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ARTÍCULO ORIGINAL

Implication of NF-κB and p53 in the expression of TRAIL-death receptors and apoptosis by apple procyanidins in human metastatic SW620 cells

María Elena Maldonado[1,2,3,4], Souad Bousserouel[1,2,3], Francine Gossé[1,2,3], Annelise Lobstein[5], Francis Raul[1,2,3]

[1] INSERM U682, Laboratory of Nutritional Cancer Prevention, Strasbourg, France
[2] Faculty of Medicine, University Louis Pasteur, Strasbourg, France
[3] Institut de Recherche contre les Cancers de l'Appareil Digestif (IRCAD), Strasbourg, France
[4] Escuela de Nutrición y Dietética, Universidad de Antioquia, Medellín, Colombia
[5] CNRS UMR7081, Faculty of Pharmacy, University Louis Pasteur, Illkirch, France

Introduction. The nuclear factor-kappaB (NF-κB) has been shown to upregulate pro-apoptotic mediators such as TRAIL-DR4/-DR5 receptors and the p53 transcription factor depending on the type of stimulus and the cell type involved. Previously, apple procyanidins (Pcy) have been shown to upregulate the expression of TRAIL-DR4/-DR5 and thereby overcoming the resistance of human colon cancer-derived metastatic SW620 cells to TRAIL.
Objectives. NF-κB and p53 were investigated for their involvement in the Pcy-triggered apoptosis of human derived-metastatic colon cancer (SW620) cells.
Materials and methods. Cell death, p53, TRAIL-DR4/-DR5 proteins were analyzed by flow cytometry. DR4/DR5 mRNA was analyzed by RT-PCR in real time. Activated p50/p65 and p53 forms were studied by ELISA and immunoblotting.
Results. Pcy-triggered cell death was prevented by specific inhibitors of NF-κB and of p53: amino-4-(4-phenoxy-phenylethylamino) quinazoline (QNZ) and pifithrin α (Pα), respectively. QNZ and Pα inhibited the Pcy-dependent activation of TRAIL-DR4/-DR5 death receptors. However, the upregulation of TRAIL-DR4 by Pcy was significantly decreased only when NF-κB and p53 inhibitors were used in combination; this effect was not observed with a single inhibitor. This effect was not observed for TRAIL-DR5 and suggested that the expression of each TRAIL-death receptor may be regulated differently.
Conclusions. These data suggested that NF-κB and p53 are partially required in Pcy-triggered apoptosis of SW620 cells by up-regulating the expression of TRAIL-DR4/-DR5. In addition, the ratio between TRAIL-DR4/-DR5 may be a determining factor in the activation of TRAIL-death receptor mediated apoptosis.

Key words: Apoptosis, colorectal neoplasms, flavonoids, tumor suppressor protein p53; receptors, TNF-related apoptosis-inducing ligand.

Implicación de NF-κB y p53 en la expresión de receptores de muerte-TRAIL y apoptosis por procianidinas en células metastásicas humanas SW620

Introducción. Se ha demostrado que el factor nuclear-κB y p53 aumentan los mediadores proapoptósicos como los receptores de muerte TRAIL-DR4/-DR5, según el estímulo y el tipo celular. Previamente demostramos que las procianidinas de manzana aumentaban la expresión de TRAIL-DR4/-DR5, superando la resistencia a TRAIL característica en células humanas metastásicas SW620 derivadas del cáncer de colon.
Objetivo. Investigar si NF-κB y p53 están involucrados en la apoptosis inducida por procianidinas en las células SW620.
Materiales y métodos. La muerte celular y las proteínas p53, TRAIL-DR4/-DR5 se analizaron por citometría de flujo. Los ARN mensajeros (ARNm) de DR4/DR5 se analizaron por RT-PCR. Las formas activadas de p50/p65 y p53 se estudiaron por ELISA e inmunodetección.
Resultados. La muerte celular activada por procianidinas fue prevenida por inhibidores específicos de NF-κB y de p53: amino-4-(4-fenoxi-feniletilamino)-quinazolina y pifitrina α, respectivamente. La quinazolina y la pifitrina α inhibieron la activación dependiente de procianidinas de TRAIL-DR4/DR5. Sin embargo, el aumento en la expresión de TRAIL-DR4 disminuyó significativamente sólo cuando la quinazolina y la pifitrina α se usaron simultáneamente; este efecto no se observó con cada uno por separado. No se observaron para TRAIL-DR5 estos efectos, lo cual sugiere que la expresión de cada receptor de muerte TRAIL puede estar regulada en forma diferente.

