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RESEARCH ARTICLES

Cyanidin attenuates PGE_2 production and cyclooxygenase-2 expression in LNCaP human prostate cancer cells

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Abstract

In the United States, the primary cancer in elderly men is prostate cancer (33% of newly diagnosed malignancies), but the prevalence is 75% lower in some Mediterranean countries. A possible explanation for the large difference in prostate cancer cases is that in Mediterranean countries the diet includes fish, olive oil and high amounts of nuts, fruits, vegetables, along with a regular intake of wine with meals several times per week. The LNCaP prostate cancer cells represent the nonaggressive androgen-dependent cell model that expresses moderate levels of cyclooxygenase-2 (COX-2). Epidemiological evidence indicates that polyphenolic compounds in diets are protective against cancer, and cyanidin and kaempferol are abundant in wine and plants. Therefore, the objective of the investigation was to determine the effects of cyanidin and kaempferol on prostaglandin E_2 (PGE_2) and COX-2 protein levels, and if peroxisome proliferator-activated receptor γ (PPARγ) and nuclear factor κB (NFκB) are involved in the expression of COX-2 in prostate cancer cells. Cyanidin and kaempferol at 1 μM reduced the level of PGE_2 in LNCaP cell cultures and also attenuated the effect of arachidonic acid on increasing the amount of PGE_2. Cyanidin reduced the levels of COX-2 protein in a dose- and time-dependent fashion. PPARγ mRNA levels were lower in cells treated after 24 h with kaempferol (0.1 and 1 μM) and cyanidin (1 μM). The reduction of COX-2 mRNA by kaempferol and cyanidin may be mediated through the actions of NFκB and PPARγ as nuclear factors that bind to the COX-2 gene promoter.

Keywords: LNCaP prostate cancer cells; Cyanidin; Kaempferol; COX-2; PUFA; PGE_2

1. Introduction

Prostate cancer in elderly males accounts for 33% of all newly diagnosed malignancies among men in the United States [1,2]. Only 10–15% of prostate cancers are classified as familial [3], indicating that environmental factors, such as diet, play an important role in the development of this disease. Some Mediterranean countries, such as Greece, Italy and Spain, with a different dietary lifestyle than the United States, have up to 75% less incidence of prostate cancer compared to that in America [4]. The Mediterranean diet consists of weekly consumption of oily fish, use of olive oil, high intakes of nuts, fruits and vegetables, and red wine with meals [5,6].

Prostate cancer is mainly associated with the outer (peripheral) tissues of the prostate [7–9], where it may begin as focal atrophies associated with chronic inflammation [8,10,11]. All grades of prostate cancer express cyclooxygenase-2 (COX-2); and there is a positive correlation between the amount of COX-2 expressed and the tumor grade and aggressiveness of cancer [12]. In cell culture studies, LNCaP cells represent the nonaggressive androgen-dependent model of prostate cancer, expressing moderate levels of COX-2. In addition, LNCaP cells transfected with a COX-2 gene proliferate faster in vitro and promote tumor growth in vivo [13].

Epidemiological evidence suggests that dietary components such as fatty acids and flavonoids (polyphenolic, nonnutrient components of fruits and vegetables) play an important role in the prevention of chronic diseases such as cancer [14–16]. Anthocyanins and flavonols are two of the

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; EPA, eicosapentaenoic acid; NFκB, nuclear factor kappaB; PGE_2, prostaglandin E_2; PPAR, peroxisome proliferator-activated receptors; PPRE, peroxisome proliferator receptor element; PUFA, polyunsaturated fatty acids; Q-PCR, quantitative polymerase chain reaction.

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six main groups of flavonoids [17]. Cyanidin 3-glucoside is the most common anthocyanin, which is present in 90% of fruits and is responsible for the bluish color in grapes and berries [18]. Kaempferol is a flavonol present in onions and tea, and although abundant, its presence is not as high as that of quercetin [19]. The n-3 polyunsaturated fatty acids (PUFA) have been implicated in a protective role against cancer, especially that of the breast [20,21], by decreasing chronic inflammation and reducing proinflammatory prostanoids [22] such as prostaglandin E_2 (PGE_2) produced from arachidonic acid (AA) by COX-2. High levels of PGE_2 have been found in several cancer sites, including that of prostate cancer [23–25].

Other factors involved in cancer and inflammation are the peroxisome proliferator-activated receptors (PPARs), which are ligand-activated transcription factors that have an activation site on the promoters of multiple genes after dimerization with retinoid X receptor. There are three types of PPARs, α, β or δ, and γ, each with a unique tissue distribution [26]. In cell culture studies, the activation of PPARγ by endogenous ligands (the prostanoid 15d-PGJ_2 and dietary factors such as PUFA) or by synthetic ligands (troglitazone or ciglitazone) [27] reduced cell growth in some types of cancer, such as that of colon and lung [28,29]. PPARγ has been found in prostate cancer biopsies and in human prostate cancer cell lines such as LNCaP, PC-3 and DU-145 [30,31]. Patients with advanced prostate cancer on a phase II clinical trial displayed prolonged stabilization of prostate-specific antigen (PSA) when treated with troglitazone [30].

The activation of PPARs by 15d-PGJ_2 or synthetic ligands may decrease the amount of COX-2 and PGE_2 via nuclear factor κB (NFκB) [32]. Evidence indicates that the PPARγ agonist 15d-PGJ_2 can inhibit NFκB-dependent transcription by two mechanisms, both of them reduce the amount of NFκB in the nucleus of cells [33,34]. A decrease in the nuclear level of NFκB may reduce transcription of the COX-2 promoter, which has two NFκB binding sites [35].

