

Phytochemical Combinations Modulate the Activation of Nrf2 and Expression of SOD in Pancreatic Cancer Cells More Efficiently Than Single Plant Components [†]

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Abstract: Pancreatic adenocarcinoma mainly occurs in elderly people. Thus, the management of pancreatic cancer in the aging population is becoming increasingly relevant. In this preliminary study we evaluated the effect of selected phytochemicals and their combinations on the expression and activation of Nrf2 transcription factor in the human pancreatic cancer cell line MIA-Pa-Ca-2. Treatment for 24 h with xanthohumol (X), resveratrol (RES), indole-3-carbinol (I3C) or phenethyl isothiocyanate (PEITC) had no effect on the expression and activation of Nrf2, or the expression of the *SOD* gene controlled by Nrf2. However, combinations of these phytochemicals significantly increased Nrf2 activation and subsequently the expression of *SOD*. The most efficient were the mixtures of resveratrol and glucosinolates degradation products, I3C and PEITC. These results indicate that combinations of phytochemicals resembling that occurring in natural diets may efficiently modulate the signaling pathways, whose proper function is important for pancreatic cancer prophylaxis or improving the results of conventional therapy.

Keywords: Nrf2; SOD; phytochemicals; pancreatic cancer; elderly people

1. Introduction

Pancreatic cancers possess the worst prognosis, having one of the highest mortality rates [1]. Advancing age is a high risk factor for this type of cancer, and more than 60% of new cases and over 70% of cancer mortalities occur in elderly people [2]. Thus, searching for an alternative approach for both prevention and therapy of these tumors is therefore necessary. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor involved in the regulation of expression of antioxidative enzymes such as superoxide dismutase (SOD) [3]. Our previous studies have shown that naturally occurring compounds from chalcones group (xanthohumol) [4], stilbenes (resveratrol) [5], products of glucosinolates degradation (indole-3-carbinole, phenethyl isothiocyanate) [6] have the ability to modulate expression of *Nrf2* and *SOD* in HepG2 and HaCaT cells. Recent studies indicate that using combinations of naturally occurring chemopreventive agents, instead of using the individual phytochemical is a more efficient strategy of cancer chemoprevention [7]. The aim of this study was to evaluate the effect of combinations of the different classes of phytochemicals on the Nrf2 activation and expression of *SOD* in human pancreatic carcinoma cells.

2. Materials and Methods

2.1. Cell Culture and Treatment

Human pancreatic cancer cells (MIA-Pa-Ca-2, ATCC, Rockville, MD, USA) were cultured in standard conditions. Cells were treated with 1 μ M phenethyl isothiocyanate (PEITC), indole-3-carbinol (I3C), resveratrol (RES), xanthohumol (X) and their combination for 24 h.

2.2. Preparation of RNA, Cytosolic and Nuclear Fraction

Total RNA from cells was isolated with the GeneMatrix Universal DNA/RNA/Protein Purification Kit (EurX, Gdańsk, Poland). The cytosol and nuclear extracts from the MIA-Pa-Ca-2 cell line were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Research, Milpitas, CA, USA).

2.3. Real-Time PCR Analysis

For cDNA synthesis RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada) was used. The real-time PCR assay was performed using Maxima SYBR Green qPCR Master Mix (Fermentas, Burlington, Ontario, Canada) with specific primers for each gene. The relative change in the expression of *Nrf2* and *SOD* was calculated using the Pfaffl method. *PBGD* and *TBP* served as reference genes.

2.4. Western Blot Analysis

Nuclear and cytosolic fractions (100 μ g of protein) were analyzed on 10% and 12% SDS-PAGE and transferred to nitrocellulose membrane Immobilon P (Millipore). The membranes were then incubated with primary antibodies against Nrf2, SOD (Santa Cruz Biotechnology, Paso Robles, CA, USA) and secondary antibody with the alkaline phosphatase-labeled IgG. The amount of immunoreactive product in each lane was determined by scanning and evaluating with the Quantity One programme (BioRad, Hercules, CA, USA). Values were expressed as relative quantity (RQ) per mg of protein.

2.5. Nrf2 Activation Elisa Assay

Nrf2 activation was evaluated by an enzymatic immunoassay using commercial kit (Transcription Factor ELISA Assay Kit Active Motif, Rixensart, Belgium). The nuclear extract was incubated in the wells with oligonucleotide sequence which contain the ARE consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3'). Wells were then washed and incubated with the antibody against Nrf2. Subsequent addition of an HRP-conjugated secondary antibody allowed the colorimetric readout of the conjugate at 450 nm.

2.6. Statistical Analysis

One-way ANOVA test was applied for the statistical analysis. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, with $p < 0.05$.