Conclusiones. Estos datos sugieren que NF-κB y p53 se requieren parcialmente en la apoptosis de células SW620 inducida por procianidinas mediante el aumento en TRAIL-DR4/-DR5. La proporción de DR4/DR5 podría ser un factor determinante en la activación de la apoptosis por vía de TRAIL-DR4/-DR5.

Palabras clave: apoptosis, neoplasias colorrectales, flavonoides, proteína p53 supresora de tumor, receptores del ligando inductor de apoptosis relacionado con el FNT

The nuclear factor kappa beta (NF-κB) is a transcription factor that plays an important role in tumor formation and progression by regulating the transcription of genes that promote cell proliferation, survival, inflammation, invasion (1), as well as apoptosis (2,3). The NF-κB is composed of homo- and heterodimeric complexes of members of the NF-κB family. Five subunits occur in mammals: p50, p65, c-Rel, p52, RelB. These proteins share a 300-amino acid sequence in the N-terminal region known as the Rel homology and mediate DNA-binding, protein dimerization, and nuclear localization. The p50/p65 is the most common heterodimer found in the NF-κB signaling pathway. NF-κB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins. Treatment of cells with various inducers results in the phosphorylation, ubiquitination and subsequent degradation of IκB proteins favouring the formation of NF-κB dimers, which subsequently translocate to the nucleus, where they activate appropriate target genes (4).

In a number of systems, NF-κB has a demonstrable anti-apoptotic function (5). NF-κB inhibited TNF-mediated apoptosis in Jurkat T cells, primary rat and human fibroblasts, and in MCF-7 breast carcinoma cell lines (5-7). NF-κB also protect against chemotherapy-mediated apoptosis in a number of malignant cell lines (8). In contrast, convincing evidence has highlighted a paradoxical pro-apoptotic role for NF-κB (2,3,9-12). These observations raise the possibility that sites in pro- or anti-apoptotic genes may exhibit different preferences for particular subunits of the NF-κB dimer, and that NF-κB may have signal-specific effects on cell survival. NF-κB has been implicated in the up-regulation of pro-apoptotic genes such as Bax (13), Fas, FasL (14), and TRAILDR4/-DR5 (15,16) genes. NF-κB activation may also cooperate with p53 to induce apoptosis (9,17) by the up-regulation of these genes by p53 (18,19). However, the regulation of NF-κB transcriptional activity that leads to the up-regulation of pro-apoptotic genes is unclear.

The p53 protein is a transcription factor that can act as a tumor suppressor and can be induced by DNA damage, whereas in unstressed cells, p53 levels remain low. The p53 protein plays a key role in cell cycle arrest and in the induction of apoptosis (20,21). Tumor suppressor protein p53 also up-regulates the expression of TRAIL-death receptors DR4 and DR5, since p53-binding sites have been found in the genomic locus of DR4 and DR5 (19,21,22). TRAIL-death receptors can induce an apoptotic signal after binding with their specific ligand TRAIL in a wide variety of tumor cell lines, but normal cells are relatively resistant to TRAIL (23).

Apple products have been tested in experimental animal models to treat chemically or genetically-induced tumors of the colon. They have provided a first indication of cancer chemopreventive efficacy *in vivo* which is attributed in great part to flavonoids (24-29). Apples are an important source of flavonoids, one of the main polyphenols present in human diet. Flavanols represent the major subclass of flavonoids containing monomers (epicatechin and catechin) and polymeric forms (procyanidins, Pcy). The Pcy are oligomeric and polymeric polyphenols formed by the association of several monomeric units of flavan-3-ols (catechin and epicatechin) (30,31). The regular consumption of Pcy-containing foods has been associated with a reduced risk of various types of cancers (32). Flavonoids are able to increase the expression of pro-apoptotic mediators and prevent or delay tumor development. In this regard, the induction of apoptosis appears as one of the most important targets in a chemopreventive approach (33).

In previous studies, we reported that apple Pcy triggered apoptosis in human colon carcinoma-derived metastatic TRAIL-resistant SW620 cells through the activation of TRAIL-DR4/DR5 receptors (34,35). Therefore, since NF-κB and p53

Correspondence:
María Elena Maldonado, Laboratorio de Alimentación y Nutrición Humana, Sede de Investigación Universitaria SIU, Universidad de Antioquia, Carrera 53 Nº 61-30, Medellín, Colombia.
Teléfono: (574) 219 6498 y 219 6400, extensión 6669
mariaele@quimbaya.udea.edu.co

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have binding sites in TRAIL-DR4/-DR5 promoter regions, the current investigation was directed to determining whether NF-κB and p53 are involved in Pcy-triggered apoptosis.