Although few studies describe the effects of flavonoids on PPARγ, the isoflavone genistein above 1 μM promoted adipogenesis by acting as a PPARγ ligand in mouse bone marrow cells [36]. Another experiment showed that apigenin, chrysin and kaempferol acted like PPAR ligands for murine macrophage-like cells (RAW 264.7) and decreased COX-2 transcription [37]. Therefore, we hypothesize that flavonoids and PUFA can alter the expression of the COX-2 gene in LNCaP cancer cells to reduce prostanoid synthesis and cell viability. Hence, the objective of our investigation was to examine the actions of cyanidin and kaempferol on cell viability, and flavonoids combined with selected PUFA on the production of PGE_2 and expression of COX-2 in prostate cancer cells. This study also determined if flavonoids alter PPARγ and NFκB mRNA levels in cell cultures that are associated with the regulation of the COX-2 promoter.

2. Materials and methods

2.1. Materials

Cultures of LNCaP human prostate cancer cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA) containing antibiotic–antimycotic and trypsin/EDTA solutions (Invitrogen). The HEPES, β-actin antibody and peroxidase-conjugated donkey antimouse polyclonal antibody, fatty acid-free bovine serum albumin (BSA), methanol, isooctane and chloroform were obtained from Sigma (St. Louis, MO). Other materials included fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), MTS assay kit (Cell Titer 96 AQ_{ueous}; Promega, Madison, WI), M-PER and NE-PER (Pierce Biotechnology, Rockford, IL), protein assay kit, 10% Tris–HCl SDS-PAGE gels, protein loading buffer Immun-Blot PVDF membrane, and iScript (Bio-Rad, Hercules, CA).

In addition, fatty acids (>98% purity, Nu-Check-Prep, Elysian, MN), peroxidase-conjugated donkey antirabbit polyclonal antibody (Amersham Biosciences, Buckinghamshire, UK), cyanidin and kaempferol (Indofine Chemical Company, Somerville, NJ), troglitazone and ovine COX-2 protein standard (Cayman Chemical, Ann Arbor, MI), β-actin, COX-2, NFκB and PPARγ gene primers (Sigma-Genosys, The Woodlands, TX), antihuman COX-2 polyclonal rabbit antibody-PG27 (Oxford Biomedical Research, Oxford, MI), antihuman PPARγ polyclonal mouse antibody E-2 (sc-7273, Santa Cruz Biotechnology, Santa Cruz, CA), RNAqueous-4PCR kit (Ambion, Austin, TX), ECL Western Blotting detection reagent (Amersham Biosciences), PGE_2 kit (Assay Designs, Ann Arbor, MI), quantitative polymerase chain reaction (Q-PCR) plates and caps, and SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) were all obtained from the sources indicated.

2.2. Cell treatments

LNCaP cells were cultured in RPMI 1640 supplemented with 1% antibiotic–antimycotic, 10 mM HEPES, and 10% FBS at 37°C and 5% CO_2/95% air. Cells were subcultured at 3-day intervals (80% confluent) using a trypsin/EDTA solution in PBS buffer. Cells for the treatments were from passages 5–40.

Cyanidin, kaempferol and troglitazone were dissolved in DMSO, aliquoted, flushed with nitrogen gas and stored at 4°C until needed. Flavonoid concentrations used for the cell number assay ranged from 0.1 to 100 μM. For all other treatments, flavonoids were used at 0.1, 0.5 or 1 μM. Troglitazone was used at 1 and 10 μM.

Fatty acids were dissolved in 100% ethanol to a final concentration of 100 mg/ml and stored in single-use glass vials at −20°C. Fatty acid-supplemented medium was prepared by dissolving BSA in serum-free media at a concentration of 0.33 g/ml or 500 μM (2:1 ratio of PUFA to BSA). An aliquot of the free fatty acid stock was added to the BSA-serum-free media for a final concentration of 1 mM, flushed with nitrogen gas and incubated in a water

bath at 37°C for 1 h in the dark. Fatty acid concentrations used for the cell number assay ranged from 25 to 200 μM. Western blots and Q-PCR experiments were performed with 25 μM of fatty acids.

2.3. Cell number assay

Cells were in 96-well plates and left undisturbed until they reached 90–95% confluency. For treatment addition, the medium was removed and the cells were rinsed with PBS once and cyanidin and kaempferol added (concentrations ranged from 0.1 to 100 μM). After 24 h, cells were washed with serum-free, phenol red-free media. Phenol red-free (100 μl) media was added to the cells followed by 20 μl of Cell Titer (Aqueous One Solution, Promega). Cells were incubated for 30 min at 37°C with 5% CO_2 to allow color development, and the absorbance at 490 nm was read using a spectrophotometer (Spectra MAX 190, Molecular Devices, Sunnyvale, CA). Phenol red-free media alone was used as a blank. Proliferation was assessed by comparing the absorbance measurement of control cells to treated cells. The viable cells reduce MTS tetrazolium compound into a colored formazan determined at an absorbance of 490 nm.

2.4. Prostaglandin E_2

PGE_2 was measured using a kit from Assay Designs (catalog no. 900-001) following the manufacturer's instructions. One characteristic of this kit is that the cross-reactivity between PGE_2 and PGE_3 is low, only 16.3%, when compared to other kits or antibodies where the cross-reactivity is up to 43%.

2.5. Western blot

Cells treated with cyanidin, kaempferol or a combination of fatty acids and flavonoids for 4 or 24 h were collected and protein extracted. Total cell protein was isolated using M-PER cell lysis reagent. Nuclear protein was isolated using NE-PER nuclear and cytoplasmic extraction reagents following the manufacturer's recommendations. Collected protein concentration for both extraction methods was determined by the Bradford method using a spectrophotometer (Spectra MAX 190, Molecular Devices) at 595 nm.

Proteins were combined with an equal volume of the protein loading buffer. The protein extracts were separated by SDS-PAGE (10% Tris–HCl gels) using equivalent amounts of protein in each lane at 100 V constant voltage. Proteins on gels were transferred overnight to an Immun-Blot PVDF membrane under refrigerated conditions (4°C).

For Western blot, membranes with the PPARγ section were blocked with 10% nonfat dry milk (NFDM) in TBS-T, and those with COX-2 section with 10% NFDM in TBS-T for 1 h. Primary COX-2 antibody was added at a dilution of 1:4000, PPARγ at a dilution of 1:2500 and β-actin at a 1:20,000 dilution. Secondary antibody for COX-2 was antirabbit at a 1:5000 dilution, for PPARγ and β-actin, antimouse at 1:1000 and 1:30,000 dilutions, respectively. Membranes were developed using ECL Western Blotting detection reagent. Net intensity of proteins was analyzed using a Kodak 1D program (Eastman Kodak, Rochester, NY).

