)

Fluorescent Microscopy

Standard immunofluorescence techniques were used to visualize Nrf2 localization in cells. The cells were incubated with Nrf2 primary antibody (GeneTex) for 1hr. Then the cells were incubated with a rhodamine labeled secondary antibody (GeneTex) for 30 minutes. Cells were then either visualized or counterstained for three color imaging using Alexa 488 phalloidin (Thermo-Fisher) at 1:50, and NucBlue (Thermo-Fisher) at 1:50. Imaging was performed on a Nikon A1R laser scanning confocal microscope.

Bioassay for Nrf2 Activation

This assay measures the luminescence emitted by AREc32 cells that contain an ARE promoter and a firefly luciferase reporter gene in presence or absence of Protandim. [1]

Results and discussion

Nrf2 localization and timing

Primary small intestinal epithelial cells were treated with 24 µg/mL Protandim and imaged using a fluorescent antibody to Nrf2 (Fig. 1). In control untreated cells, Nrf2 staining was found to be predominantly cytoplasmic, as would be expected in a normal dividing cell (Fig. 1A). Two hours following Protandim exposure, Nrf2 signal had shifted almost entirely to the nucleus, indicating that treatment quickly induces release of Nrf2 from KEAP-1 suppression, nuclear translocation, and activation of genes bearing ARE sequences in their promoters (Figure 1B). Sub-cellular localization of Nrf2 returned to normal (primarily cytoplasmic) by 24 hours post-treatment (Fig. 1C), no matter whether the cells where in contact with Protandim for 2 or 24 hrs (Fig. 1D)

We followed the time-dependent progression of this effect in a quantitative manner by using an enzyme-linked immunosorbent assay (ELISA) using an antibody specific to Nrf2. Cells were treated with a single dose (24 µg/mL) of Protandim, and nuclear extracts collected for ELISA at various time points afterward (Fig. 2A & B). We saw that Nrf2 levels in the nucleus began rising almost immediately following Protandim exposure, and reached a maximum at 12 hours post-treatment. Nuclear Nrf2 levels then decreased, returning to pre-treatment levels by 24 hours (Fig. 2A), indicating that Nrf2 induction by Protandim is reversible.

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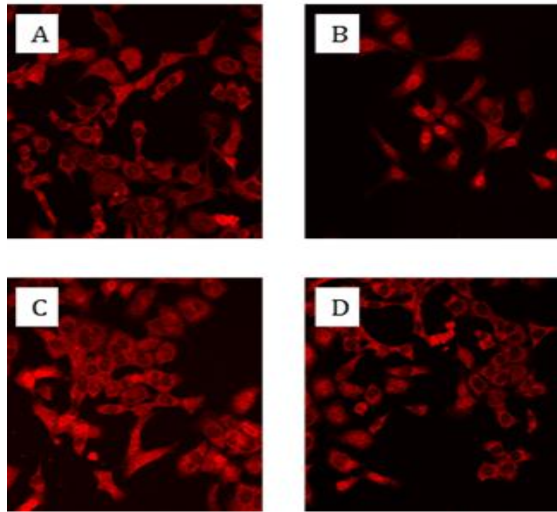
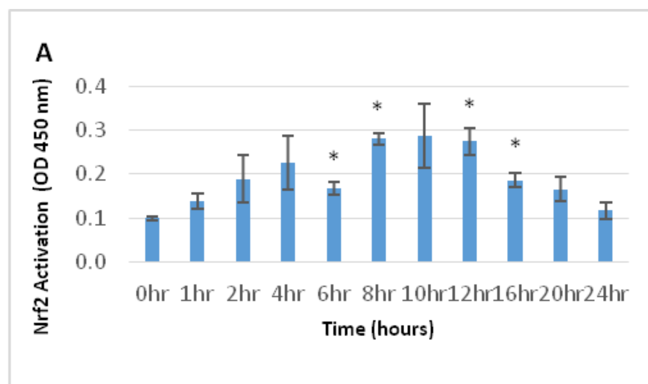


Figure 1. Effect of Protandim on subcellular localization of Nrf-2. (**A**) Untreated cells; (**B**) cells 2 hrs following Protandim exposure; (**C**) cells 24 hr exposure to Protandim, and (**D**) cells 2 hr exposure to Protandim then incubate 22 hr with no Protandim.

