Unique natural antioxidants (NAOs) and derived purified components inhibit cell cycle progression by downregulation of ppRb and E2F in human PC3 prostate cancer cells

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Abstract Prostate cancer (PCA) is the leading cause of cancer mortality among older men in Western countries. Epidemiological studies have shown correlation between a lower risk of PCA and a higher consumption of antioxidants. However, the mechanism by which antioxidants exert their effects is still unknown. In the present study, we explored the signaling mechanism through which unique natural antioxidant derived from spinach extract (NAO) exerts their beneficial effects in the chemoprevention of PCA using human PC3 cells. Probing into the effect of NAO and its derived polyphenols on cell-cycle G1 arrest, we found that they cause cell-cycle prolongation. NAO and its two derived purified components exhibited a significant increase in the level of $p21^{cip1}$ expression after 36 h of starvation, followed by 18 h of treatment with NAO in the presence of serum. In addition, under similar conditions, the expressed level of Cyclin A and CDK-2 in the PC3 cells was significantly reduced after treatment with NAO or its purified components. Immunoblot analysis demonstrated a significant increase in the hypophosphorylated form of pRb and a decrease in ppRb. NAO and its purified derived components were found to downregulate the protein expression of another member of the pRb family, p107, as well as that of E2F-1. These results suggest that NAO-induced G1 delay and cell cycle prolongation are caused by downregulation of the protein expression of ppRb and E2F in the human PCA cell line PC3.

Keywords: Prostate cancer; Antioxidant; Cell cycle; E2F; pRb

1. Introduction

Prostate cancer (PCA) is one of the most common malignancies in Western societies. Risk factors include age, race, ethnicity, hormone metabolism, and dietary factors [1,2].

The use of naturally occurring dietary agents is becoming increasingly appreciated as an effective way to manage many types of cancer in an approach known as cancer chemoprevention [3–12]. Among these natural dietary agents, polyphenols, a constituent of different classes of foods and beverages, have been intensely studied in recent years [13–23]. The efficacy of green tea polyphenols, wine antioxidant polyphenols, and silibinin in the chemoprevention of PCA was reported [3,24,25].

We recently published details on the isolation and characterization of unique natural antioxidants (NAOs) from spinach leaves [26,27]. This NAO contains polyphenols, which include phenolic acids (*p*-coumaric acid derivatives) and the glucuronic acid derivatives of flavonoids [26]. We have shown that NAO has antioxidative [26,27], antiproliferative [28], and anti-inflammatory properties [29–33] in various biological systems. Spinach flavonoids have been shown to have strong antimutagenic potential and were found beneficial in a Tg.AC mouse model [33]. Furthermore, spinach NAO has been reported to have a protective effect in various animal models for diseases such as doxorubicin-induced cardiotoxicity [30], skin papilloma [31], and lipopolysaccharide-induced septic shock [29,32].

Recently, we have investigated the efficacy of NAO in PCA chemoprevention [28]. NAO was tested in the transgenic adenocarcinoma mouse prostate (TRAMP) model. The efficacy of NAO was compared to that of the green tea polyphenol, epigallocathecin-3-gallate (EGCG), and *N*-acetylcysteine (NAC). Although significant effects were exerted on different lobes by the latter three antioxidants, when the most severe hyperplasia in all four lobes of TRAMP mice was evaluated, only NAO reduced hyperplasia at weeks 9 and 13 [28]. In addition, NAO exhibited antiproliferative activity in DU145 and PC3 cells [28].

In the present study, we explored the signaling mechanism through which NAO and its derived polyphenols exert their beneficial effects in the chemoprevention of PCA using human PC3 cells (an androgen-independent and metastatic PCA cell line). Here we demonstrate that the purified polyphenols isolated from NAO attenuate the G1-to-S transition of PC3 cells by inhibiting the expression of key elements in the cell cycle machinery which regulate G1-to-S transition.

2. Materials and methods

2.1. Materials

The human PCA cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium (Biological Industries, Inc., Kibbutz Beit Haemek, Israel)

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Abbreviations: NAO, natural antioxidant; PCA, prostate cancer; TRAMP, transgenic adenocarcinoma mouse prostate; CDK, cyclin-dependent kinase; EGCG, epigallocathecin-3-gallate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; CDKI, cyclin-dependent kinase inhibitor

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supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin, and 0.2% amphotericin. The cells were maintained at 37 °C and 5% CO_2 in a humid environment.