2.6. Quantitative polymerase chain reaction

After the treatment with either PUFA or flavonoids for the desired times, cells were washed with PBS. The mRNA was collected using the RNAqueous-4PCR kit following the manufacturer's instructions. The concentrations of mRNA were measured in a UV plate by reading the concentration at 260 nm, and the purity of the sample was determined at 260/280 nm.

The mRNA was converted to cDNA using the iScript kit and automated iCycler (Bio-Rad). The conditions for the reverse transcriptase reaction were as follows: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. Primers used for the Q-PCR reaction were the following: β-actin-fwd: CCTGGCACCCAGCACAAT, β-actin-rvs: GCCGATCCACACGGAGTACT; COX-2-fwd: GAATCATTCACCAGGCAAATTG, COX-2-rvs: TCTGTACTGCGGGTGGAACA; NFκB-fwd: GGCTACACCGAAGCAATTGAA, NFκB-rvs: CAGCGAGTGGGCCTGAGA; and PPARγ-fwd: GGCTTCATGAC AAGGGAGTTTC, PPARγ-rvs: AAACTCAAACTTGGGCTCCATAAA. The cDNA was mixed with the primers and the SYBR Green PCR master mix. Q-PCR was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Amplification of the cDNAs was analyzed, and data were presented as comparative Ct values [38,39].

2.7. Statistical analyses

The data collected were analyzed using one-way analysis of variance with the use of SAS programs (SAS Institute, Cary, NC). Student–Newman–Keuls (SNK) multiple-comparison test was performed, and differences between treatments were found significant at $P<.05$. Results are presented as means of measurements and variation expressed with their corresponding SDs.

3. Results

The addition of flavonoids (0.1–100 μM) to the media for 24 h reduced the number of LNCaP viable cells in a dose-dependent fashion (Fig. 1). Cyanidin and kaempferol at 10 μM and above significantly reduced the number of LNCaP cells when compared to the vehicle control (Fig. 1, panel A). The number of viable prostate cancer cells declined with cyanidin at 0.5 μM when compared to higher concentrations (Fig. 1, panels A and B). Treatment with kaempferol did not show this dramatic effect at 0.5 μM.

Treatment of LNCaP cells with AA (25 μM) elevated the concentrations of PGE_2 produced in culture compared to the vehicle control (Fig. 2). Kaempferol (1 μM), and not cyanidin, reduced PGE_2 levels in cell cultures compared to the vehicle control; however, either cyanidin or kaempferol when combined with AA reduced the amount of PGE_2

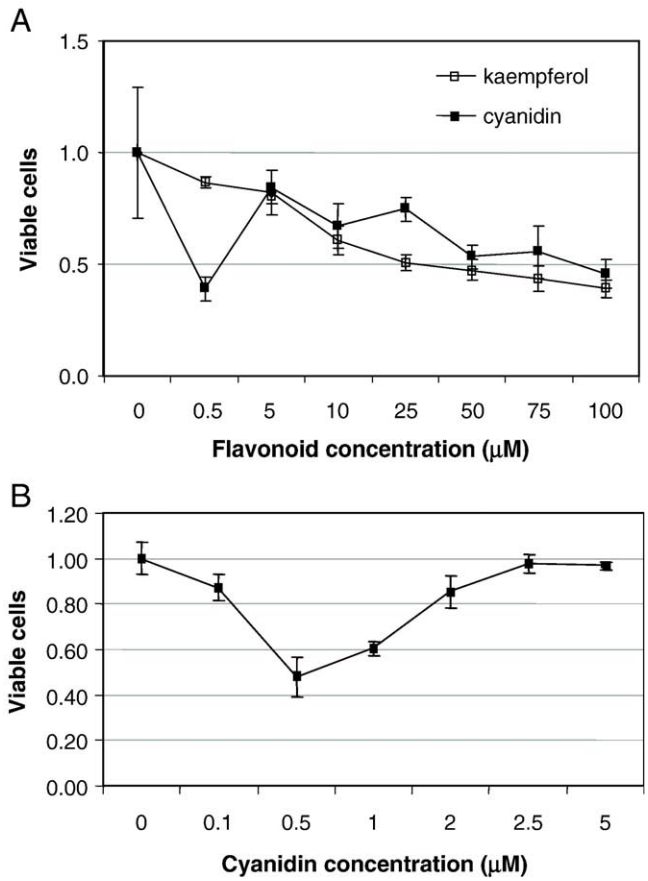

Fig. 1. LNCaP mean±SD viable cell number (standardized to the control vehicle) after treatment with different concentrations of cyanidin and kaempferol for 24 h (panel A). Mean±SD viable cell number after cyanidin treatment for 24 h (panel B). Each experiment was conducted with $n=3$ cultures.

compared to the AA treatment alone. The data indicate that these flavonoids interfere with the COX enzyme or substrate availability for PGE_2 synthesis.

The effects of both flavonoids (0.1 and 1 μM) and PUFA were examined on COX-2 protein and mRNA levels in LNCaP cells (Fig. 3). In a dose-dependent experiment, the effects of AA on COX-2 protein levels showed that at 100 μM and after 24 h, AA reduced the amount of COX-2 protein in LNCaP cells compared to the vehicle control (Fig. 3, panel A). A significant decrease in COX-2 mRNA ($P<.05$) occurred in cells treated with 25 μM of AA after 24 h (Fig. 3, panel B). Cyanidin (0.1 and 1 μM) alone and in combination with AA (25 μM) did not reduce the amount of COX-2 protein in LNCaP cells in culture after 4 h of exposure compared to the vehicle control (Fig. 3, panel C). Interestingly, the combination treatment of cyanidin with eicosapentaenoic acid (EPA) in LNCaP cells elevated the amount of COX-2 protein (Fig. 3, panel C). The addition of kaempferol at 0.1 μM with and without AA supplementation for 4 h was not effective in reducing COX-2 protein level compared to the vehicle control (Fig. 3, panel D).

