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Original Contribution

Synergistic induction of heme oxygenase-1 by the components of the antioxidant supplement Protandim

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ABSTRACT

Protandim is an antioxidant supplement that consists of five ingredients, namely, ashwagandha, bacopa extract, green tea extract, silymarin, and curcumin, each with known therapeutic properties. Protandim was formulated with the objective of combining multiple phytochemicals at low nontoxic doses to gain synergy among them. A recent clinical study demonstrated the in vivo antioxidant effects of Protandim (S.K. Nelson et al., 2006, *Free Radic. Biol. Med.* 40, 341–347). The objective of the present study was to determine if the components of Protandim induce heme oxygenase-1 (HO-1) in a synergistic manner in cultured MIN6 cells, a mouse β-cell line, and in SK-N-MC cells, a human neuroblastoma cell line. When the components of Protandim were tested alone at low doses, curcumin showed minimal induction, whereas the others were unable to induce the HO-1 promoter, assayed by transient transfection. All components together, however, produced a strongly synergistic induction of around three- to ninefold in a dose-dependent manner, greatly exceeding the sum of the parts. Similar findings were obtained for the expression of HO-1 at the mRNA and protein levels. Protandim-mediated HO-1 induction involved the presence of ARE sites in the HO-1 promoter and nuclear translocalization of the transcription factor Nrf2, which binds to ARE sites. The involvement of multiple signaling pathways, including PI3-kinase/Akt, p38MAPK, and PKCδ, in HO-1 induction seems to be the probable mechanism of synergy between the components of Protandim. There were significant increases in the levels of total glutathione in Protandim-treated cells. These findings suggest that the use of a combination of phytochemicals may be an efficient method for the induction of antioxidant enzymes.

Oxidative stress plays a significant role in the progression of many diseases, including diabetes, Alzheimer disease (AD), and atherosclerosis [1–3]. Oxidative stress generally results from an imbalance between free radicals generated during normal cellular metabolism and the free radical or oxidant scavenging capacity of the endogenous antioxidant enzymes. Although attempts at stoichiometric neutralization of free radicals with dietary antioxidant supplements have been reported to have some beneficial effects [4], there are also many reports of failure to produce a beneficial effect [5] and even reports of pro-oxidant effects [6]. Furthermore, it is clear that we rely on the endogenous antioxidant enzymes to protect our cells from oxidative stress and that consumption of so-called antioxidant compounds in low stoichiometric amounts cannot serve this role. Much attention has been focused recently on naturally occurring polyphenolic compounds that are capable of inducing antioxidant enzymes [7]. Prevention of oxidative stress by phytochemicals has evolved as a promising therapeutic approach in the treatment of several diseases [8,9].

Protandim is a dietary supplement designed to induce endogenous antioxidant enzymes. It consists of five herbal ingredients, namely, silymarin from milk thistle (*Silibum marianum*), bacopa (*Bacopa monniera*) extract, ashwagandha (*Withania somnifera*), green tea (*Camilia sinesis*) extract, and curcumin from turmeric (*Curcuma longa*). The therapeutic properties of each of these herbs have been previously reported [9–11]. However, to gain optimal beneficial effects, the individual components may have to be used at pharmacological doses not easily achieved by oral administration and that could cause toxic side effects. Therefore, Protandim was formulated with the concept of combining multiple phytochemicals at low doses to gain synergy among them. A recent study demonstrated that administration (675 mg/day for 120 days) of Protandim in human subjects resulted in 30–50% increases in the activities of the antioxidant enzymes SOD and catalase in erythrocytes [12]. Furthermore, the age-dependent increase in circulating TBARS seen before treatment was completely suppressed after intake of Protandim. No undesirable side effects

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were observed, suggesting that Protandim is a safe nutraceutical supplement.

In a recent study, we reported that curcumin, one of the ingredients of Protandim, induces the expression of heme oxygenase-1 (HO-1) in mouse β cells by a pathway involving the transcription factor Nrf2 and PI3-kinase/Akt-mediated signaling pathway [13]. EGCG, the active constituent present in green tea, has also been shown to induce HO-1 in endothelial cells [14]. However, there are no reports on the effects of the other three ingredients of Protandim on HO-1. We predicted that Protandim is likely to be a strong inducer of HO-1 because it is a phase 2 enzyme. HO-1 is an inducible rate-limiting enzyme that breaks down heme into carbon monoxide, iron, and bilirubin. HO-1 is emerging as a novel therapeutic target in several disease models [15].

The objectives of the present investigation are to (a) develop a cell culture model to characterize the mechanism of synergy between the components of Protandim and (b) determine if Protandim induces HO-1 in a synergistic manner through activation of multiple signaling pathways. We used MIN6 cells, a mouse β-cell line, and SK-N-MC cells, a neuroblastoma cell line, to test the effects of Protandim to determine if the supplement has potential beneficial effects in diabetes and Alzheimer disease, respectively. We demonstrate that the components of Protandim induce HO-1 through synergistic actions on multiple signaling pathways.

Materials and methods

Materials

Minimum essential medium Eagle, fetal bovine serum (FBS), streptomycin, and penicillin were obtained from Life Technologies (Rockville, MD, USA) and Gemini Bio Products (Woodland, CA, USA). HO-1 antibody and Akt inhibitor IV were purchased from Calbiochem (La Jolla, CA, USA). Nrf2 (H-300) antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The enzyme inhibitors SB203580, SP600125, rottlerin, LY294002, and U0126 were purchased from Biomol (Plymouth Meeting, PA, USA). Plasmids for transfection experiments were purified using Qiagen's (Valencia, CA, USA) Maxi kit. LipofectAMINE 2000 reagent was obtained from Invitrogen Life Technologies. The dual-luciferase assay kit was purchased from Promega (Madison, WI, USA). Anti-rabbit IgG linked to Cy3 was obtained from Jackson ImmunoResearch (West Grove, PA, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Enriched fractions of Protandim

The dietary supplement (675 mg) Protandim (LifeVantage Corp., Littleton, CO, USA) consists of five ingredients: 150 mg *W. somnifera* powder (ashwagandha), 150 mg *B. monniera* (45% bacosides), 225 mg *S. marianum* (70–80% silymarin), 75 mg *Ca. sinesis* (green tea, 98% polyphenols and 45% (−)-epigallocatechin-3-gallate), and 75 mg curcumin (95%) from turmeric (*Cu. longa*). The alcohol extract of Protandim was prepared by shaking 675 mg of Protandim with 16.875 ml of 95% ethanol overnight at 4 °C and centrifuging at 5000 rpm (4 °C) for 5 min, and the extract (40 mg/ml) was stored at −80 °C. For studies on synergy, the individual components present in 675 mg of Protandim were extracted in the same volume of alcohol by a similar procedure. Parallel preparations of Protandim extracts were also prepared with one of the components omitted. The addition of the ethanolic extract of complete Protandim to the cell culture medium to produce a Protandim concentration of 10 μg/ml resulted in the following concentrations of each of the putative active components: withanolides from *W. somnifera*, 0.07 μM; bacopasides from *B. monniera*, 1.1 μM; silymarin from *S. marianum,* 5.5 μM; (−)-epigallocatechin-3-gallate from *Ca. sinesis*, 1.1 μM; and curcumin from *Cu. longa*, 2.8 μM. Controls were treated with the same volume of alcohol used in the treated groups.