Materials and methods

Isolation and characterization of apple procyanidins

Polyphenols were purified from a cider apple (*Malus domestica*, variety Antoinette) as reported by Souquet *et al*. (36). In brief, apples were reduced to a homogeneous powder which was extracted by water:ethanol:acetic acid (975:1000:25). After filtration, evaporation under vacuum and freeze drying, the crude extract was dissolved in 2.5% acetic acid and separated by preparative HPLC (Lichrospher RP 18, 12 µm, Merck, Darmstadt, Germany) to remove sugars and other non-phenolic polar compounds. Polyphenols were eluted with a acetonitrile:water:acetic acid (300:700:25) solution. Fractions containing polyphenols were evaporated and freeze-dried. The polyphenols were fractionated on a Fractogel column (36,37). Pcy were characterized and quantified by reverse-phase HPLC after thioacidolysis. On a weight basis, the Pcy-fraction contained 70% Pcy, consisting of 95% (-)- epicatechin and 4% (+)-catechin. The mean degree of polymerization approached seven. The Pcy fraction was almost totally devoid of monomeric catechins and other phenols (<2%). Pcy was diluted in dimethylsulfoxide (DMSO) and used at 80 µg/ml final concentration.

Cell culture and treatments

SW620 cells were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured in 75 cm^2 Falcon flasks in Dulbecco's modified Eagle's medium containing 25 mM glucose, 2 mM L-glutamine, 10% heat-inactivated (56°C) horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids (Invitrogen Corp., Cergy Pontoise, France). Incubations were carried out at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was replaced every 48 h. For all experiments, horse serum was reduced to 3%, and the medium was supplemented with 5 µg/ml transferrin, 5 ng/ml selenium and 10 µg/ml insulin (ITS-defined medium; Gibco, Invitrogen, Cergy-Pontoise, France). Cells were exposed to the different compounds 24 h after seeding and incubated for 24 or 48 h. The DMSO final concentration in culture medium was 0.1% for control and treated cells. The stock solution of pifithrin α (Pα) (Calbiochem, Nottingham, United Kingdom), an inhibitor of the transcriptional activity of p53, was diluted in DMSO and tested at 30 µM. The 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ) (Calbiochem, Nottingham, United Kingdom), an inhibitor of the transcriptional activation of nuclear factor-kappa B, was diluted in DMSO and tested at 30 nM. Cells were pretreated for 30 min with Pα with and without QNZ before addition of Pcy to culture medium.

Flow cytometric analysis of sub G0/G1 cell population

The amount of dying and dead cells of the cell populations was determined by flow cytometry analysis and corresponded to the percentage of hypodiploid cells present in the sub-G0/G1 region as shown previously (28,32). Cells were seeded in culture dishes ($1x10^6$ cells per 10 cm internal diameter or $2x10^5$ cells per 2.5 cm internal diameter) and treated with DMSO 0.1% (control) or Pcy (80 µg/ml) with or without QNZ (30 nM) and Pα (30 µM) for 24 h or 48 h. Cells were pretreated for 30 min with Pα with or without QNZ before addition of Pcy or DMSO to culture medium. Control and treated cells were harvested by trypsinization, centrifuged and washed twice with ice-cold PBS 0.1 M, pH 7.2 at 1,800 X g for 5 min at 4°C, and fixed in 1 ml methanol:PBS (9:1, v/v) at -20°C for at least 30 min. The fixed cells were washed twice with ice-cold PBS and cell pellets were resuspended in 100 µl PBS containing 0.25 mg/ml RNAse A and 0.1 mg/ml propidium iodide (PI) (Sigma-Aldrich, Munich, Germany), incubated in darkness at 37°C for 30 min. Cell cycle phase distribution was analyzed using a FACScan flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA, USA). Data from 10,000 events per sample were collected and analyzed using the CellQuest software. Apoptosis was quantitatively measured as the percentage of hypodiploid cells in the sub-G0/G1 region using the Cell Fit analysis program (Beckton Dickinson, USA).

Measure of activated p50/p65 subunits of NF-kB

Cells were harvested by scraping, washed twice in PBS and stored at -20°C. NF-κB release activation was quantified with the TransAM NF-κB Family Transcription Factor Assay Kit (Active Motif Europe, Rixensart, Belgium) in a 96-well plate according to the manufacturer's instructions. This system is an ELISA method for detecting active form of NF-κB contained in nuclear extract; NF-κB specifically

binds to an immobilized oligonucleotide in the ELISA plate that contains the NF-κB consensus site (5'-GGGACTTTCC-3'). The Raji nuclear extract was provided as positive control. Nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif Europe, Rixensart, Belgium). The nuclear extracts of SW620 cells were incubated overnight, followed by addition of primary antibodies specifically used to detect an epitope on p50 and p65–epitopes accessible only when NF-κB is activated and bound to its target DNA. Horseradish peroxidase-conjugated Streptavidin was then used for colorimetric detection and assessed by reading absorbance at 450 nm. Values were expressed as optical density (OD).