NAO is a brown powder. It is a water-soluble antioxidant composed of a mixture of natural molecules extracted and purified from spinach (*Spinacia oleracea*) leaves and containing mainly aromatic polyphenols [26]. For preparation, spinach leaves were homogenized with an equal amount of H_2O (w/w). The supernatant was collected and purified by ultrafiltration using a 3K-pore-size membrane, and centrifuged at $20\,000\times g$ for 10 min. The supernatant was collected and used as an NAO in this study.

2.2. Cell proliferation and viability assay

The effect on cell proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, MO) assay based on the ability of live cells to cleave the tetrazolium ring to a molecule in active mitochondria that absorb at 570 nm [34]. 5×10^3 PC3 cells were grown on a 96-well microliter plate and incubated with polyphenols [glucurinated flavonoids (5–20 μM) and *p*-coumaric acid derivatives (15–60 μM)] or NAO (0.8, 1.6, and 3.2 mg/ml) in RPMI-10% FCS. After 24, 48, and 72 h, the medium was changed and 130 μl/well of fresh RPMI-1640 complete media were added. Next, 20 μl of MTT reagent (5 mg/1ml PBS) was added to each well, and the cells were further incubated at 37 °C for 2 h. To determine lysis of the cells, 100 μl of *N*,*N*-dimethyl formamide solution (50% final concentration of *N*,*N*-dimethyl formamide and 20% sodium dodecyl sulfate, pH 4.7) was added to each well for an additional 7 h, followed by reading on a scanning multiwell spectrophotometer.

For the trypan blue dye assay, 1×10^6 PC3 cells were plated in triplicate (for each time point) into 100-mm culture dishes and treated with NAO (0.8, 1.6, and 3.2 mg/ml) in RPMI-10% FCS for 24, 48, and 72 h. The viable cells were determined by trypan blue dye assay. The experiment was repeated three times.

2.3. DNA cell cycle analysis

PC3 cells were grown to 70% confluence, detached using a solution of 0.1% trypsin, counted, and plated at a density of 1×10^6 cells in 100-mm culture dishes. The cells were starved for 36 h (G0 phase), after which they were treated with polyphenols [glucurinated flavonoids (5–20 μM) and *p*-coumaric acid derivatives (15–60 μM)] or 3.2 mg/ml NAO, in RPMI complete medium for 8–24 h and then spun at 300 g. The pellet was resuspended in 250 μl PBS and 1 ml ethanol for 1 h at 4 °C. After centrifugation at $300\times g$ for 5 min, the pellet was washed twice with PBS, suspended in 400 μl of PBS and 400 μl of propidium iodide (50 μg/ml final concentration) for 15 min, and analyzed using flow cytometry.

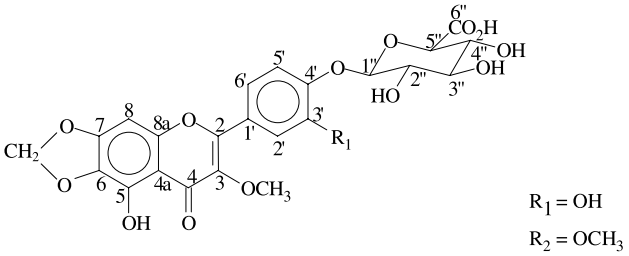
2.4. Western blot analysis

The PC3 cells were grown to 70% confluence, detached using a solution of 0.1% trypsin, counted, and plated at a density of 1×10^6 cells in 100-mm culture dishes. The cells were starved for 36 h, after which they were treated with purified polyphenols derived from NAO [glucurinated flavonoids (40 μM) or *p*-coumaric acid derivatives (120 μM) or a combination of glucurinated flavonoids (20 μM) and *p*-coumaric acid derivatives (60 μM) in RPMI-1640 complete media for 18 h] or with NAO (0.8, 1.6, and 3.2 mg/ml). The media were then aspirated and the cells washed with cold PBS. The cells were scraped and washed twice by centrifugation at $500\times g$ for 5 min at 4 °C. The pellet was resuspended in lysis buffer supplemented with proteases and phosphotase inhibitors and incubated for 1 h at 4 °C. The lysate was collected by centrifugation at $14\,000\times g$ for 40 min at 4 °C, and the supernatant (total cell lysate) was stored at −20 °C. For Western blot analysis, 25–40 μg protein were resolved over 7.5% or 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (1–5% non-fat dried milk/1% Tween 20 in PBS) for 1 h at room temperature, incubated with appropriate monoclonal (human reactive anti-$p21^{cip1}$, anti-CDK-2, and anti-pRb) or polyclonal (human reactive anti-Cyclin A, anti-p107, and E2F-1) primary antibodies in blocking buffer overnight at 4 °C. The blot was then incubated with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate and detected by chemiluminescence and autoradiography using X-ray film.