HO-1 promoters

Several HO-1 promoter constructs linked to a firefly luciferase reporter gene were generated as described previously [16]. The full-length promoter construct pHO15luc was generated by cloning a 15-kb promoter fragment of the mouse *Ho-1* gene into the luciferase reporter gene vector pSK1luc. The HO-1 promoter contains multiple antioxidant response elements (AREs) in the enhancer regions E1 and E2. A 600-bp (*Sac*I/*Sac*I) fragment (E1) of pHO15kluc was deleted to generate the plasmid pHOluc-ΔE1. The plasmid pHOluc-ΔE2 was generated by deletion of the 161-bp *Afl*II/*Bsr*BI fragment (E2). Deletion of both of these fragments resulted in the construct pHOluc-(ΔE1 + ΔE2).

Culture of MIN6 and SK-N-MC cells

SK-N-MC cells, a neuroblastoma cell line, were maintained in minimum essential medium Eagle supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in 5% CO_2/humidified air. MIN6 cells, a mouse pancreatic β-cell line obtained from Dr. Jun-ichi Miyazaki (Kyoto University, Japan), were cultured in DMEM containing 5.6 mM glucose, 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 50 μM β-mercaptoethanol at 37 °C in a humidified atmosphere of 5% CO_2. A low-serum (0.1%) medium was used while exposing the cells to Protandim.

Transfection procedure

MIN6 or SK-N-MC cells were cultured to 70% confluence in 12-well dishes. Plasmid (1.5 μg) and LipofectAMINE 2000 reagent (3 μl) were separately diluted in 100 μl of Opti-MEM and incubated for 5 min at room temperature. After being mixed, they were incubated at room temperature for another 20 min and the mixture was added to the cells. A constitutively active *Renilla* luciferase (pRL-TK-Luc) was included in the plasmids to correct for transfection efficiency. The transfected cells were cultured in low-serum (0.1%) medium with appropriate treatment for 12 h. The treated cells were washed with cold PBS and then lysed in 100 μl of passive lysis buffer (Promega). After freezing and thawing, the lysates were centrifuged (10,600 *g;* 20 min) to collect the supernatant. Firefly luciferase and *Renilla* luciferase activities from transfected cells were measured using the Dual-Glo Luciferase Assay System (Promega). HO-1 promoter activity is defined as the ratio of firefly luciferase to *Renilla* luciferase activity.

Immunocytochemistry

SK-N-MC cells were cultured on coverslips to 70% confluence. They were incubated in the absence or presence of Protandim (40 μg /ml) for 6 h. The treated cells were fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were washed with PBS and permeabilized by treatment with 0.2% Triton X-100 and 5% BSA in PBS for 90 min at room temperature. They were incubated in the presence of Nrf2 antibody (1:200) at 4 °C overnight. After being washed with PBS, the cells were exposed to anti-rabbit IgG linked to Cy3 along with 4,6-diamidino-2-phenylindole (DAPI; 2 g/ml; nuclear staining) for 90 min at room temperature. The cells were then washed in PBS, mounted on slides with mounting medium, and examined by fluorescence microscopy.

Western blot analysis

After treatment with Protandim extracts, MIN6 and SK-N-MC cells were washed with ice-cold PBS. Cells were lysed with mammalian protein extraction reagent (Pierce, Rockford, IL, USA) containing phosphatase inhibitors and protease inhibitor cocktail. The protein content of the lysate was measured [17]. Diluted samples containing equal amounts of protein were mixed with 2× Laemmli sample buffer and subjected to electrophoresis in 12% SDS–polyacrylamide gels. After transfer to polyvinylidene difluoride membranes, the membranes were blocked with TBST [20 mM Tris–HCl (pH 7.9), 8.5% NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk at room temperature for 1 h and exposed to primary antibodies (1:1000) in TBST containing 5.0% BSA at 4 °C overnight. After being washed in blocking solution, the membranes were exposed to secondary antibodies conjugated to alkaline phosphatase and developed with CDP-Star reagent (New England Biolabs, Beverly, MA, USA). The intensity of the protein bands was visualized using Fluor-S Multi-Imager and Quantity One software from Bio-Rad. All densitometric values obtained for the HO-1 protein were normalized to β-actin levels obtained on the same blot.

RNA isolation and real-time quantitative RT-PCR

MIN6 and SK-N-MC cells cultured in 100-mm dishes were exposed to 40 or 20 μg/ml Protandim, respectively, for 24 h. RNA was isolated by Qiagen's RNeasy column method. The levels of HO-1 mRNA were examined by real-time quantitative RT-PCR using TaqMan probes. The PCRs were monitored in real time in an ABI Prism 7700 sequence detector (Perkin–Elmer Corp./Applied Biosystems). The sequences of the primers and probes for HO-1 were as follows: mouse (MIN6 cells),