Flow cytometric analysis of intracellular p53 expression

Cells ($1x10^6$) were seeded and treated as described for the sub-G0/G1 cell population. Cells were harvested by trypsinization, washed twice with ice cold PBS with 2% BSA and fixed with 4% paraformaldehyde for 30 min at 4°C. Cells were permeabilized by twice treating the cell pellet with 200 µl of wash buffer containing 0.5% Tween-20 and 0.2% BSA. After washing, cells were incubated with FITC-conjugated mouse anti-human p53 monoclonal antibody anti-p53 (Ab-1, IgG2a Clone PAb425) (1:100) (Calbiochem, Nottingham, United Kingdom) or FITC-conjugated mouse IgG1 monoclonal isotype control antibody (BD Biosciences, USA) for 30 min at 4°C. After washing the pellet twice in wash buffer, the fluorescence of 10,000 cells were analyzed using a FACScan flow cytometer (excitation 488 nm, emission pass filter FL-1: 530 nm) and CellQuest software (FACScan, BD Biosciences, USA).

Western blot analysis of p53 and p53 at Ser15

Cells were harvested by trypsinization after treatment and lysed for 15 min at 4°C in a lysis buffer (Tris-HCl 50 mM pH 7.5, NaCl 150 mM, EDTA 5 mM, DTT 1 mM, Triton X-100 1%). The lysed cells were centrifuged for 30 min at 16,000 X g at 4°C, and the total protein content was determined by Lowry assay. Electrophoresis of cell lysate proteins (80 µg) was performed in 15% SDS-polyacrylamide gels for 100 min at 80V and proteins were transferred onto nitrocellulose membranes (BioRad Laboratories, Marnes-la-Coquette, France) 80 min at 100V. After addition of a blocking solution (BSA 3%; Tween 20 0.1%, Tris-HCl 10 mM pH 7.5, NaCl 0.1 M) for 1 h at room temperature, the membranes were incubated with rabbit-polyclonal anti-human p53 and anti-human phospho-p53 (Ser15) antibodies (Abcam, Paris, France) at 1:1000 and 1.500 dilutions, respectively, or mouse anti-human beta-actin at 1:2000 (Chemicon Int., Hampshire, U.K.). Subsequently, the membranes were incubated with 0.02 µg/ml HRP-conjugated goat anti-rabbit IgG (Pierce**,** Perbio Science, Brebières, France), and the proteins visualized by Super Signal West Pico Chemiluminescent Substrate System (Pierce, France). Intensity of bands was analyzed using BioRad Quantity One 1-D Analysis Software version 4.2.1 (BioRad Laboratories, France).

Detection of cell surface expression of DR4 andDR5 receptors

Cells ($1x10^6$) were seeded and treated as described for the sub-G0/G1 cell population. Cells were harvested by trypsinization, washed twice with ice cold PBS and incubated for 30 min at 4°C with anti-human-DR4 (mouse IgG1, clone HS101, 1:50) or anti-human-DR5 (mouse IgG1, clone HS201, 1:50) monoclonal antibodies (Alexis Biochemicals Corp., Stockholm, Switzerland). For DR4 and DR5 detection, cells were washed with ice-cold PBS and incubated with a secondary antibody anti-mouse IgG1-FITC (clone STAR81F, 1:50) for 30 min at 4°C in darkness, the excess was removed by two washings. Isotype control mouse IgG1-FITC antibody (BD Pharmingen, San Jose CA, USA) was used as negative control, incubated for 30 min at 4°C in darkness. After washing with PBS, cells were resuspended in PBS, and surface expression of 10,000 events per sample was analyzed with a FACScan flow cytometer and CellQuest software (Beckton Dickinson, USA).