3. Results

3.1. The Effect of Single Phytochemicals and Their Combinations on the Expression of Nrf2

Quantitative analysis (Figure 1A) of Nrf2 transcript and nuclear protein levels showed that phytochemicals alone did not affect the expression of Nrf2 in MIA-Pa-Ca-2 cells. However, the treatment with the combinations of I3C and RES increased the amount of Nrf2 transcript and nuclear protein by 34% (Figure 1A,B). Moreover, this combination as well as the combination of RES and PEITC and to less extent X with PEITC significantly (by 80–53%, respectively) enhanced the Nrf2

activation measured in terms of the amount of Nrf2 contained in DNA binding complex. Nrf2 consensus site-ARE was immobilized on the ELISA microplates as bait (Figure 1C).

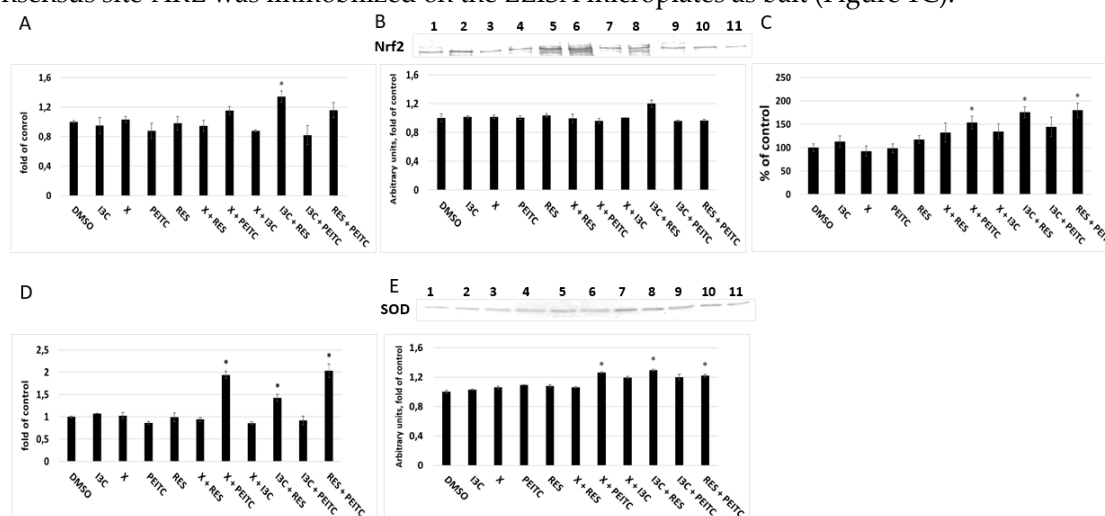


Figure 1. The effect of phytochemicals and their combinations on Nrf2 and SOD expression in MIA-Pa-Ca-2 cells. (A) Nrf2 transcripts calculated as mRNA level in comparison with control cells (expression equals 1). (B) Representative immunoblots for the analysis of the nuclear level of Nrf2 protein. Lane 1—control; lane 2—I3C 1 μ M; lane 3—X 1 μ M; lane 4—PEITC 1 μ M, lane 5—RES 1 μ M; lane 6—X + RES 1 μ M; lane 7—X + PEITC 1 μ M; lane 8—X + I3C 1 μ M; lane 9—I3C + RES 1 μ M; lane 10—I3C + PEITC 1 μ M; lane 11—RES + PEITC 1 μ M. Results of Western blot analysis were expressed as nuclear protein level in comparison with control cells (expression equals 1). (C) Binding of Nrf2 to ARE-containing oligonucleotide, the values were calculated and compared with control level, equals 100%. (D) SOD transcript levels. (E) Representative immunoblots for the analysis of SOD protein. The data are presented as mean $\pm$ SEM. The asterisk (*) above the bar denotes statistically significant differences to the control group, $p < 0.05$.

3.2. The Effect of Phytochemicals and Their Combinations on the Expression of SOD

The increased activation of Nrf2 as result of treatment with I3C and RES and two other combinations (RES+PEITC; X+PEITC) led to enhanced expression of the SOD gene. The increased expression was confirmed both on mRNA transcript and at protein level (Figure 1D, E).

4. Discussion

Nrf2 regulates the expression of many antioxidant enzymes including SOD, which catalyze the dismutation of superoxide anion free radical into molecular oxygen and hydrogen peroxide. The enzyme can be considered as an anti-inflammatory agent which also prevents precancerous cell changes. SOD levels drop with aging and thus elderly people become more prone to oxidative stress-related diseases including cancer [8]. The increased expression of SOD found in our study as a result of treatment with phytochemicals, common diet ingredient combinations, suggest their cancer chemopreventive potential. On the other hand, in pancreatic cancer, the model that was used in this study, the increased expression of SOD as result of Nrf2 activation may protect against side effects of drugs such as anthracyclines, often used in pancreatic cancer treatment [9].

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Conflicts of Interest: The authors declare no conflict of interest.

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