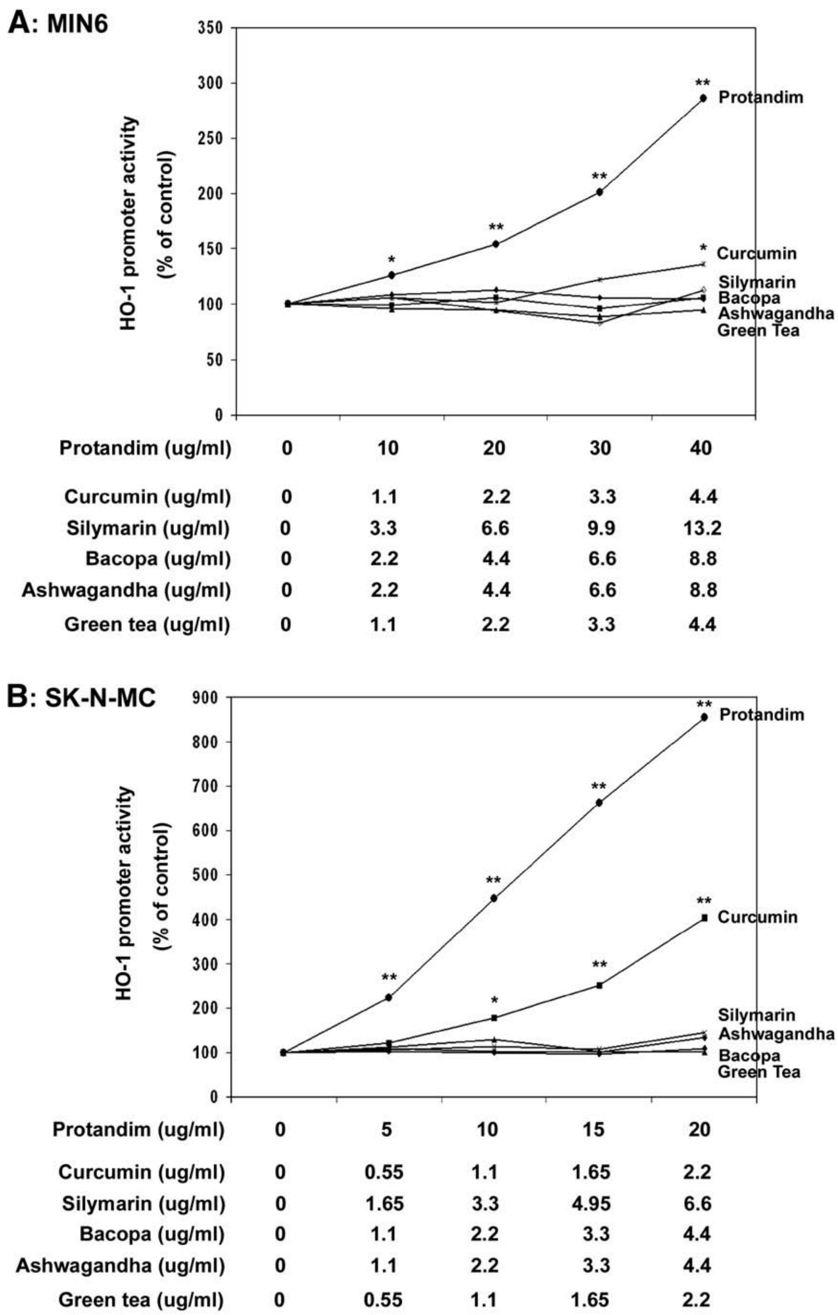

Fig. 1. Synergistic induction of the HO-1 promoter by Protandim components. The luciferase reporter gene pHO15luc was generated by cloning a 15-kb promoter fragment of the mouse *Ho-1* gene into the vector pSK1luc. (A) MIN6 and (B) SK-N-MC cells cultured in 12 dishes to 70% confluence were transfected with pHO15luc and a constitutively active *Renilla* luciferase (pRL-TK-Luc; to correct for transfection efficiency). Six hours after transfection, the cells were exposed to the alcohol-soluble fraction of Protandim or its constituents at increasing concentrations for another 18 h. Cell lysates were prepared for the assay of luciferase activity. The results are the means of four independent observations. *$p<0.01$ and **$p<0.001$ vs untreated control.

forward primer GTGATGGAGCGTCCACAGC, reverse primer TGGTGGCCTCCTTCAAGG, TaqMan probe 5′-6FAM-CGACAGCATGCCC-CAGGATTTGTC-TAMRA-3′; human (SK-N-MC cells), forward primer AGGCCAAGACTGCGTTCCT, reverse primer GGTGTCATGGGTCAG-CAGCT, TaqMan probe 5′-6FAM-TCAACATCCAGCTCTTTGAGGAGTTG-CAG-TAMRA-3′.

Glutathione assay

Total glutathione content in Protandim-treated cells was determined by a standard colorimetric method [18]. The treated cells were rinsed with ice-cold PBS, scraped off from the 100-mm plate, and suspended into 250 μl of ice-cold phosphate buffer (0.1 M, pH 7.4). The cell suspension was vortexed for 20 s, followed by sonication and centrifugation (2500 rpm for 5 min at 4 °C). The cell lysate was mixed with an equal volume of 10% sulfosalicyclic acid and the denatured protein was removed by centrifugation (20 min). One hundred microliters of supernatant was treated with 450 μl of 5,5′-dithiobisnitrobenzoic acid in 0.1 M phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm along with glutathione standards treated in the same way and the cellular total glutathione content was expressed as nmol/mg protein.

Statistical analyses

Data are expressed as means ± SE. Statistical analysis in this study was performed by one-way ANOVA with Dunnett's multiple comparison test.

Results

Synergy between the components of Protandim in the induction of the HO-1 promoter

The induction of HO-1 by Protandim was tested by transient transfection of its promoter linked to the luciferase reporter gene in MIN6 cells, a mouse insulinoma cell line, and in SK-N-MC cells, a human neuroblastoma cell line. The alcohol-soluble fraction of Protandim induced the HO-1 promoter in a dose-dependent manner. A maximum induction of 3-fold was seen at 40 μg/ml in MIN6 cells,

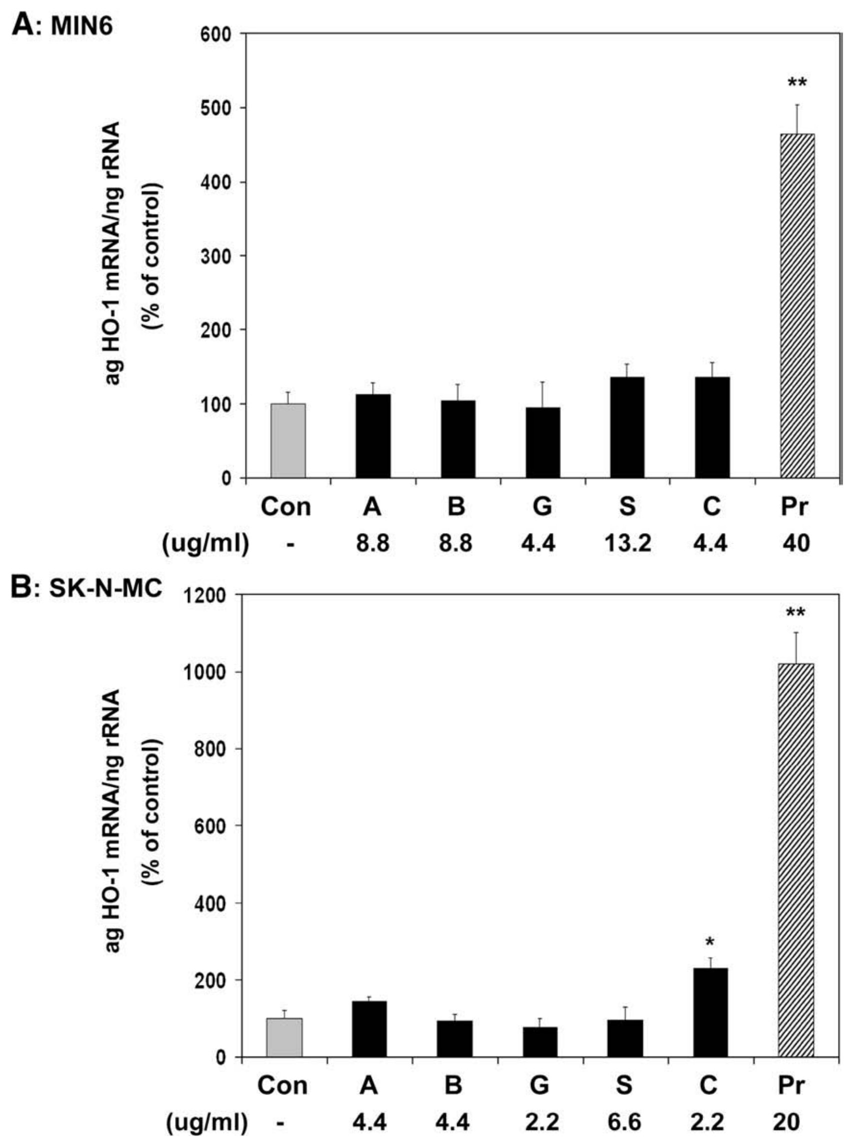

Fig. 2. Synergy in the induction of HO-1 at the mRNA levels by Protandim components. (A) MIN6 cells and (B) SK-N-MC cells cultured in 100-mm dishes were exposed to Protandim (Pr) or its components (A, ashwagandha; B, bacopa; G, green tea; S, silymarin; C, curcumin) at the indicated concentrations for 24 h. RNA was isolated using Qiagen's RNeasy column method. The mRNA levels of HO-1 were determined by real-time quantitative RT-PCR using a TaqMan probe and expressed in attograms (ag). The results are the means ± SE of four independent observations. *$p < 0.01$ and **$p < 0.001$ compared to untreated control.

whereas an 8.5-fold increase was seen in SK-N-MC cells at a lower dose of 20 μg/ml (Fig. 1). The decreased induction of HO-1 in the β-cell line is not surprising because this cell type is known to express antioxidant enzymes at low levels [1,19]. Protandim consists of five ingredients, namely, ashwagandha, bacopa, green tea, silymarin, and curcumin. Therefore, in parallel, we tested the effects of the alcohol-soluble fraction of each of these components present in the corresponding dose of Protandim. Except for curcumin, the individual ingredients did not induce the HO-1 promoter significantly. Curcumin induced the HO-1 promoter by 36 and 300% in MIN6 and SK-N-MC cells, respectively, at concentrations present in the maximum dose of Protandim. Therefore the effects of Protandim on HO-1 promoter are more than the sum of the effects of individual components, suggesting excellent synergy among the phytochemicals. Our attempts to match the maximum induction of Protandim by increasing the dose of the individual components failed because of toxicity at higher concentrations (results not shown).