Total RNA extraction and RT–PCR detection of DR4 and DR5 mRNA transcripts

To determine whether cell surface expression of DR4/DR5 at the surface level was associated with levels of DR4/DR5 RNA, the expression of their respective transcripts was quantified by RT-PCR and data were analyzed by a relative quantification method (38). Relative quantitation described the change in expression of the target gene (DR4 or DR5) relative to untreated cells (control group) under the same conditions for detection of cell surface expression of DR4/DR5. Total RNA was extracted using an RNeasy Mini kit (QIAGEN, VWR, Denmark) following manufacturer's instructions. RNA was reversely transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). TaqMan gene expression assays were used to measure transcription levels of the

selected genes (DR5, Hs00366272_m1; DR4, Hs00269492_ml; Applied Biosystems, Foster City, CA). Beta-actin was applied as an endogenous control (catalog no. Hs99999903_m1; Applied Biosystems). Real time quantitative PCR was performed by using TaqMan Universal PCR master mix (Applied Biosystems) and ABI Prism 7500 Sequence Detection System (Applied Biosystems Sequence detector) in triplicate wells. The data were analyzed by a comparative threshold cycle (C_T) method. Values of the Δ cycle threshold (ΔCt) were obtained by normalizing the average Ct value of each treatment compared to its opposite endogenous control (β-actin) and then calculating 2^{-DDCt} for each treatment. The statistical analyses were as previously described (38).

Statistical analysis

The data were presented as mean ± standard error (SE) from three independent experiments. Comparisons between groups were done by one- and two-way ANOVA. Comparison between treated and not treated with Pcy was done by two-tailed paired t-test. Results were considered significant when $p<0.05$. For the quantitative RT-PCR data Tukey's multiple comparisons post-test was used to determine significance at the $p<0.05$ level. These analyses were done with the GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California, USA).

Results

Cell death induction by apple Pcy

Propidium iodide allowed the characterization of cell distribution in each phase of the cell cycle (G0/G1, S or G2/M) by measurement of cellular DNA content. Induction of cell death caused DNA degradation, and therefore dead cells exhibited a DNA content lower than 2n. These cells were detected by flow cytometry in the sub-G0/G1 region as reported previously (34). This approach permitted the determination of the amount of dead or dying cells in a population, but gave no information on the cell death process (39). Figure 1 shows that the hypodiploid population increased significantly ($p<0.05$) from 1% in the untreated control group to 16% after 48 h exposure to apple Pcy (80 µg/ml).

Effect of Pcy on the activation of p50/p65 NF-κB subunits

NF-κB is a factor implicated in apoptotic cell death of several types of cancer cells. The above experiments were designed to determine if Pcy was able to activate the NF-κB. As shown in figure 2A, the optical density (O.D) exhibited by active forms of p50 and p65 proteins in nuclear extracts of SW620 cells was significantly increased after Pcy (80 µg/ml) treatment compared to untreated cells for 24 h and 48 h.

Effects of Pcy on p53 expression and activation

The role of p53 in Pcy-induced apoptosis was determined in SW620 cells, a cell line which expresses a mutant form of p53 (40,41). Total p53 protein levels were measured by flow cytometry using a specific monoclonal antibody (PAb425) that recognized both mutant- and wild-type p53. A significantly increased expression of total p53 protein was observed after the Pcy treatment (figure 2B). The effect of Pcy on the activation of p53 protein was tested by western blot as well. Activation of p53 tumor-suppressor function was critically dependent on the phosphorylation at the Ser[15] residue. As shown in figure 2C, Pcy-

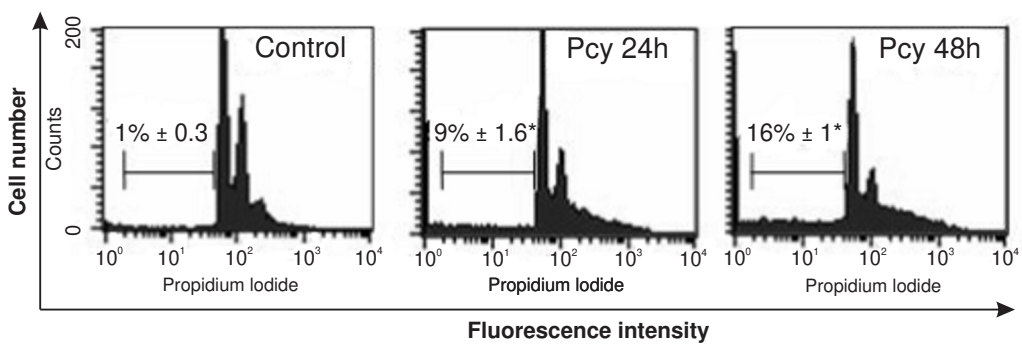

Figure 1. Effect of Pcy on cell death in SW620 cells. Analysis of hypodiploid cells by flow cytometry after Pcy (80 µg/ml) treatment of cells for 24 and 48h. Data are presented as mean percentage ± SE of cells in the sub-G0/G1 region. The flow cytometry histograms are representative of three or more experiments. Treatments were significant at $p<0.05$.

increased levels of total p53 were correlated with a progressive increase of the phosphorylated active form of p53 at Ser[15] by 2-fold over untreated cells.