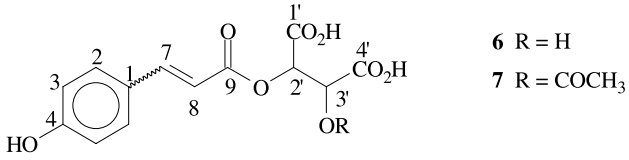
2.5. HPLC analysis and separation

The concentration of solids in the NAO supernatant was about 3%, as determined by reflectometry. The supernatant was concentrated to 25% by evaporation at 80 °C. One ml of this supernatant was mixed with 9 ml acetone AR for 5 min by stirring. This mixture was centrifuged at $20\,000\times g$ for 10 min. The supernatant was collected, and the acetone was evaporated at 85 °C; this residue was used for HPLC analysis and separation. Four compounds were purified from NAO: two glucurinated flavonoids and two *p*-coumaric acid derivatives.

Flavonoid derivatives (GF):



p-Coumaric acid derivatives:



One mg of NAO contains about 2% of both purified materials.

3. Results

3.1. Effect of purified polyphenols derived from NAO on PCA cell proliferation and cell viability

Recently, we have elucidated the efficacy of NAO in slowing down the spontaneous tumorigenic progression in TRAMP male mice [28]. The mechanisms involved in the chemopreventive effect of NAO and its purified derived components, are not yet understood. The main goal of the present study was to shed some light on these mechanisms using the PCA cell line system.

The effect of the purified polyphenols and the spinach extract NAO at various concentrations on cell proliferation of human PCA cell line was studied after 24, 48, and 72 h of cell growth (Fig. 1). It is clearly seen that in polyphenol-treated PC3 cells, inhibition of cellular proliferation occurred in a dose- and time-dependent manner. Similar data were obtained with NAO (Fig. 2). These data were also supported by the results of the trypan blue assay for studying cell viability (Fig. 3A). The trypan blue exclusion assay demonstrates that NAO treatment resulted in a dose- and time-dependent inhibition of cell growth.

Fig. 3B shows that the inhibition of cell growth is not a result of cell death. The relatively high proportion of dead cells found in the control sample 48 h after seeding probably reflects anoikis [35]. After 72 h, the proportion of dead cells is reduced by more than half (data not shown).

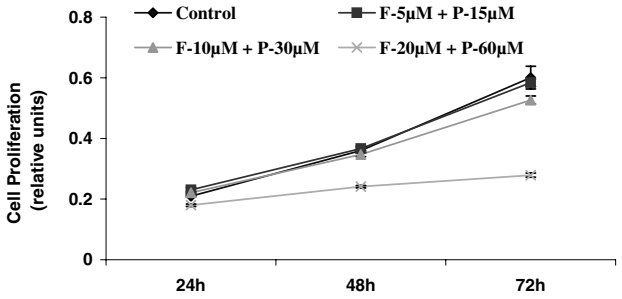


Fig. 1. Dose- and time-dependent effect of the combination of purified compounds derived from NAO on the proliferation of human PC3 cell line. PC3 cells at 5×10^3 cells/well were cultured with purified compounds [glucurinated flavonoids (F) (5–20 μM) and *p*-coumaric acid derivatives (P) (15–60 μM)] in RPMI-10% FCS. Cell proliferation on each day was determined by MTT assay, and experiments were terminated on day 4. Each time point represents quadruplicate samples ± S.E., and the growth curve was derived from three repeated experiments.

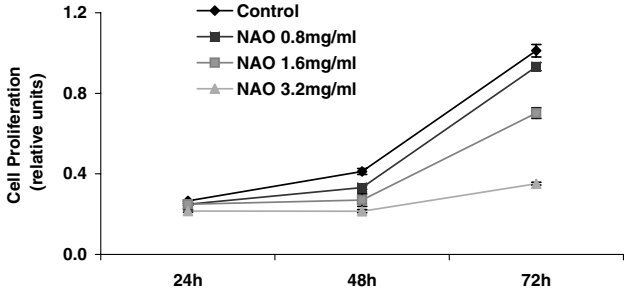


Fig. 2. Dose- and time-dependent effect of NAO on proliferation of human PC3 cell line. PC3 cells at 5×10^3 cells/well were cultured with NAO (0.8, 1.6, and 3.2 mg/ml) in RPMI-10% FCS. Cell proliferation on each day was determined by MTT assay, and experiments were terminated on day 4. Each time point represents quadruplicate samples ±S.E., and the growth curve was derived from three repeated experiments.