Synergistic induction of HO-1 at the transcriptional level by Protandim

We further examined the expression of HO-1 at the mRNA level in MIN6 and SK-N-MC cells incubated in the presence of Protandim or its components at the respective maximum doses used for HO-1 promoter assays. HO-1 mRNA levels were determined by real-time

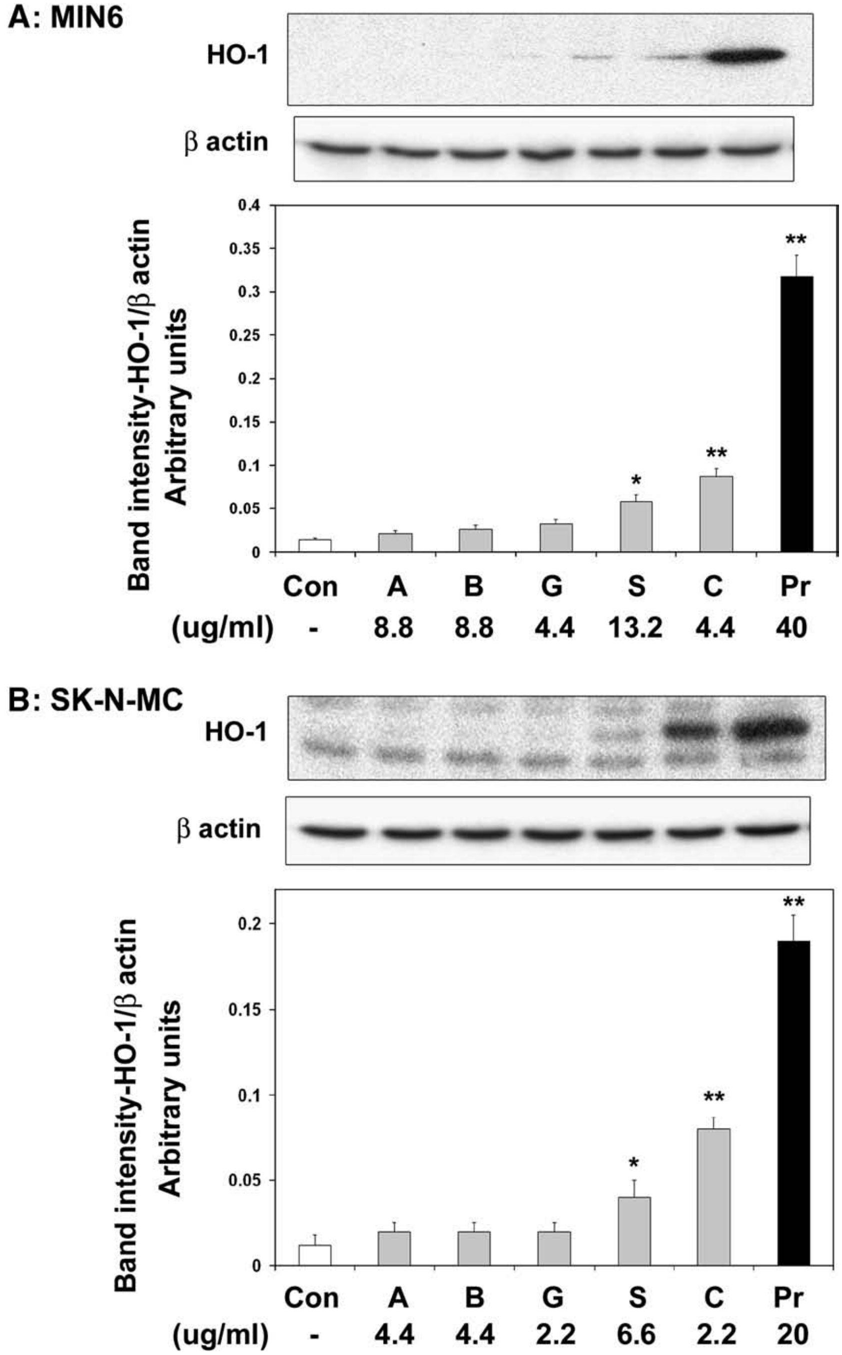

Fig. 3. Synergistic increase in HO-1 protein by Protandim components. (A) MIN6 and (B) SK-N-MC cells were exposed Protandim (Pr) or its components (A, ashwagandha; B, bacopa; G, green tea; S, silymarin; C, curcumin) for 24 h. The cell lysates were processed for Western blot analysis of HO-1. The blots were reprobed for β-actin. The intensities of bands were quantitated by scanning in a MultiImager using Quantity One software (Bio-Rad), and HO-1 expression was corrected for β-actin levels. *$p<0.01$ and **$p<0.001$ compared to untreated control.

quantitative RT-PCR analysis using a TaqMan probe. In MIN6 cells, when the extracts of the individual components present in 40 μg/ml Protandim were tested, silymarin and curcumin had a minimal effect on HO-1 induction (1.5-fold), whereas the other three components treated individually failed to induce HO-1. However, treatment with Protandim extract increased the expression of HO-1 by 4.6-fold (Fig. 2A). In SK-N-MC cells, Protandim effected a 10-fold induction and curcumin a 2-fold induction, whereas the other components present in Protandim did not have any significant effect on HO-1 expression (Fig. 2B). These observations are similar to the findings on the activation of the HO-1 promoter (Fig. 1).

Synergy among the components of Protandim in HO-1 induction at the protein levels

Protandim-mediated induction of HO-1 at the protein levels was examined by Western blot analysis. In both MIN6 cells and SK-N-MC cells, the HO-1 levels increased by 15- to 20-fold when the cells were incubated in the presence of Protandim at the respective optimal doses (Fig. 3). Among the individual components, silymarin (3- to 4-fold) and curcumin (6-fold) showed significant induction of HO-1. Overall, the extent of HO-1 induction by Protandim was significantly more compared to induction at the promoter and mRNA levels, especially in MIN6 cells. This observation suggests that Protandim might improve the stability and translation of HO-1 mRNA. The results presented thus far have been for MIN6 and SK-N-MC cells to suggest that Protandim could reduce oxidative stress in diabetes (β cells) and during neurodegeneration. The results for the subsequent experiments are presented for SK-N-MC cells alone to avoid redundancy, even though similar observations were obtained with MIN6.