Protein levels of PPARγ were determined in LNCaP cells upon treatment with cyanidin (0.1 and 1 μM) for 4 and 24 h. In these experiments, the protein levels in the treated cell cultures were not different from the vehicle controls (data not shown).

Based on the aforementioned results, further experiments were performed to determine the effects of cyanidin (0.5 and 1 μM) on the mRNA levels for COX-2, NFκB and PPARγ in LNCaP cells after 2 and 24 h of treatment (Fig. 4). Exposure of cells to 1.0 μM of cyanidin significantly reduced the mRNA levels for COX-2 after 24 h, but not 2 h, compared with the vehicle control (Fig. 4, panel A). The cyanidin treatment had no effect on the mRNA levels for NFκB (Fig. 4, panel B); however, after 24 h, cyanidin reduced PPARγ mRNA in cell cultures compared to the vehicle control (panel C).

Experiments with flavonoids on the gene targets in LNCaP cells were repeated using cyanidin and kaempferol at 0.1 and 1.0 μM and troglitazone as a ligand for PPARγ following 2 and 24 h of exposure to the treatments (Fig. 5). After 24 h of treatment with cyanidin and kaempferol (both at 0.1 μM), the level of COX-2 mRNA was lower compared to the value for the vehicle control cell cultures (Fig. 5, panel A). None of the treatments reduced the amount of mRNA for NFκB and PPARγ in LNCaP cells (panels B and C) compared to the vehicle control. Both cyanidin and

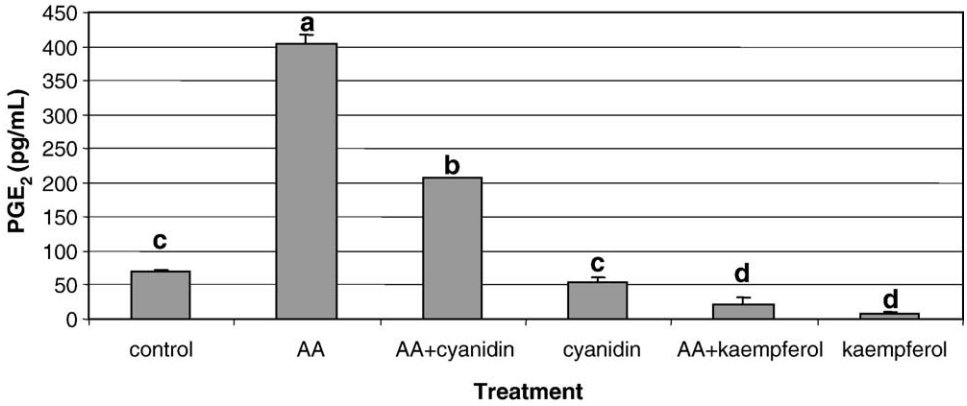

Fig. 2. PGE_2 levels (mean±SD) in LNCaP cell cultures after treatment for 24 h with PUFA (AA at 25 μM) and flavonoids (1 μM) or the combination of AA and flavonoid treatments ($n=2$). Bars with different letters (a–d) are significantly different ($P<.05$, SNK).