3.2. Effect of NAO-derived polyphenols on cell cycle

Since the effect of the polyphenols derived from NAO on cell proliferation may be due to cell cycle regulation, we examined this effect on cell cycle perturbations using flow cytometric analysis. The effect of these antioxidants on PC3 cells was studied after starving the cells for 36 h, followed by serum addition. No cells were found in the sub-G1 population region of the cell cycle, which eliminates apoptosis as a possible mechanism of action. Moreover, the hypothesis that the purified polyphenols or NAO act through apoptosis induction was negated since no morphological alterations were detected, as shown by confocal microscopy after either labeling cells with acridine orange and ethidium bromide or detection with the Annexin V Fitc (Apoptosis Detection Kit) (data not shown).

Probing into the effect of the polyphenols on cell-cycle G1 arrest, we did a time-dependent study on a synchronized population, following the status of the cells at different times points after they re-entered the cell cycle. The data obtained are presented in Figs. 4 and 5. They clearly show that the purified spinach polyphenols inhibit the exit of the cells from the G1 state. This phenomenon was more significant after 18 h

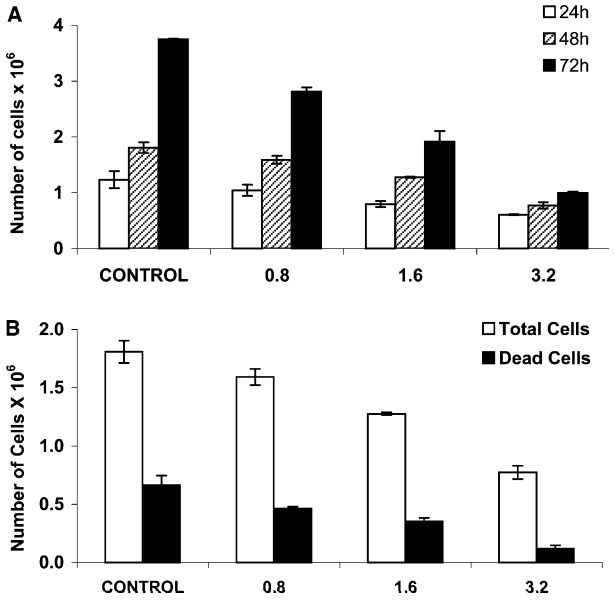


Fig. 3. (A) Dose- and time-dependent effect of NAO on PC3 cell growth. (B) Viability of PC3 cells following NAO treatment for 48 h. PC3 cells at 1×10^6 cells/dish were incubated with NAO (0.8, 1.6, and 3.2 mg/ml) in RPMI-10% FCS, and the total number of cells and the number of viable cells were measured by trypan blue dye assay. The data are represented as number of cells $\times 10^6$. Data shown are mean values ± S.E. determined from three independent experiments.

of treatment with NAO or its purified components. For that reason, we focused on the 18-h treatment for further analysis. The passage of the PC3 cells to the G1 phase was significantly delayed by NAO at a concentration of 3.2 mg/ml after 24 h of treatment, as compared to the control.

3.3. Effect of the polyphenols on cell protein expression

Using Western blot analysis, we next assessed the effect of NAO-derived polyphenols on the expression of proteins that might be involved in the control of cellular proliferation. Cell cycle progression is regulated via irreversible transitions propelled by CDKs and cyclins [36]. Therefore, we reasoned that an observed G1 delay by the polyphenols or NAO could be due to a decrease in kinase activity of CDKs and cyclins. CDK activity is regulated positively by cyclins and negatively by CDKIs [36,37]. We assessed the effect of the polyphenol treatment on cyclin-dependent kinase inhibitor (CDKI) $p21^{cip1}$ level in PC3 cells. The glucurinated flavonoids and *p*-coumaric acid derivatives exhibited a significant increase in the level of $p21^{cip1}$ expression after 36 h of starvation, followed by 18 h of treatment with the purified polyphenols (Fig. 6). A similar effect was obtained with NAO (Fig. 6). In addition, we found that under similar conditions, the expressed level of Cyclin A and CDK-2 in the PC3 cells was significantly reduced after treatment with the polyphenols or NAO (Fig. 6).