Effect of quinazoline (QNZ) and pifithrin α (Pα) on Pcy-induced cell death

NF-κB and p53 transcriptional factors were investigated for their involvement in the Pcy-induced cell death of SW620 cells. Flow cytometry was used to visualize the effects of a specific inhibitor of NF-κB activation (6-amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ)) and a specific inhibitor of the transcriptional function of p53 (pifithrin α (Pα)) on the amount of hypodiploid cells induced by Pcy. As shown in figure 3, the Pcy-induced cell death was reduced by 60% and by 78% with QNZ and Pα, respectively. In contrast, a further increase in the amount of hypodiploid cells induced by Pcy was observed after a simultaneous inhibition of NF-κB and p53 compared to cells treated with Pcy/QNZ and Pcy/Pα. Taken together, these data suggested that NF-κB and p53 were partially required in the Pcy-induced cell death in SW620 cells.

Effect of Pcy on TRAIL death receptors regulated by NF-κB and p53 proteins

Pcy has been shown previously to trigger apoptosis in SW620 cells involved the up-regulated expression and activation of TRAIL-DR4/-DR5 (34,35). The current study aimed to show if Pcy-enhanced expression of the TRAIL-death receptor in SW620 cells was regulated by NF-κB and p53. The cell surface expression of TRAIL-DR4/-DR4 was analyzed by flow cytometry using specific antibodies after 48 h of treatment with Pcy single or combined with the inhibitors QNZ or/and Pα. As shown in figure 4A, the ability of Pcy to up-regulate cell surface expression of TRAIL-DR4 receptor was reduced by 59%, 46% and 80% with QNZ, Pα and QNZ/Pα combined treatments, respectively. In contrast, QNZ, with or without Pα, reduced to a lesser extent the amount of cells expressing at their cell surface TRAIL-DR5 in the presence of Pcy (figure 4A). These observations were confirmed by measuring DR4 and DR5 transcript levels by real time RT-PCR (figure 4B). These data suggested that DR4 and DR5 receptors were regulated differently by Pcy or Pα and QNZ. The TRAIL-DR4

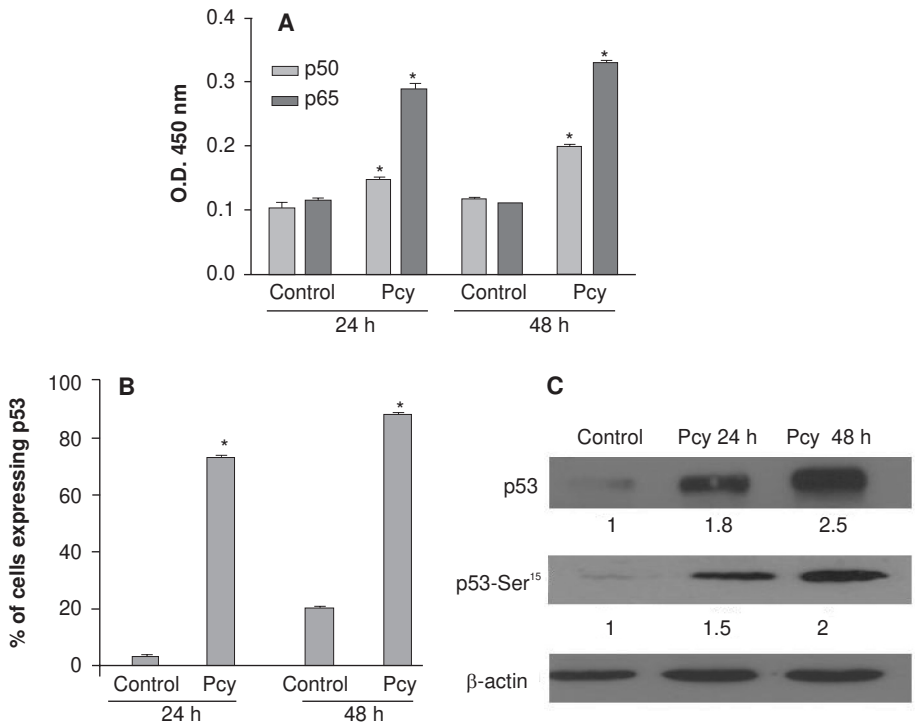

Figure 2. (A) Effect of Pcy on the levels of p50/p65 NF-κB subunits in nuclear extracts of SW620 cells. (B) Intracellular detection of total p53 protein by flow cytometry analysis. The histograms represent the percentage of cells expressing total p53 protein. (C) Effect of Pcy on total and phosphor-Ser[15] p53 protein in whole lysates (80 µg) of SW620 cells treated for 48h. Data are presented as mean ± SE of three separate experiments. Treatments were significant at $p<0.05$.