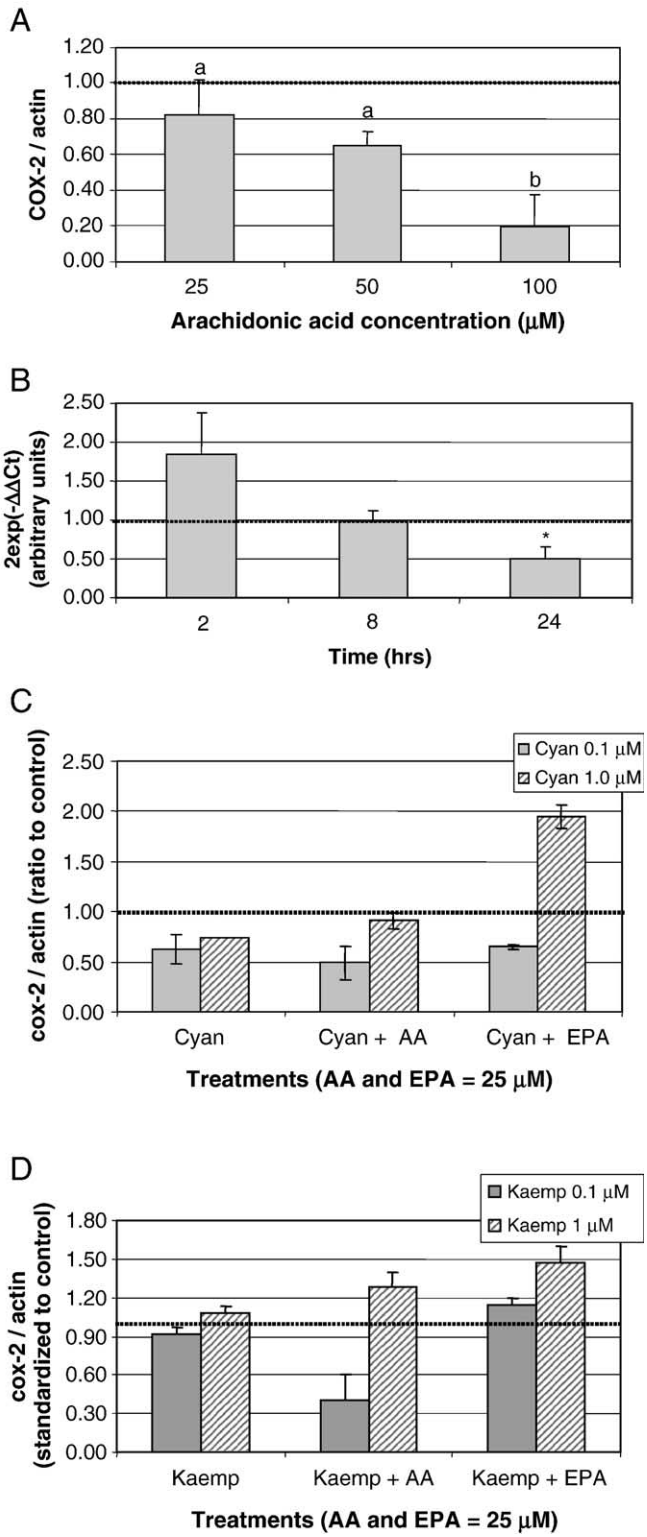

Fig. 3. Values (mean±SD, $n=3$) for COX-2 protein or mRNA levels in LNCaP cell cultures treated with AA or flavonoids compared to the vehicle control (represented by the dotted line, set at 1.0). Bars with different letters (a, b) or an "*" indicate significant difference ($P<.05$, SNK). Values for COX-2 represent treatment with AA for 24 h (panel A). Values for COX-2 mRNA levels at 2, 8 and 24 h after treatment with 25 μM of AA (panel B). Values for COX-2 protein levels after 4-h treatment with cyanidin (Cyan) and cyanidin plus fatty acids (AA and EPA at 25 μM) (panel C). Values for COX-2 protein levels after 4-h treatment with kaempferol (Kaemp) or kaempferol plus fatty acids (panel D).

kaempferol at 0.1 μM showed a lower level of PPARγ mRNA after 24 h when compared to these treatments after 2 h of exposure to the flavonoids.

Three consistent responses were observed in cultures of LNCaP cells treated with flavonoids. First, the number of viable cells was reduced when cultures were exposed to 0.5 μM of either cyanidin or kaempferol (greater than 0.5 μM). Second, a decrease in PGE_2 production in cell cultures occurred when exposed to both flavonoids (24 h) and in combination with AA compared to AA alone. Third, COX-2 mRNA levels were lowered in cells cultured with cyanidin at 0.1 and 0.5 μM after 24 h of exposure, and with kaempferol at 0.1 μM for 24 h. Other significant observations from these experiments are that the dose of flavonoid and duration of exposure were critical for the desired action in cultures of LNCaP cells. For example, cyanidin at 0.1 and 0.5 μM was effective in reducing PPARγ mRNA, but the

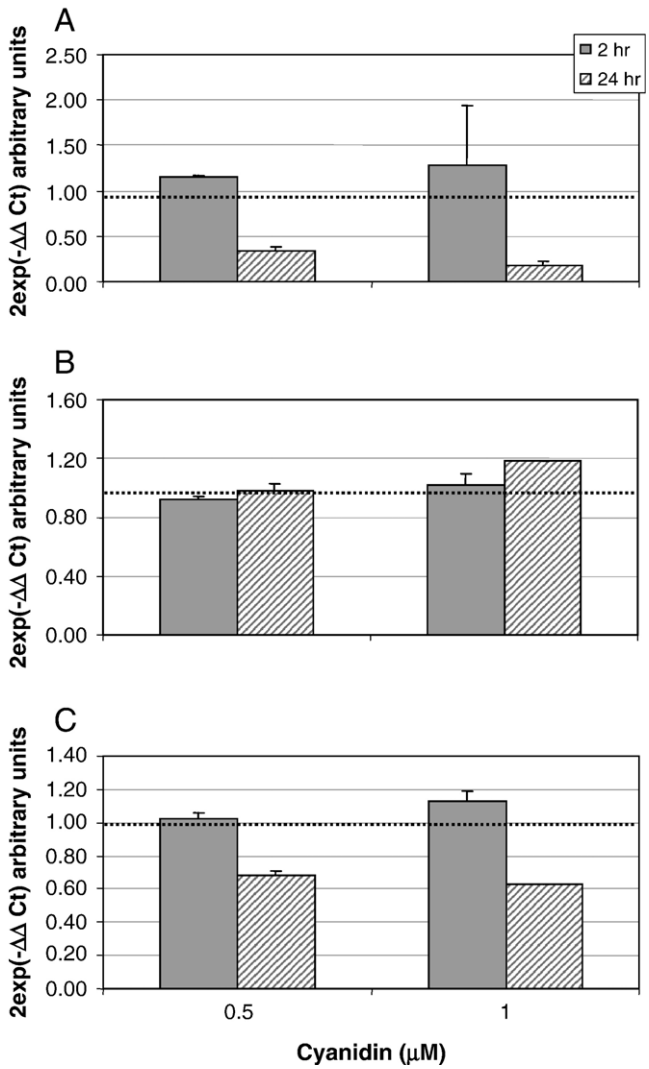

Fig. 4. Values (mean±SD, $n=3$) for COX-2 mRNA (panel A), NFκB mRNA (panel B) and PPARγ mRNA (panel C) in LNCaP cell cultures treated with cyanidin after 2 and 24 h. The vehicle control values are represented by the dotted line (unit 1).