Proteins from the retinoblastoma (Rb) family and from the E2F family of transcription factors are thought to play a key role in the regulation of G1>S phase transition in the cell cycle. In view of the data obtained from the cytometric study of the cell cycle, which clearly indicated a G1>S delay (Fig. 4), we explored the possible involvement of the pRb-E2F pathway during polyphenol-mediated cell cycle delay. It is well documented in the literature that the binding of phosphorylated

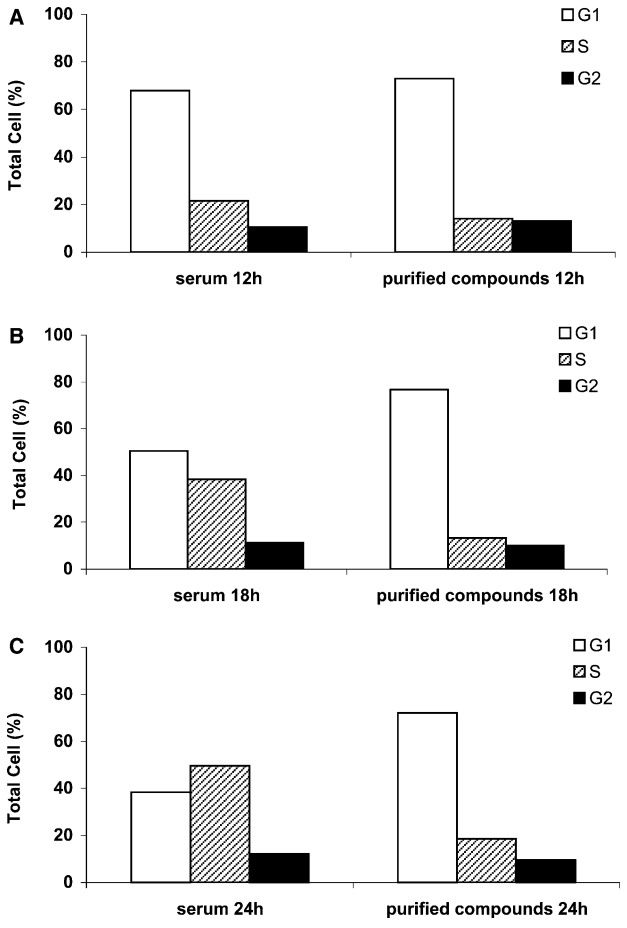


Fig. 4. Effect of the purified compounds derived from NAO on cell cycle G1 delay. A time-dependent study followed the status of the cells after treatment with the purified compounds. PC3 cells at 1×10^6 cells/dish were synchronized by serum starvation for 36 h after which they were treated with serum and a combination of the purified compounds [glucurinated flavonoids (20 μM) and *p*-coumaric acid derivatives (60 μM)] for 12, 18, and 24 h and analyzed by flow cytometry. The data shown are representative of three independent experiments with similar findings.

members of the Rb family of proteins is essential for the activation of members of the E2F family of transcription factors [38]. Therefore, the effect of the polyphenols and NAO on the expression of Rb proteins in PC3 cells was assessed. The phosphorylation of Rb proteins is an important event in the progression of the cell cycle at G1>S transition. Immunoblot analysis demonstrated a significant increase in the hypophosphorylated form of pRb and a decrease in ppRb (Fig. 6). pRb is largely found in hypophosphorylated form in early G1 phase. The purified derived components and NAO were found to downregulate the protein expression of another member of the Rb family, p107, as well as that of E2F-1 (Fig. 6). We demonstrated that the effect of NAO treatment on the expression of several members of the cell cycle machinery in PC3 cells was dose-dependent (Fig. 7). We also studied the effect of the purified polyphenols and NAO on other proteins involved in cell cycle regulation such as p27, cyclin D1, Cyclin E, and CDK 4/6. We did not find any significant changes in the level of these proteins due to the various treatments (data not shown).

It is clear that the polyphenol or NAO treatment significantly reduced the expression level of phosphorylated Rb proteins. These results suggest that the polyphenol-induced G1 delay and cell cycle prolongation are caused by downregulation of the protein expression of pRb and E2F in the human PCA cell line PC3.

4. Discussion

We have previously reported that an aqueous spinach leaf extract contains a powerful system composed of NAOs, which we named NAO [39]. Recently, the main components of NAO were isolated, chemically analyzed, and characterized as derivatives of flavonoids and *p*-coumaric acid [26]. The biological potential of NAO was elucidated in various in vivo and in vitro model systems [32].