Removal of any one of the components affects the efficiency of Protandim

To demonstrate synergy by a different approach, we tested the effects of Protandim in SK-N-MC cells by omitting one component at a time. When silymarin or curcumin was omitted, the induction of the HO-1 promoter was almost completely lost (Fig. 4A). Omission of ashwagandha, bacopa, or green tea from Protandim significantly

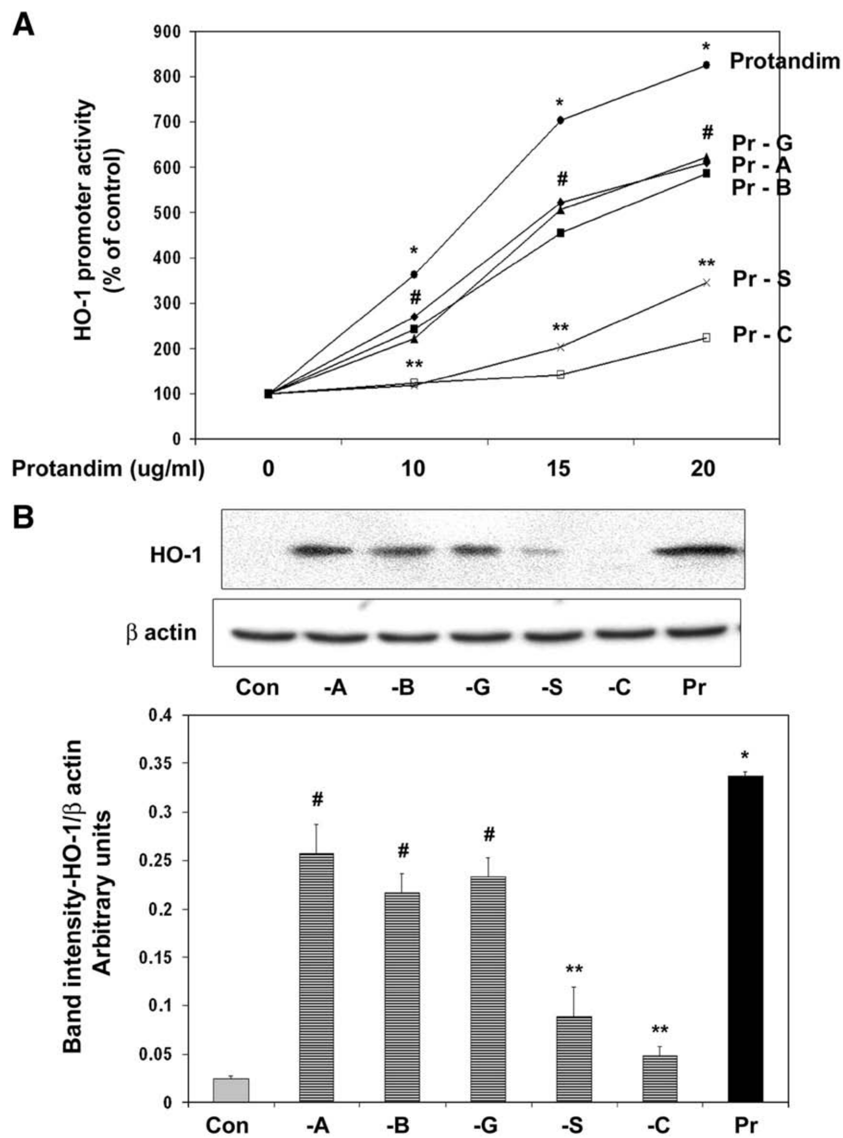

Fig. 4. Contribution by Protandim components in the synergistic induction of HO-1. (A) SK-N-MC cells were transfected with a full-length promoter of HO-1 linked to a firefly luciferase reporter and constitutively active *Renilla* luciferase. Six hours after transfection, the cells were exposed to the alcohol-soluble fraction of Protandim or of Protandim minus one of its components for 18 h. Cell lysates were prepared for the assay of luciferase activity. (B) SK-N-MC cells were cultured in the absence or presence of Protandim or Protandim minus one of its components for 24 h. The cell lysates were processed for Western blot analysis of HO-1. The band intensities were quantified by scanning and HO-1 expression was corrected for β-actin levels. −A, minus ashwagandha; −B, minus bacopa; −G, minus green tea; −S, minus silymarin; −C, minus curcumin; Pr, Protandim. The results are the means of four independent observations. *$p<0.001$ vs untreated control; #$p<0.01$ and **$p<0.001$ vs Protandim at the corresponding doses.

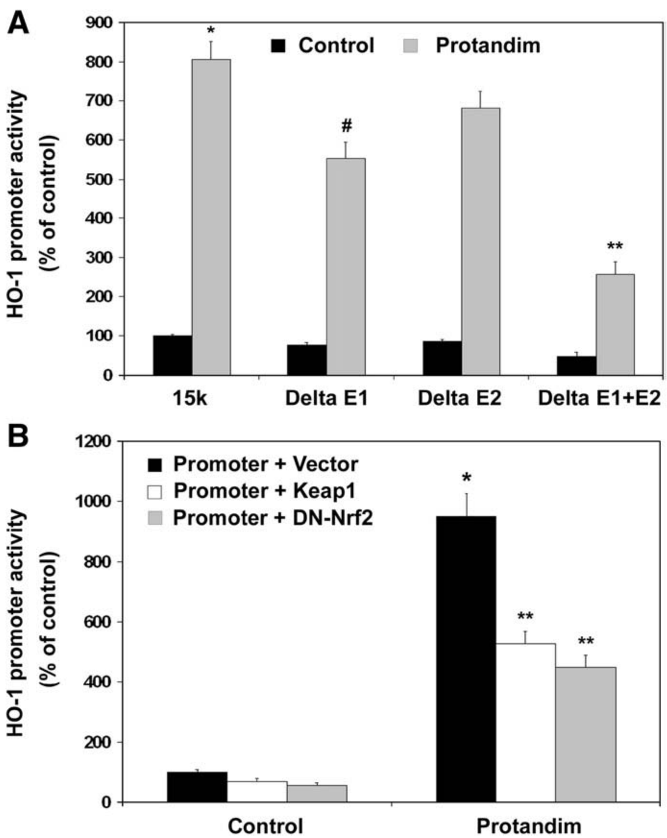

Fig. 5. ARE site- and Nrf2-dependent induction of the HO-1 promoter by Protandim. (A) The plasmids ΔE1 and ΔE2 were obtained by deletion of a 600-bp *Sac*I/*Sac*I fragment and a 161-bp *Afl*II/*Bsr*BI fragment, respectively, from the 15-kb promoter fragment of the mouse *Ho-1* gene and were cloned into the luciferase reporter gene. SK-N-MC cells cultured in 12-well dishes to 70% confluence were transfected with the indicated HO-1 promoter constructs linked to firefly luciferase along with constitutively active *Renilla* luciferase using the LipofectAMINE 2000 reagent. After 6 h of transfection, the cells were exposed to 20 μg/ml Protandim for another 18 h. Cell lysates were prepared and luciferase activities were measured. The ratios of the activities of firefly luciferase and *Renilla* luciferase were determined. The results are means ± SE of four independent observations. *$p<0.001$ vs untreated control; #$p<0.01$, **$p<0.001$ with respect to full-length promoter activation by Protandim. (B) SK-N-MC cells were transfected with (1) the full-length promoter of HO-1 linked to the firefly luciferase reporter and vector (pEF) or (2) the promoter/reporter construct plus an expression construct for Keap1 or (3) the promoter/reporter construct plus a dominant negative Nrf2 expression construct. After 6 h of transfection, the cells were exposed to 20 μg/ml Protandim for 18 h. Cell lysates were prepared for the assay of luciferase activity. The results are means ± SE of four independent experiments. *$p<0.001$ vs untreated control; #$p<0.001$ with respect to vector control.

reduced its induction of HO-1 by 25 to 40% ($p<0.01$; Fig. 4A). It is interesting to note that these three ingredients when tested alone did not cause any significant induction, whereas omitting them from the combination reduced the activity of Protandim significantly. Similar decreases at the protein level as determined by Western blot analysis were observed when each component was omitted (Fig. 4B). These observations further confirmed that a combination of phytochemicals at low doses can induce the antioxidant enzyme HO-1 efficiently.