When a second dose of Protandim was administered to cells 12 hours after the first, Nrf2 levels were not seen to further accumulate in the nucleus, indicating that maximal effect was achieved with a dose of 24 µg/mL (Fig. 2B). This dose repetition did however lead to maintenance of Nrf2 in the nucleus at a level above baseline, but below peak. Levels began to return to normal by 28 hours (16 hours after the second dose). Maintenance of Nrf2 in the nucleus, therefore, requires constant presence of Protandim; the effect can be abrogated by ceasing exposure. These data support twice-daily administration of Protandim for sustained submaximal Nrf2 induction.



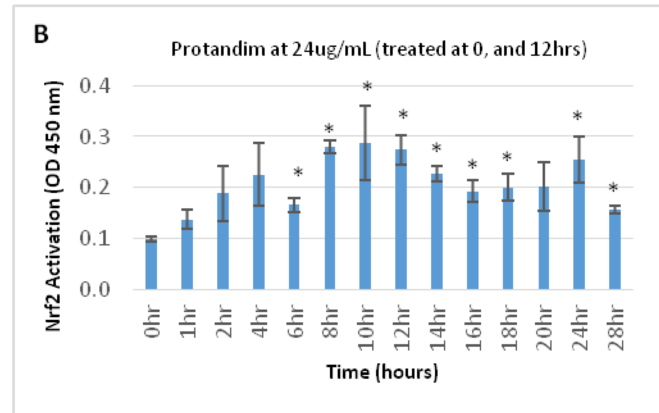


Figure 2. Nrf2 Activation after primary small intestinal epithelial cells were exposed to Protandim. (Done in duplicate) * statistically significant at $p \leq 0.05$ (two-tailed, paired t-test with $\alpha_{0.05}$)

ARE activation and timing

Furthermore, we showed that this translocation led to an increase in transcription from the Antioxidant Response Element in AREc32 cells. (Fig. 3). Luciferase enzyme activity begins to manifest at 8 hours, but did not become fully induced until 24 hours with statistical differences at 12 and 24 hours versus baseline.

Luciferase activity lags behind nuclear localization described above, which may be related to the time required for assembly on the promoter, transcription, translation and post-translational processing of luciferase.

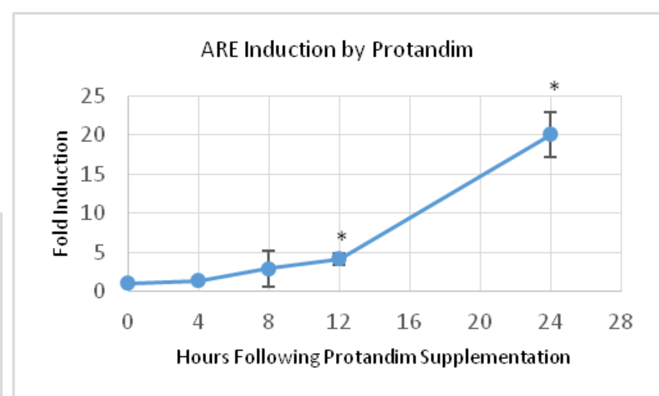


Figure 3: Induction of luciferase in AREc32 cells by Protandim. Data are represented as the proportion of luminescent signal versus untreated control.

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* statistically significant at $p \leq 0.05$ (two-tailed, paired t-test with $\alpha_{0.05}$)

Cytotoxic effect on cancer cell lines

As Protandim was developed in an effort to supplant the overuse of supplemental antioxidants, it was important to us to show that Protandim use did not lead to cancer cell promotion. Two colon carcinoma cell lines, HCT-116 and CaCo-2 were treated with varying amounts of Protandim for 24 hours, and subjected to a cell viability assay. In both cell lines, Protandim was not associated with an increase in cell viability or number, and as the dose increased, viability decreased, suggesting that Protandim was toxic to these tumor cells. Further experiments are needed to test the basal expression of Nrf2 in those cancer cells and determine if the treatment with Protandim further increases the Nrf2 activation, thus rendering the cells more resistant to oxidative stress and whether Protandim affects proliferation and invasion of cancer cells.

Acknowledgements

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