In a more recent study, we elucidated the beneficial effect NAO has in preventing PCA in mice using the TRAMP model [28]. In this model, the efficacy of NAO in reducing the severity of hyperplasia on the various prostate lobes was compared to that of other antioxidants. NAO exerted a significant preventative effect on the dorsal and lateral lobes after 9 and 13 weeks of treatment. In addition, plasma peroxide levels in TRAMPs were reduced following oral administration of NAO. Moreover, in NAO-treated PC3 PCA cells, inhibition of cellular proliferation occurred.

In the present study, we focused on the effect of the purified polyphenols isolated from NAO on cell proliferation of human PCA cell lines, and tried to elucidate the signaling mechanism through which they exert their effect. In addition, we studied, in parallel, the effect of NAO in these systems since NAO has the potential to be used as a natural preventive combination against PCA.

First, we discovered that in the PC3 PCA human cell line, the inhibition of cell proliferation is dose- and time-dependent (Figs. 1 and 2).

Since preliminary results indicated that NAO can cause G1 arrest [28], we carefully explored these pathways by following the dependence of the cell cycle on time kinetics both with the control and the polyphenol-treated cells (Fig. 4). This clearly demonstrated that the purified fractions and NAO affect the proliferation of the PCA cells by prolonging the cell cycle. Thus, they delay the mitosis of the cancer cells by increasing the level of the cells at the G1 phase.

Pathways that link the cell cycle machinery to the transcription apparatus largely control cell cycle progression. Consequently, transcriptional components play a central role in the regulation of the cell cycle. Members of the E2F transcription factor family have been characterized as growth-stimulating proteins [40,41]. E2F and particularly E2F-1 function to enhance cell growth by advancing quiescent cells through G1 into S phase [40]. Typically, E2F-1 activity is inhibited during G1 by binding to the Rb tumor suppressor gene product [42–44]. pRB belongs to a family of proteins designated as "pocket proteins" [45], which include Rb p107. Members of this family suppress cell growth, at least in part, by inhibiting E2F-dependent transcription of genes whose products are required for DNA synthesis and/or cell cycle progression [41,45]. The ability of pRb to bind E2F depends on its phosphorylation status. Specifically, pRb is phosphorylated in G0/G1 and becomes overtly hyperphosphorylated at