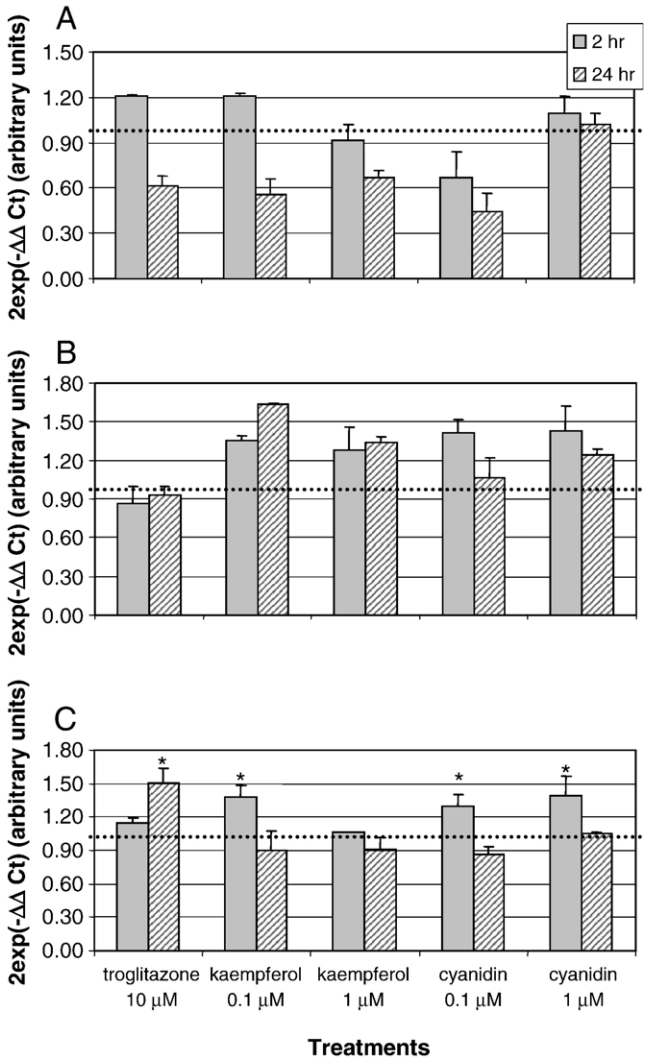

Fig. 5. Values (mean±SD, $n=3$) for COX-2 mRNA (panel A), NFκB mRNA (panel B) and PPARγ mRNA (panel C) in LNCaP cell cultures treated with troglitazone, kaempferol and cyanidin after 2 and 24 h. The vehicle control values are represented by the dotted line (unit 1). In panel C, the "*" represents a significant difference between the two time points for the PPARγ mRNA values ($P<.05$, SNK).

response was variable at 1 μM. This may be explained to some extent by the observation that exposure of cells to 0.5 μM of cyanidin lowered the number of viable cells compared to a higher concentration of this compound. From these data and those on mRNA levels, the action of cyanidin on LNCaP cells appears to be dose dependent.

4. Discussion

The evidence is mounting that a regular intake of foods containing flavonoids decrease chronic disease risk [40]. Our study showed that cyanidin and kaempferol reduced the number of viable prostate cancer cells after 24 h of exposure when compared to the vehicle control cells. Other investigators reported a decrease in viable PC-3 and LNCaP cells when treated with <10 μM of genistein, daidzein, coumestrol and equol for 6 days [41] or with increasing concentrations (10–100 μM) of epigallocatechin gallate [42]. Cyanidin also reduced the number of human colon HT-29 and HCT-116 cells, demonstrating an IC_{50} of 63 and 85 μM, respectively [43]. In mice consuming either a diet containing tart cherries, anthocyanins or cyanidin, researchers observed significantly fewer and smaller cecal adenomas when compared to the number in mice given the control diet [43]. Our results showed that cyanidin at 0.5 μM significantly reduced the number of LNCaP cells when compared to the control, and others found that quercetin, catechin and epicatechin, extracted from red wine and added to PC-3 and LNCaP cultures at ≤1 μM for 5 days, reduced cell numbers [44].

Studies on prostate cancer have examined the actions of flavonoids on the androgen receptor and PSA production [45–47]. Based on the literature, no study in prostate cancer cells investigated the effects of flavonoids on COX-2 protein and mRNA levels, or the related transcription factors. Most experiments that have evaluated flavonoid actions on COX-2 were conducted in macrophages. For example, rat macrophages treated with apigenin, genistein or kaempferol (12.5 μM) and lipopolysaccharide (LPS) for 24 h resulted in lower COX-2 protein levels when compared to cells treated only with LPS. In contrast, ECGG, myricetin and quercetin did not have the same effect on COX-2 in macrophages [48]. These results are, in part, comparable to those observed in our study where COX-2 protein levels were decreased after 24 h of cyanidin exposure compared to 2 h.

In our experiments, the addition of kaempferol, kaempferol and AA, and cyanidin and AA to cultures of LNCaP cells reduced PGE_2 production. The effect of flavonoids on reducing prostanoid synthesis occurred at a much lower concentration of cyanidin (1 μM) than previously reported (40 μM) in analytical studies [49,50]. The difference in concentrations might be related to lowered flux for prostanoid synthesis in cell cultures compared with inhibition of COX-2 enzyme activity.

Multiple factors regulate the promoter of the COX-2 gene. Our hypothesis that flavonoids reduce prostanoid synthesis may be mediated, in part, by changes in two nuclear factors (PPARγ and NFκB) associated with control of the COX-2 promoter and subsequent elaboration of mRNA and protein levels of COX-2. Recent studies suggest that an interaction between PPARγ and COX-2 is involved in the expression of cancer-related genes and the transcription factors of carcinogenesis [51]. In support of this relationship, adipocytes treated with the potent synthetic PPARγ ligand, rosiglitazone, increased PPARγ mRNA levels in a time- and dose-dependent manner [52,53]. We observed an increase in PPARγ mRNA level in LNCaP cells after 24-h exposure to troglitazone. One study reported the effects of troglitazone addition to LNCaP cells, which resulted in decreased cell growth, but the authors did not evaluate any actions on PPARγ mRNA [30]. In our experiments, the addition of 10 μM of troglitazone for 24 h prevented cell growth; however, at a lower concentration

(1 μM), cells continued to grow normally (data not shown), suggesting an important dose response.

Synthetic PPARγ ligands have been shown to down-regulate the expression of COX-2 in HT-29 human colon cancer cells [51]. Liang et al. [37] suggested that kaempferol, apigenin and chrysin acted as dietary PPARγ ligands since these flavonoids activated PPARγ binding to the peroxisome proliferator receptor element (PPRE) in a luciferase assay and decreased relative COX-2 promoter activity of macrophages. Their studies showed that flavonoids might not directly bind to the PPARγ binding site and instead act as an allosteric effector. Our data indicate that the reduction in COX-2 mRNA in LNCaP cell cultures after 24 h by cyanidin (0.1 and 0.5 μM) may be, in part, associated with the decrease in PPARγ (Fig. 4). More specific gene studies are needed to demonstrate the relationship.