Protandim induces HO-1 mainly through Nrf2

HO-1 is known to be induced by a number of transcription factors, including Nrf2, c-jun, NF-κB, and CREB [20–22]. Several studies have reported that Nrf2 plays a major role through the E1 and E2 regions, each of which contains several AREs. To determine if the synergy among the components of Protandim is due to the involvement of multiple transcription factors, we tested the effects of deletion of the ARE site-containing E1 and E2 regions. Deletion of E1 resulted in a 30% decrease ($p<0.01$) in the induction of the HO-1 promoter (Fig. 5A). Deletion of E2 did not decrease Protandim-mediated induction significantly. When both E1 and E2 were deleted, the activation of the HO-1 promoter by Protandim decreased by 70%. This observation suggested that Protandim induces HO-1 primarily through the AREs although the involvement of other response elements in the HO-1 promoter cannot be ruled out. Next, to determine the role of the transcription factor Nrf2, which binds to ARE sites, we took a cotransfection approach. Nrf2 is normally present in the cytoplasm bound to Keap1. Inducers of promoters with ARE sites dissociate Nrf2 from Keap1 and allow it to translocate to the nucleus. When HO-1-luc was cotransfected with a plasmid encoding Keap1, Protandim-induced HO-1 promoter activity decreased by 45% because overexpression of Keap1 can be expected to retain more of Nrf2 in the cytoplasm (Fig. 5B). Overexpression of dominant negative Nrf2 with a deleted transactivation domain also decreased HO-1 promoter activation by

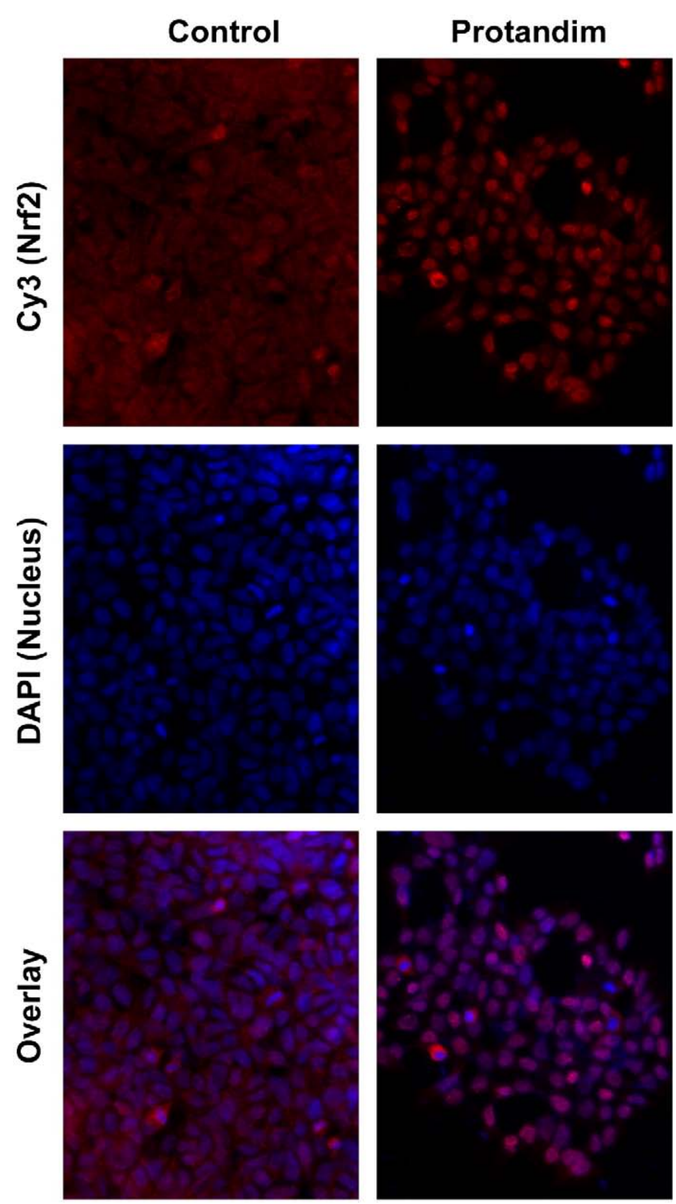

Fig. 6. Nuclear translocation of Nrf2 by Protandim. SK-N-MC cells cultured on coverslips were exposed to 20 μg/ml Protandim. After 6 h, cells were fixed in 4% paraformaldehyde, permeabilized, and immunostained for active Nrf2 (Cy3; red). The nuclei were stained with DAPI (blue). Images were examined by fluorescence microscopy. The merge of Cy3 and DAPI is shown as an overlay. The images presented are representative of multiple fields from three independent experiments.

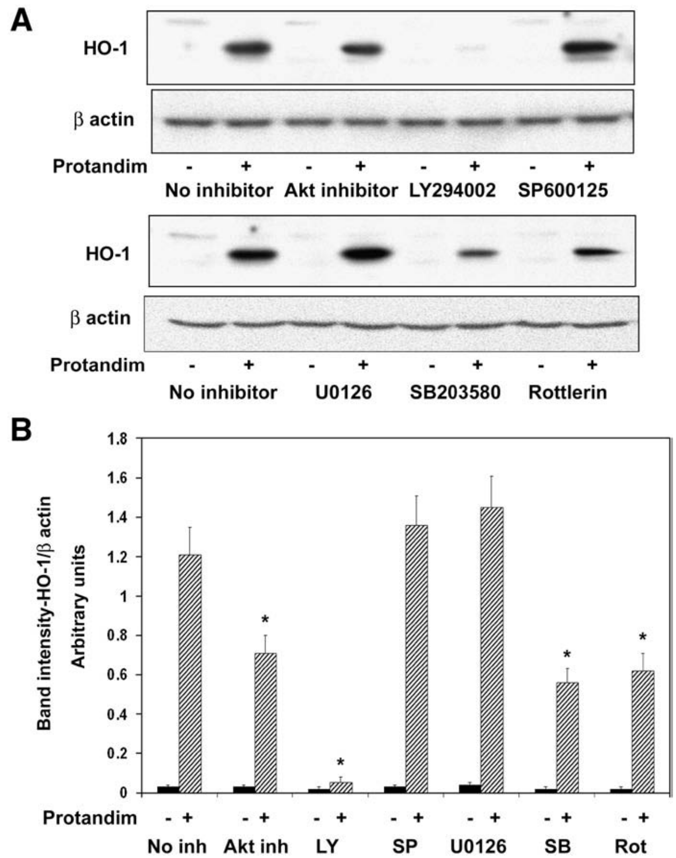

Fig. 7. Role of multiple signaling pathways on induction of HO-1 expression by Protandim. SK-N-MC cells were preincubated in the presence of 250 nM Akt inhibitor IV, 30 μM LY294002, 1 μM rottlerin, 10 μM U0126, 20 μM SB203580, or 20 μM SP600125 for 30 min followed by exposure to 20 μg/ml Protandim for 24 h. (A) Cell lysates were electrophoresed and immunoblotted for HO-1. The blots were then reprobed with the antibody for β-actin. A representative of four blots is presented for each inhibitor. (B) The intensities of the bands were quantified by densitometry using Fluor-S MultiImager and Quantity One software from Bio-Rad. HO-1 levels were corrected for β-actin expression. *$p<0.001$ compared to untreated control. #$p<0.001$ with respect to Protandim-treated cells in the absence of inhibitors.