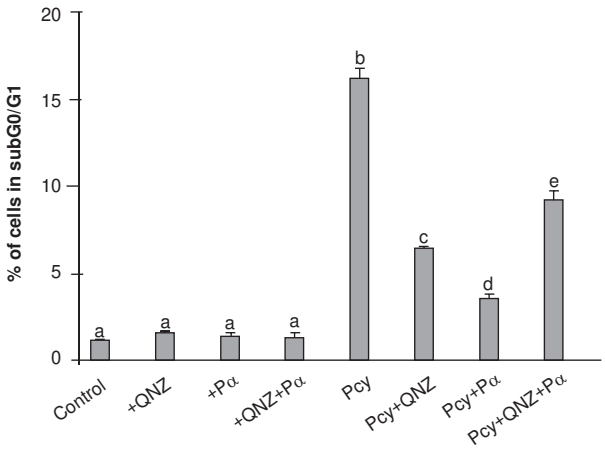

Figure 3. Effects of 6-amino-4-quinazoline (QNZ) and pifithrin α (Pα) on Pcy-induced cell death. Columns not sharing the same superscript letter differ significantly: a≠b≠c≠d≠e, $p<0.05$.

expression may be dependent on the activation of NF-κB and p53 by Pcy, whereas TRAIL-DR5 expression may be regulated by other transcription factors also activated by Pcy.

Discussion

The NF-κB transcription factor plays an important role in tumor formation and progression, in contrast to p53 which plays a key role in the induction of apoptosis (20,21). However, NF-κB may have divergent effects on cell survival and cell death, depending on the cell type and on specific activating signals (10-16). In the current study, NF-κB and p53 transcription factors were investigated for their potential roles in Pcy-induced cell death of SW620 cells. This was accomplished by using QNZ, a specific inhibitor of the transcriptional activation of NF-κB, and by using Pα.an inhibitor of the transcriptional activity of p53. The results suggested that Pcy-induced cell death in SW620 cells required partly the transcriptional function of NF-κB and p53 for the following reasons: (i) Pcy increased the levels of active NF-κB p50/p65 nuclear subunits, (ii) Pcy up-regulated the expression of p53 and its activated/phosphorylated form, and (iii) the type of cell death significantly inhibited by QNZ and Pα was apoptosis by a Pcy-dependent activation of TRAIL-DR4/-DR5 death receptors.

These results suggested that Pcy can induce an apoptosis dependent on NF-κB- and- p53, because Pcy combined with QNZ or Pα reduced significantly the pro-apoptotic effects of Pcy. However, the simultaneous inactivation of NF-κB and p53 in the presence of Pcy was less effective in reducing the amount of apoptotic cells. These observations suggested that Pcy was also able to trigger apoptosis in SW620 cells by activating mechanisms independently of NF-κB and p53 that not involve regulation of pro-apoptotic genes such as TRAIL-DR4/DR5. For example, an apoptotic mechanism that may be independent of NF-κB and p53 is the Pcy-triggered ROS production. This mechanism is related to the Pcy-mediated activation of polyamine catabolism favoring mitochondrial dysfunction (34,35).

In a previous study, Pcy was found to up-regulate the expression of TRAIL-DR4/-DR5 receptors in SW620 cells, leading to the activation of TRAIL-death receptor mediated apoptosis (34,35). Because both NF-κB and p53 were involved in apoptosis, the additional role of a Pcy-induced activation of NF-κB and p53 was investigated for its necessity in the activation of TRAIL-DR4/-DR5 death receptors of SW620 cells. The significant reduction of the Pcy-induced activation of DR4/DR5 in presence of QNZ or Pα suggested that activation of NF-κB or p53 was required in this process. These events may be associated with the Pcy-increased levels of active NF-κB p50/p65 subunits in nuclear extracts of SW620 cells, since the p65 subunit can induce the expression of TRAIL-DR4/-DR5 death receptors (12,17,42). Furthermore, the inhibition of p65 activation has been shown to abolish the expression of TRAIL-death receptors induced by retinoid-related molecules in human prostate and breast carcinoma cells (17,43).

The potentiation of Pcy-triggered apoptosis in SW620 cells by the polyamine oxidase inhibitor, MDL, has been reported to involve a depletion of the intracellular polyamine pool. This depletion leads to an activation of the extrinsic apoptotic pathway by up-regulating TRAIL-death receptor expression (34). These events may be associated to the increased basal level of NF-κB proteins and induced NF-κB nuclear translocation [~~, and activated its sequence specific DNA binding as~~] observed in breast cancer MCF-7 cell line (44) and intestinal IEC-6 cells that were depleted in polyamines (45).