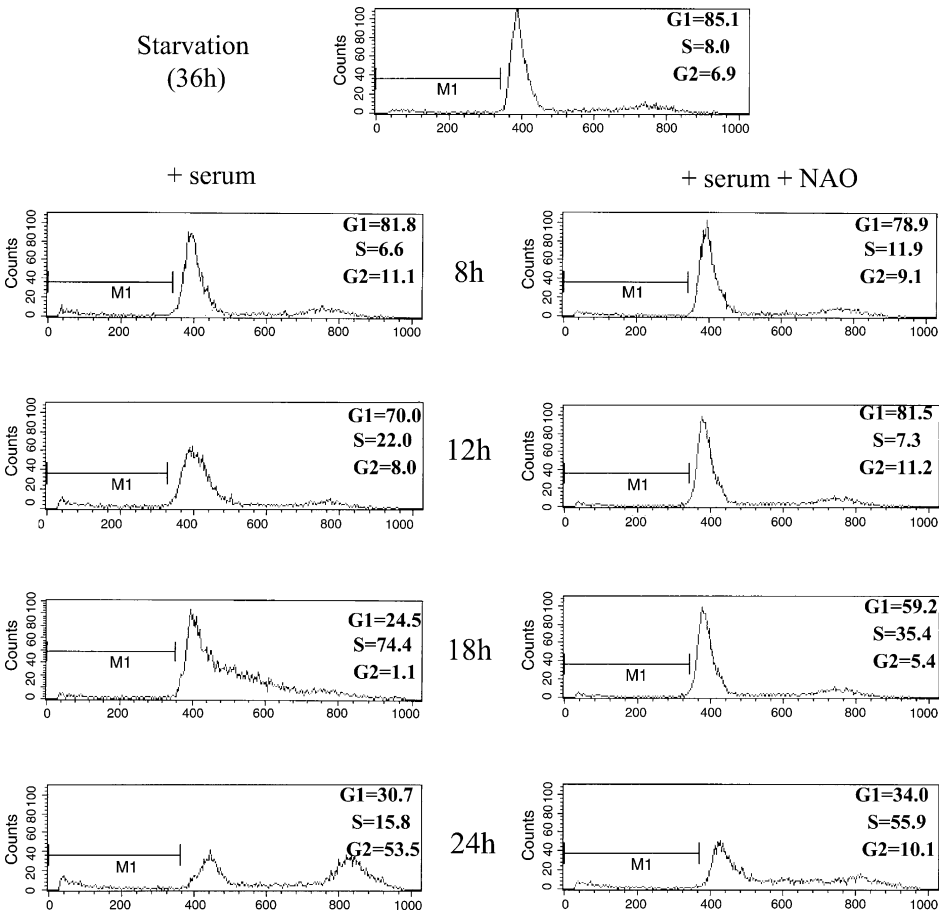


Fig. 5. Effect of NAO on cell cycle G1 delay. A time-dependent study followed the status of the cells after NAO treatment. PC3 cells at 1×10^6 cells/dish were synchronized by serum starvation for 36 h after which they were treated with serum and 3.2 mg/ml NAO for 8, 12, 18, and 24 h and analyzed by flow cytometry. The data shown are representative of three independent experiments with similar findings.

or near the G1/S boundary. pRb phosphorylation, caused by cyclin–CDK complexes, results in the release of "free" E2F and thereby activates genes required for DNA synthesis, such as DNA polymerase and thymidine kinase [46].

Cyclin A:CDK-2 complex phosphorylates Rb and its kinase activity is rate-limiting for entrance into the S phase [47]. Cyclins, their associated kinases (CDKs), and cyclin inhibitory proteins like $p21^{cip1}$, are integral components in the coordinated progression of the cell cycle [48–50]. $p21^{cip1}$ is a ubiquitous inhibitor of cyclin kinases and a critical regulator of cell cycle progression [48,50–52]. $p21^{cip1}$ has been shown to independently bind CDK-2, Cyclin A, and proliferating cell nuclear antigens [52].

The association of $p21^{cip1}$ with Cyclin A:CDK-2 inhibits kinase activity [50] and interferes with transcriptional activation events mediated by this complex [51,53].

The ability of the spinach polyphenols and NAO to attenuate G1-to-S transition is probably a consequence of their ability to interfere with several components which regulate the cell cycle. At the protein level, these antioxidants inhibit the elevation of several key proteins which function in the transition from G1-to-S, including Cyclin A, CDK-2, and E2F. Moreover, the polyphenols and NAO elevate the protein level of $p21^{cip1}$, which inhibits Cyclin A:CDK-2 activity. All together, these changes are probably responsible for sustaining the hypophosphorylated state of pRb that is seen in cells treated with the polyphenols or NAO. This effect, together with the reduced level of E2F protein, which is needed as an active transcription factor at the G1/S boundary, explain the delay in S phase entrance seen in the presence of these NAOs. Nevertheless, an as-yet unknown mechanism somehow overrides these effects since the cells do eventually enter the S phase. For this reason, the polyphenols and NAO cause prolongation of the cell cycle instead of G1 arrest. The mechanism by which these natural compounds reduce the protein levels of Cyclin A, CDK-2, or E2F is still an enigma. In order to see if NAO interferes with mitogenic signals elicited by serum-derived growth factors, we designed the experiments in a way that enabled us to compare the mitogenic response of quiescent cells when serum is added in the presence or absence of NAO. In fact, serum addition caused an elevation in the expression of Cyclin A, CDK-2, and E2F while NAO prevented this effect in a dose-dependent manner, suggesting a possible disruption of the transduction of the mitogenic signal. One publicized study mentioned that coumaric acid, which is one of the components of NAO, is a tyrosine kinase inhibitor and might block the activity of tyrosine kinase receptors [54]. Our results showed that, besides *p*-coumaric acid, glucorinated flavonoids were also effective, indicating that NAO activity resides in more than one component. Recently, several studies with the antioxidant vitamin E and the polyphenolic flavonoid silibinin showed similar results concerning their anti-proliferation effects on