53% (Fig. 5B). These observations further suggest that Protandim-mediated HO-1 induction proceeds primarily through Nrf2.

Nuclear translocalization of Nrf2 by Protandim in SK-N-MC cells

Next we tested by immunofluorescent staining whether Nrf2 undergoes nuclear localization after treatment of SK-N-MC cells with Protandim (Fig. 6). Nrf2 stained with Cy3 was present mostly in the cytoplasm of untreated cells. Culture of these cells with the alcohol-soluble fraction of Protandim (20 μg/ml) for 6 h resulted in the appearance of Cy3 signal mostly in the nucleus. The red fluorescent stain of Nrf2 overlapped with DAPI stain (blue) for nucleus, suggesting nuclear localization. This observation, along with the findings from the HO-1 promoter assay with ARE-site-deleted constructs (Fig. 5A) and the cotransfection experiments (Fig. 5B), suggests that the components of Protandim act mainly through the transcription factor Nrf2. We had made similar observations with curcumin, an essential component of Protandim, in MIN6 cells previously [13]. These findings also suggest that the synergistic effect of Protandim components is not likely to be primarily through the involvement of multiple transcription factors.

Multiple signaling pathways are involved in the Protandim-mediated induction of HO-1

Having shown that Protandim induces HO-1 mainly through Nrf2, we searched for other potential sites of synergy. Translocation of Nrf2 to the nucleus can be triggered by phosphorylation on serine 40 [23]. Involvement of multiple signaling pathways in Nrf2 phosphorylation and HO-1 induction have been previously reported [16,24–26]. We hypothesized that the synergistic action of the phytochemicals present in Protandim could take place through activation of multiple signaling pathways. To identify the signaling pathway(s) involved in HO-1 promoter induction by Protandim, we used different pharmacological inhibitors that specifically block each of the following pathways: 5-(2-benzothiazolyl)-3-ethyl-2-[2-(methylphenylamino) ethenyl]-1-phenyl-1*H*-benzimidazolium iodide (Akt inhibitor IV) for Akt, LY294002 for PI3-kinase, SP600125 for JNK, U0126 for MEK/ERK, SB203580 for p38MAPK, and rottlerin for PKCδ. Involvement of PI3-kinase/Akt was suggested by a significant ($p<0.001$) decrease in the Protandim-induced increase in HO-1 protein levels in the presence of LY294002 and Akt inhibitor IV (Fig. 7). We had previously reported that curcumin and its analogues induce HO-1 by activating this pathway [13]. Interestingly, in that study p38MAPK and PKCδ were not involved in HO-1 induction by curcumin, whereas the Protandim-mediated increase in HO-1 expression decreased by 50% in the presence of SB203580, an inhibitor of p38MAPK, and rottlerin, an inhibitor of PKCδ. Therefore, the components of Protandim other than curcumin could be contributing to HO-1 induction through the p38MAPK and PKCδ pathways. No significant effect on HO-1 induction by Protandim was observed in the presence of U0126 or SP600125, suggesting that MEK/ERK and JNK may not play a role in Protandim-stimulated HO-1 expression. Our observations in this experiment suggest that the synergy among the components of Protandim in the induction of HO-1 through Nrf2 could be due to the involvement of multiple signaling pathways.

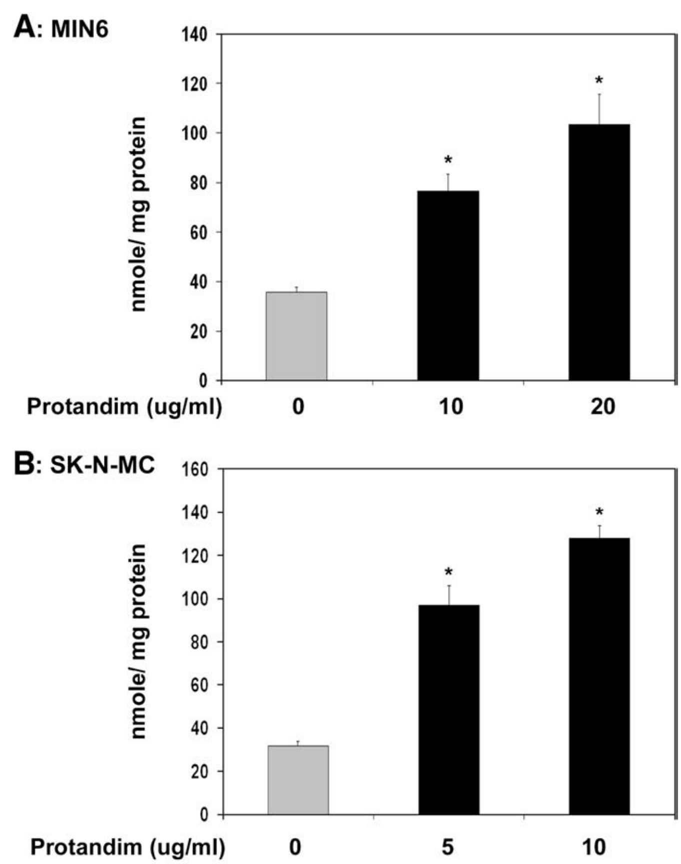

Fig. 8. Protandim-mediated increase in cellular total glutathione content. (A) MIN6 and (B) SK-N-MC cells cultured in 100-mm dishes to 70% confluence were exposed to the indicated concentrations of Protandim for 24 h. Cell lysates were prepared for the assay of total glutathione. *$p<0.001$ compared to untreated control.

Elevation of glutathione content in Protandim-treated cells

To determine the functional outcome of induction of an antioxidant enzyme by Protandim, we examined the cellular content of glutathione, which scavenges free radicals. There were significant ($p<0.001$) increases in the levels of total glutathione after exposure to Protandim (Fig. 8). In MIN6 cells, a two- to threefold increase was observed after treatment with 10–20 μg/ml Protandim. SK-N-MC cells showed higher sensitivity to Protandim as in the case of HO-1. Elevation of glutathione content by two- to fourfold was observed in the presence of 5–10 μg/ml concentrations of Protandim.

Discussion

The nutraceutical supplement Protandim has been shown to reduce the age-dependent increase in the accumulation of circulating products of lipid peroxidation in healthy subjects [12]. In this study, we demonstrate that the phytochemical ingredients present in Protandim exert synergy in inducing HO-1, a cytoprotective phase 2 enzyme, in cultured MIN6 and SK-N-MC cells. The effect of Protandim was significantly more than the sum of the effects of individual components. Omission of any one of the ingredients, including those that did not have any independent effect, reduced the activity of Protandim significantly. Curcumin was the most active component of this supplement with respect to HO-1 induction. The induction by Protandim involved the presence of ARE sites in the HO-1 promoter and the nuclear localization of the transcription factor Nrf2. Involvement of multiple signaling pathways mediated by PI3-kinase/Akt, p38MAPK, and PKCδ seems to be the probable mechanism for the synergy among the components of Protandim. Furthermore, Protandim elevated the glutathione content of cells, a marker of the cellular defense against oxidative stress. This study suggests that induction of antioxidant enzymes by a combination of phytochemicals at low doses is an efficient and safe approach to reducing oxidative stress in chronic diseases.