Huang et al. [54] reported that some berry extracts could reduce the activity of NFκB, possibly mediated via the inhibition of IκBα phosphorylation. In addition, the flavonoid apigenin (1–20 μM) showed a reduction in the luciferase activity of the COX-2 and NFκB promoter of transfected macrophages [48]. Thus, it is suggested that the modulation of the COX-2 gene may occur through nuclear factors, such as NFκB, that binds to the COX-2 promoter and not due to a direct effect of the flavonoid on the COX-2 promoter. In our study, cyanidin and kaempferol did not affect the levels of NFκB mRNA in LNCaP cell cultures.

In rat adipocytes there was no increase in the luciferase activity in transfected cells when treated with cyanidin at concentrations from 0.1 to 100 μM, meaning that there is no enhanced binding of PPARγ to the luciferase PPRE [55]. In the same study, there was an increase in PPARγ mRNA in cells after treatment with 100 μM of cyanidin for 24 h when compared to the control cells. Assuming that this observation is possible in other cell types, it is likely that flavonoids may inhibit the activation of the PPRE site on the COX-2 gene, thus preventing the transcription of the gene, and by consequence, lower COX-2 protein. We did observe an increase in PPARγ mRNA with cyanidin treatment in LNCaP cells at 2 h compared to 24 h, and when compared to those used in the study by Tsuda et al. [55], our levels were more likely physiological.

In summary, our research showed that cyanidin and kaempferol reduced LNCaP cell viability and PGE_2 production. When cultures of prostate cancer cells were exposed to cyanidin at 0.1 μM, COX-2 mRNA was reduced after 2 and 24 h, and COX-2 protein at 2, 8 and 24 h. The response of cells to cyanidin was specific at 0.1 μM and was not observed with 1.0 μM. Cyanidin consistently caused a decrease in PPARγ mRNA in LNCaP cultures without a change in NFκB mRNA. In contrast, kaempferol did not affect the mRNA of these two factors. Although this research did not address the issue of whether cyanidin and kaempferol bind to PPARγ, additional studies using transfected cells would be beneficial in order to understand the actions of these compounds on prostate cancer cells. A possible action for cyanidin in controlling prostate cancer is to interfere with COX-2 enzyme activity and its expression. The potent effect of cyanidin on AA responses in LNCaP cells is of considerable interest since n-6 PUFA are the major source of essential fatty acids in the diet. It is clear that cyanidin and keampferol as a class of dietary factors in fruits and vegetables offer a potential benefit in minimizing COX-2 gene expression and subsequent gene products associated with cancer and inflammation, although the precise action requires more research.

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References

[1] US Cancer Statistics Working Group. United States Cancer Statistics: 1999 incidence. Atlanta, GA: Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute; 2002.
[2] Crawford ED. Epidemiology of prostate cancer. Urology 2003; 62:3–12.
[3] Narod S. Genetic epidemiology of prostate cancer. Biochim Biophys Acta 1999;1423:1–13.
[4] Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2000: Cancer incidence, mortality and prevalence worldwide. IARC Cancer Base No. 5. Lyon, France: IARC Press; 2001.
[5] Trichopoulou A, Lagiou P, Kuper H, Trichopoulos D. Cancer and Mediterranean dietary traditions. Cancer Epidemiol Biomarkers Prev 2000;9:869–73.
[6] Simopoulos AP. The Mediterranean diets: what is so special about the diet of Greece? The scientific evidence. J Nutr 2001;131:3065–73.
[7] De Marzo AM, Marchi VL, Epstein JI, Nelson WG. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. Am J Pathol 1999;155:1985–92.
[8] Franks LM. Atrophy and hyperplasia in the prostate proper. J Pathol Bacteriol 1954;68:617–21.
[9] Shah R, Mucci NR, Amin A, Macoska JA, Rubin MA. Postatrophic hyperplasia of the prostate gland: neoplastic precursor or innocent bystander? Am J Pathol 2001;158:1767–73.
[10] Liavag I. Atrophy and regeneration in the pathogenesis of prostatic carcinoma. Acta Pathol Microbiol Scand 1968;73:338–50.
[11] Yoshimura R, Sano H, Masuda C, Kawamura M, Tsubouchi Y, Chargui J, et al. Expression of cyclooxygenase-2 in prostate carcinoma. Cancer 2000;89:589–96.
[12] Lee L-M, Pan C-C, Cheng C-J, Chi C-W, Liu T-Y. Expression of cyclooxygenase-2 in prostate adenocarcinoma and benign prostatic hyperplasia. Anticancer Res 2001;21:1291–4.
[13] Fujita H, Koshida K, Keller ET, Takahashi Y, Yoshimito T, Namiki M, et al. Cyclooxygenase-2 promotes prostate cancer progression. Prostate 2002;53:232–40.
[14] WHO/FAO Expert Consultation. Diet, nutrition and the prevention of chronic diseases. WHO Technical Report Series 916. Geneva (Switzerland): World Health Organization; 2003.
[15] de Lorgeril M, Salen P. Modified Cretan Mediterranean diet in the prevention of coronary heart disease and cancer. World Rev Nutr Diet 2000;87:1–23.