The current study showed that Pcy significantly increased the expression of p53 and its phosphorylated form at Ser^{15}. These events may favor the Pcy activation of TRAIL-death receptors in SW620 cells despite the presence of a less susceptible p53 mutated form in SW620 cells (21). Indeed, in SW620 cells, p53 exhibits two point mutations: the Pro309→Ser (P309S) and

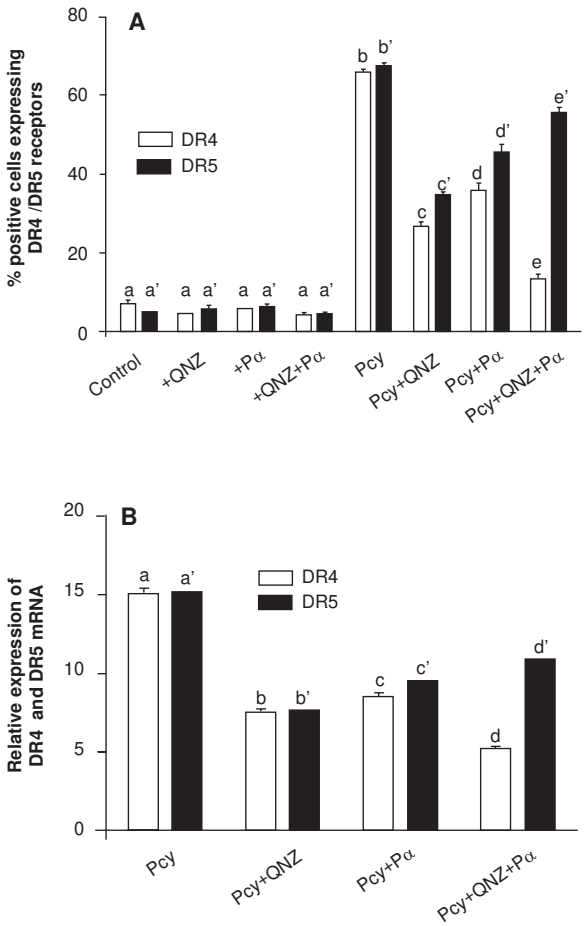

Figure 4. Effects of 6-amino-4-quinazoline (QNZ) and pifithrin α (Pα) on the expression of TRAIL-DR4/-DR5 receptors in Pcy-treated SW620 cells. (A) Cell surface expression of DR4/DR5. For each cell receptor, columns not sharing the same superscript letter differ significantly: a≠b≠c≠d≠e and a'≠b'≠c'≠d'≠e', **p*<0.05. (B) Relative expression levels of DR4 and DR5 mRNA analyzed by real time RT-PCR after 48 h treatments with Pcy (80 µg/ml). Histogram represents the increased expression of treated over non-treated cells. Data are presented as mean ± SE of three separate experiments, **p*<0.0001. Tukey's multiple comparisons post-test was used to determine statistical differences between groups of treatments. For DR4 or DR5, columns not sharing the same superscript letter differ significantly: a≠b≠c≠d and a'≠b'≠c', *p*<0.05.

the Arg273→His (R273H). However, the observed inhibition of the Pcy-induced TRAIL-DR4/-DR5 expression by Pα suggested that p53 exhibited a residual transcriptional activity in SW620 cells. This phenomenon has been described in other cancer lines through protein-protein interactions (17,21).

When Pcy-treated SW620 cells were exposed simultaneously to both inhibitors (QNZ and Pα), TRAIL-DR4 expression was significantly inhibited in contrast to treatments with the inhibitors applied singly; these effects were not observed for TRAIL-DR5 and suggested that the expression of each TRAIL-death receptor may be regulated differently. The DR4 promoter region contains several AP-1 binding sites, which are targets for c-Jun N-terminal kinase pathway. These sites can be activated by several chemotherapeutic agents (46). In contrast the DR5 promoter region has two Sp1 sites which are able to up-regulate DR5 transcription (47).

In conclusion, the current data have demonstrated that Pcy-triggered apoptosis occurred partly via the activation of NF-κB and p53 transcription factors that are directly involved in the upregulation of TRAIL-DR4/-DR5 death receptors. In addition, the DR4 and DR5 receptors appeared to be regulated differently and that the ratio between DR4 and DR5 expression may play an important role in the control of TRAIL-death receptor mediated apoptosis.

Conflicts of interest

The authors declare that they did not incur in any conflict of interest during the present study.

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