In response to oxidative stress and xenobiotic insult, phase 2 enzymes are induced as part of the cellular defense. The electrophiles generated by phase 1 enzymes (such as cytochrome P450s) are scavenged by phase 2 enzymes, including HO-1, γ-glutamyl cysteine ligase, glutathione *S*-transferase, and NAD(P)H:quinone oxidoreductase [27]. These enzymes contain ARE sites in their promoter region and are induced by the transcription factor Nrf2. Because Protandim induces HO-1 through Nrf2, we can anticipate that it could induce other phase 2 enzymes as well, although the degree of induction is likely to vary depending on the number of ARE sites in the promoter region. Coordinated induction of a family of enzymes with antioxidant and detoxification properties is likely to have therapeutic value. HO-1, in particular, has emerged as an important mediator of cellular defense against wide-ranging tissue injuries and has been suggested to be a therapeutic target in various disease models [15,28,29]. In addition to its antioxidant action by degradation of heme, HO-1 also exerts beneficial effects through the by-products of heme degradation, namely CO and biliverdin [15]. The cytoprotective actions of HO-1 in pancreatic β cells, which are known to express antioxidant enzymes at low levels, have been well documented. For example, induction of HO-1 in mouse islets by protoporphyrin improves islet function and survival after transplantation [30]. HO-1 upregulation leads to protection of β cells from cytokines and Fas [30–32]. Overexpression of HO-1 in rat islets reduces lymphocyte infiltration in the transplanted islets, suggesting anti-inflammatory effects [33].

In this study, we used a neuroblastoma cell line (SK-N-MC) and a mouse β-cell line (MIN6) to test the induction of HO-1 by Protandim. Our main objective was to determine if Protandim could be used as an antioxidant supplement in the context of neurodegenerative diseases and in diabetes. The brain is vulnerable to oxidative stress because of its high glucose-driven metabolic rate, high polyunsaturated fatty acid content, and high enzymatically active transition metal content [34]. The brain (2–3% of body weight) consumes 20% of the oxygen supply to the body, and 1–2% of the total oxygen consumed will form reactive oxygen species. Oxidative stress and accumulation of free radical-induced damage are important features of aging. Markers of oxidative stress are found in aged rats, especially in those with impaired spatial learning [35]. Lipid peroxidation, DNA oxidation products, and markers of protein oxidation accumulate in AD brains as a result of oxidative stress [36–39]. Tg2576 mice, a mouse model for AD, treated with a combination of antioxidant/anti-inflammatory agents have decreased protein carbonyls and decreased Aβ levels [40], suggesting that oxidative stress precedes AD pathology.

The pancreatic β cells are particularly vulnerable to oxidative stress-induced injury owing to low-level expression of antioxidant enzymes [1,19]. Oxidative stress is known to play an important role in β-cell dysfunction and loss in both types of diabetes. In type 1 diabetes, the cytokines released from immune cells that infiltrate islets generate free radicals, including nitric oxide [41]. In type 2 diabetes, although insulin resistance is considered to be the primary defect, glucotoxicity resulting from chronic hyperglycemia is known to cause β-cell dysfunction and loss through generation of free radicals [42]. Thus antioxidant therapy is likely to be beneficial in improving β-cell mass in diabetes. Furthermore, oxidative stress plays an important role in the loss of β cells in transplanted islets [43]. Islets are subjected to oxidative stress during isolation and storage and after transplantation. Overexpression of antioxidant enzymes in islets ex vivo has been shown to improve their function after transplantation [44,45].

The biological actions of curcumin, silymarin, and EGCG have been extensively studied. Very limited information is available regarding the other two ingredients, namely, ashwagandha and bacopa. Both are used in Ayurvedic medicine and studies have demonstrated their beneficial effects. Alcoholic extracts of ashwagandha administered to rats exert neuroprotective effects against 6-hydroxydopamine-induced oxidative stress [46]. The markers of oxidative stress were improved by ashwagandha. Several studies have demonstrated the antioxidant effects of extract of *B. monniera* in vivo [47–49]. The active glycosides from this herb have been isolated and characterized [50].

Although curcumin showed the maximum effects in the induction of HO-1, it would be difficult to predict the same with other antioxidant enzymes, especially those not regulated by Nrf2. For example, superoxide dismutase and catalase, observed to be induced by Protandim in a previous study [12], do not have ARE sites in their promoter regions. Different components are likely to play a primary role with respect to different end points of oxidative stress. As indicated previously, the composition of Protandim was designed based on the vast amount of studies carried out with those phytochemicals. The in vitro cell culture model used in this study could be used to determine the role of different components of Protandim on diverse end points. We will also be able to design different combinations of phytochemicals depending on the objective with respect to different disease conditions.

Phosphorylation of Nrf2 on serine 40 results in its dissociation from Keap1 and translocation to the nucleus [23]. Inducers of Nrf2-driven phase 2 enzymes have been reported to use multiple signaling pathways for Nrf2 phosphorylation. For example, signaling mediated by PI3-kinase [13,24], MEK/ERK [51], p38MAPK [16], JNK [26], and protein kinase C [52] has been shown to play a role in the induction of HO-1. In the present study, we observed a significant decrease in Protandim-mediated HO-1 induction when PI3-kinase, Akt, PKCδ, or p38MAPK was inhibited (Fig. 7). In our previous report with curcumin, we did not observe a significant role for p38MAPK, and PKCδ played a minor role in the case of demethoxy curcuminoids [13]. Therefore it seems that components other than curcumin might be contributing to HO-1 induction through p38MAPK and PKCδ. The concomitant stimulation of parallel signaling pathways seems most likely to be the source of the observed synergy among the components of Protandim.

The findings described in this study suggest that Protandim induces HO-1 through activation of Nrf2 by a mechanism involving multiple signaling pathways. Nrf2 is also known to induce several other antioxidant enzymes, including enzymes involved in the synthesis of glutathione. Glutathione synthesis is regulated by γ-glutamyl cysteine ligase, which consists of a regulatory subunit (GCLM) and a catalytic subunit (GCLC). The expression of both GCLM and GCLC is regulated by the Keap1–Nrf2–ARE pathway [53,54]. Significant increases in the cellular glutathione content were observed in Protandim-treated cells (Fig. 8), suggesting the induction of enzyme(s) involved in glutathione synthesis. This observation is of therapeutic significance because glutathione deficiency contributes to oxidative stress and plays an important role in the pathogenesis of many diseases [55].

Several studies have examined a possible link between consumption of diets rich in flavonoids and protection from diseases associated with oxidative stress [56,57]. However, doubts have been raised because of the low plasma concentrations of individual compounds after consumption through diet. These concentrations are significantly low compared to those used in in vitro studies. Furthermore, when higher pharmacological doses are used to demonstrate their effects in vivo, they cause toxic side effects. It is possible that the beneficial effects of dietary phytochemicals could result from the synergy between those compounds when used at low doses. Ayurvedic medicine also suggests synergy between components from one or more herbal preparations [58]. This possibility is evident from the findings of this study. The dose–response effect of Protandim on HO-1 promoter activity also gives a sigmoidal curve, which is a marker for synergy (Fig. 1). Even at 10 μg/ml, Protandim is able to induce the HO-1 promoter. Curcumin, the primary inducer of HO-1, is present at a concentration of 1.05 μg/ml, or 2.8 μM, in a 10 μg/ml extract of Protandim. This is significantly lower than the concentration of 20 μM required to demonstrate a noticeable effect in our recent study [13]. The ability of curcumin to induce HO-1 at such low concentrations in the presence of other ingredients strongly suggests that there is synergy among the phytochemicals.

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