[16] Middleton Jr E, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000;52:673–751.
[17] Bravo L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 1998;56:317–33.
[18] Macheix JJ, Fleuriet A, Billot J. Fruit phenolics. Boca Raton (Fla): CRC Press; 1990.
[19] Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000;63:1035–42.
[20] Terry PD, Rohan TE, Wolk A. Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. Am J Clin Nutr 2003;77:532–43.
[21] Rose DP, Connolly JM. Omega-3 fatty acids as cancer chemopreventive agents. Pharmacol Ther 1999;83:217–44.
[22] Larsson SC, Kumlin M, Ingelman-Sundberg M, Wolk A. Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. Am J Clin Nutr 2004;79:935–45.
[23] Gupta S, Srivastava M, Ahmad N, Bostwick DG, Mukhtar H. Overexpression of cyclooxygenase-2 in human prostate adenocarcinoma. Prostate 2000;42:73–8.
[24] Uotila P, Valve E, Marikainen P, Nevalainen M, Nurmi M, Härkönen P. Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. Urol Res 2001;29:25–8.
[25] Yang YJ, Lee SH, Hong SJ, Chung BC. Comparison of fatty acid profiles in the serum of patients with prostate cancer and benign prostatic hyperplasia. Clin Biochem 1999;32:405–9.
[26] Gelman L, Fruchart JC, Auwerx J. An update on the mechanisms of action of the peroxisome proliferator-activated receptors (PPARs) and their roles in inflammation and cancer. Cell Mol Life Sci 1999;55:932–43.
[27] Bocos C, Gottlicher M, Gearing K, Banner C, Enmark E, Teboul M, et al. Fatty acid activation of peroxisome proliferator-activated receptor (PPAR). J Steroid Biochem Mol Biol 1995;53:467–73.
[28] Elstner E, Muller C, Koshizuka K, Williamson EA, Park D, Asou H, et al. Ligands for peroxisome proliferator-activated receptor gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc Natl Acad Sci 1998;95:8806–11.
[29] Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, et al. Differentiation and reversal of malignant changes in colon cancer through PPARgamma. Nat Med 1998;4:1046–52.
[30] Mueller E, Smith M, Sarraf P, Kroll T, Aiyer A, Kaufman DS, et al. Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. Proc Natl Acad Sci 2000;97:10990–5.
[31] Segawa Y, Yoshimura R, Hase T, Nakatani T, Wada S, Kawahito Y, et al. Expression of peroxisome proliferator-activated receptor (PPAR) in human prostate cancer. Prostate 2002;51:108–16.
[32] Inoue H, Tanabe T, Umesono K. Feedback control of cyclooxygenase-2 expression through PPARgamma. J Biol Chem 2000;275:28028–32.
[33] Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, et al. 15-Deoxy-Delta 12,14-prostaglandin J_2 inhibits multiple steps in the NF-kappa B signaling pathway. Proc Natl Acad Sci U S A 2000;97:4844–9.
[34] Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, et al. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. Nature 2000;403:103–8.
[35] Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem 2000;69:145–82.
[36] Dang ZC, Audinot V, Papapoulos SE, Boutin JA, Lowik CWGM. Peroxisome proliferator-activated receptor gamma (PPARgamma) as a molecular target for the soy phytoestrogen genistein. J Biol Chem 2003;278:962–7.
[37] Liang YC, Tsai SH, Tsai DC, Lin-Shiau SY, Lin JK. Suppression of inducible cyclooxygenase and nitric oxide synthase through activation of peroxisome proliferator-activated receptor-gamma by flavonoids in mouse macrophages. FEBS Lett 2001;496:12–8.
[38] Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 2001;25:386–401.
[39] Applied Biosystems. Relative quantitation of gene expression. ABI PRISM 7700 sequence detection system 1997; User bulletin #2:1–36.
[40] Block G. The data support a role for antioxidants in reducing cancer risk. Nutr Rev 1992;50:207–13.
[41] Mitchell JH, Duthie SJ, Collins AR. Effects of phytoestrogens on growth and DNA integrity in human prostate tumor cell lines: PC-3 and LNCaP. Nutr Cancer 2000;38:223–8.
[42] Yu HN, Yin JJ, Shen SR. Growth inhibition of prostate cancer cells by epigallocatechin gallate in the presence of Cu(2+). J Agric Food Chem 2004;52:462–6.
[43] Kang SY, Seeram NP, Nair MG, Bourquin LD. Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. Cancer Lett 2003;194:13–9.
[44] Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, et al. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. Nutr Cancer 2000;37:223–33.
[45] Davis JN, Kucuk OM, Sarkar FH. Expression of prostate-specific antigen is transcriptionally regulated by genistein in prostate cancer cells. Mol Carcinog 2002;34:91–101.
[46] Xing N, Chen Y, Mitchell SH, Young CYF. Quercetin inhibits the expression and function of the androgen receptor in LNCaP prostate cancer cells. Carcinogenesis 2001;22:409–14.
[47] Rosenberg Zand RS, Jenkins DJA, Brown TJ, Diamandis EP. Flavonoids can block PSA production by breast and prostate cancer cell lines. Clin Chim Acta 2002;317:17–26.
[48] Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945–52.
[49] Seeram NP, Zhang Y, Nair MG. Inhibition of proliferation of human cancer cells and cyclooxygenase enzymes by anthocyanidins and catechins. Nutr Cancer 2003;46:101–6.
[50] Seeram NP, Schutzki R, Chandra A, Nair MG. Characterization, quantification, and bioactivities of anthocyanins in *Cornus* species. J Agric Food Chem 2002;50:2519–23.
[51] Yang WL, Frucht H. Activation of the PPAR pathway induces apoptosis and COX-2 inhibition in HT-29 human colon cancer cells. Carcinogenesis 2001;22:1379–83.
[52] Gauthier A, Vassiliou G, Benoist F, McPherson R. Adipocyte low density lipoprotein receptor-related protein gene expression and function is regulated by peroxisome proliferator-activated receptor gamma. J Biol Chem 2003;278:11945–53.
[53] Fontaine C, Dubois G, Duguay Y, Helledie T, Vu-Dac N, Gervois P, et al. The orphan nuclear receptor Rev-Erb{alpha} is a peroxisome proliferator-activated receptor (PPAR) {gamma} target gene and promotes PPAR{gamma}-induced adipocyte differentiation. J Biol Chem 2003;278:37672–80.
[54] Huang C, Huang Y, Li J, Hu W, Aziz R, Tangs M, et al. Inhibition of benzo(a)pyrene diol-epoxide-induced transactivation of activated protein 1 and nuclear factor {kappa}B by black raspberry extracts. Cancer Res 2002;62:6857–63.
[55] Tsuda T, Ueno Y, Aoki H, Koda T, Horio F, Takahashi N, et al. Anthocyanin enhances adipocytokine secretion and adipocyte-specific gene expression in isolated rat adipocytes. Biochem Biophys Res Comm 2004;316:149–57.