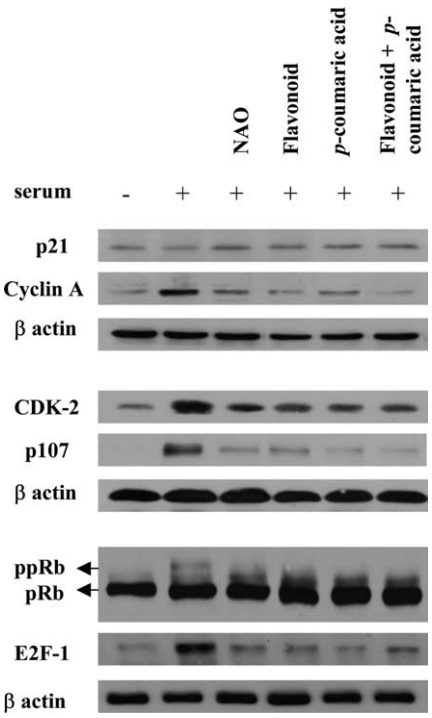


Fig. 6. Effect of NAO and its derived purified compounds on the level of $p21^{cip1}$, Cyclin A, CDK-2, p107, pRb, and E2F-1 in PC3 cells. PC3 cells at 1×10^6 cells/dish were starved for 36 h, and then treated with either NAO (3.2 mg/ml) or one of the two NAO-derived derivatives, glucurinated flavonoid (40 μM) and *p*-coumaric acid (120 μM) or the combination of glucurinated flavonoids (20 μM) and *p*-coumaric acid derivatives (60 μM), in complete media for 18 h. Total cell lysates were prepared, and 40 μg protein was subjected to SDS–PAGE, followed by immunoblot analysis. The data shown are representative of three independent experiments with similar findings.

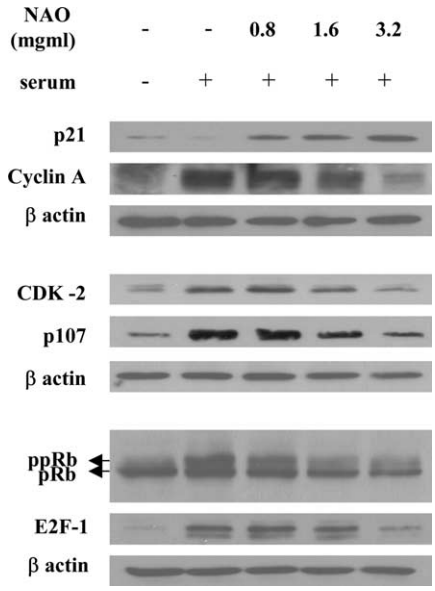


Fig. 7. Dose-dependent effect of NAO treatment on the expression of a number of key members of the cell cycle machinery in PC3 cells. PC3 cells at 1×10^6 cells/dish were starved for 36 h, and then treated with NAO (0.8, 1.6, and 3.2 mg/ml) in complete media for 18 h. Total cell lysates were prepared, and 40 μg protein was subjected to SDS–PAGE, followed by immunoblot analysis. The data shown are representative of three independent experiments with similar findings.

PCA cells. The signaling pathway includes elevation in the hypophosphorylated form of pRb at the expense of the phosphorylated form of Rb, reduction at the protein level of CDKs, and cyclin typical to G1 phase of the cell cycle together with enhanced expression of the CDK inhibitor $p21^{cip1}$ [55–57].

EGCG, a polyphenol derived from green tea, was recently reported as affecting the expression level of different E2F isoforms leading to G1 arrest and apoptosis. These activities resemble our results, but differ in the final outcome, where, unlike EGCG, the NAO-derived polyphenols attenuate rather than arrest growth and do not culminate in apoptosis. A possible explanation for the difference between the NAO-derived polyphenols and EGCG with regard to the induction of apoptosis may lie in the dissimilar effects that these two substances have on E2F-1 expression. While EGCG had only a minor effect on the protein level of E2F-1 [4], NAO-derived polyphenols caused a steep reduction in the E2F-1 protein level. E2F-1, besides being involved in G1-to-S transition, plays a role in apoptosis by enhancing, at transcription level, the expression of caspases; so that E2F-1 directly couples the cell cycle with the cell death machinery [58]. Therefore, inhibition of E2F-1 expression by NAO-derived polyphenols might partially explain the absence of apoptosis in NAO-derived polyphenol-treated cells. The absence of the apoptotic response in cells treated with NAO was also validated by testing NAO-treated cells for apoptotic response using the Annexin V Fitc-based apoptosis detection kit (data not shown).

Prolongation of the cell cycle by the natural polyphenols or NAO might provide several benefits in terms of tumor eradication or prevention. First, it reduces the amount of cell doubling and, through this, might decrease the number of genetic changes acquired by tumor cells with time. Second, a lowered rate of proliferation minimizes tumor size, helping the immune system in its effort to eradicate the tumor. Many tumors are lethal due to the fact that they develop in an aggressive manner, killing their host in a short time period. Attenuation of growth might convert those tumors from acute to chronic and prolong the life expectancy of the patients. Third, the fact that the polyphenols or NAO did not induce apoptosis suggests that it will not harm healthy, normal cells. Taken together, the above-mentioned properties qualify NAO as a potential chemopreventive agent, which might also be beneficial in treating existing tumors alone or in combination with other chemotherapeutic agents. Systemic research on NAO effects on a battery of different human tumors is now being performed.

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