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European Journal of Pharmacology 517 (2005) 158 – 164

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Aspirin inhibits NF-κB activation in a glycolysis-depleted lung epithelial cell line

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Received 20 May 2005; accepted 24 May 2005
Available online 28 June 2005

Abstract

Inhibition of glycolysis at the phosphofructo-1-kinase step slows cell growth. For this reason, overexpression of fructose-2,6-bisphosphatase is a potential target for antineoplasic treatments. However, therapeutic objectives may be compromised by side effects of glycolysis restriction, including enhanced resistance to oxidants and tumor necrosis factor-alpha (TNF-α), as well as increased activity of the nuclear factor kappa B (NF-κB). In this study we evaluated aspirin as an adjuvant drug for glycolysis restriction by overexpression of fructose-2,6-bisphosphatase. The effect of aspirin on antioxidant defences and NF-κB activity were evaluated both in control cells and in fructose-2,6-bisphosphatase-overexpressing cells. Interestingly, aspirin-induced inhibition of NF-κB activity was greater in transfectants with restricted glycolysis than in control cells. Our results indicate that aspirin is a suitable complement to therapy based on glycolysis restriction to overcome resistance associated with increased NF-κB activity and oxidative stress.

Keywords: Glycolysis; 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fructose 2,6-bisphosphate; Pentose phosphate pathway; Aspirin; Apoptosis; Glutathione peroxidase; Glutathione; Nuclear factor kappa B

1. Introduction

Tumor cells are highly dependent on glycolysis, even under normoxia (Warburg effect). In most tumor cells this increased glycolysis correlates with overproduction of fructose-2,6-bisphosphate, the most powerful regulator of 6-phosphofructo-1-kinase (Okar et al., 2001). For this reason, the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, responsible for the synthesis and degradation of fructose-2,6-bisphosphate, is a potential target for antineoplastic treatments (Chesney et al., 1999). We previously studied the effect of overexpressing the fructose-2,6-bisphosphatase domain of the liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoenzyme on the regulation of cell metabolism in stably transfected lung epithelial Mv1Lu cells (Perez et al., 2000). These cells showed glycolysis restriction at the 6-phosphofructo-1-kinase step and decreased proliferation (Perez et al., 2000), which agreed with the proposed potential of this step for targeting in antitumor therapy (Chesney et al., 1999). However, as a side effect of this treatment, glycolysis restriction at the 6-phosphofructo-1-kinase step increased pentose phosphate pathway flux, an alternative energy source for cell maintenance. The enhanced activity of the pentose phosphate pathway increased the antioxidant capacity, in terms of glutathione (GSH) content and antioxidant enzyme activities, as well as increasing the activity of the transcription factor nuclear factor kappa B (NF-κB). The increment of antioxidant defences and expression of anti-apoptotic genes associated with NF-κB activity reduced the effect of cytokines, reactive oxygen species and drugs that may compromise antineoplastic treatments (Boada et al., 2000, 2002).

Oxidative stress and inhibition of NF-κB activity are mediators of aspirin-induced apoptosis in tumor cells, such as lung and colon cancer cells (Castonguay et al., 1998;

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doi:10.1016/j.ejphar.2005.05.024

Shao et al., 2000; Stark et al., 2001; Yao et al., 2000)(Hammarstrom and Gage, 2000; Menshikova and Salama, 2000; Stark et al., 2001). For these reasons, aspirin, as well as other non-steroidal anti-inflammatory drugs (NSAIDs), were proposed as antitumor drugs, either alone or in cooperation with other strategies (Brzezinska et al., 2000; Menshikova and Salama, 2000).

Here we attempt to ascertain the efficiency of aspirin as an adjuvant to therapeutic strategies designed to inhibit glycolysis at the 6-phosphofructo-1-kinase step. We evaluate the efficiency of aspirin in overcoming the increments of antioxidant defences and NF-kB activity that appear as a side effect of glycolysis restriction in fructose-2,6-bisphosphatase-overexpressing cells (pFBPase-2 cells) (Boada et al., 2002). Our results show that aspirin reduces antioxidant defences and induces apoptosis in a similar dose-dependent manner in both transfectants, whereas pFBPase-2 cells show higher aspirin-induced inhibition of NF-κB activity than control cells.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum were obtained from Biological Industries (Kibbutz Reit, Haewek, Israel). Annexin V/FITC was from Bender MedSystems (Vienna, Austria). Acetyl salicylic acid, or aspirin, was purchased from Sigma Chemical (St. Louis, MO, USA). Enzymes and biochemical reagents were from Boehringer Mannheim (Mannheim, Germany) or Sigma Chemical (St. Louis, MO, USA). Other materials and chemicals, of the highest quality available, were provided by standard suppliers.

2.2. Stable cell transfection and culture

The mink lung epithelial cell line Mv1Lu (CCl-64, American Type Culture Collection) was stably transfected with a plasmid containing only the bisphosphatase domain of the rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase enzyme, as described elsewhere (Perez et al., 2000). These cells (pFBPase-2 cells) had a lower content of fructose-2,6-bisphosphate (5 pmol/mg protein) than control cells (25 pmol/mg protein) obtained by transfection of the empty pcDNA3 plasmid into the same cell line (Perez et al., 2000). Cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 Units/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418 in 100 mm culture dishes. For measurements, DMEM was supplemented with 0.5% (v/v) foetal bovine serum to avoid the protective effect of serum.

2.3. Flow cytometric analysis

For synchronisation, Mv1Lu cells were cultured in DMEM supplemented with 10% foetal bovine serum for 4 days, up to confluence. After this period, cells were harvested, counted and plated (2.5×10^5 cells) into 60 mm dishes, for 24 h. The culture medium was then removed and replaced with fresh medium supplemented with 0.5% fetal bovine serum to initiate the treatment with 1 to 5 mM aspirin, or vehicle alone (0.5% ethanol). This range of aspirin concentrations is similar to the peak plasma concentration of aspirin in patients treated for inflammation, whereas the tissue concentration may be even higher. After 24, 48 and 72 h, cells were harvested, washed twice in PBS and once in binding buffer containing 140 mM NaCl, 10 mM Hepes and 2.5 mM $CaCl_2$, at pH 7.4. Thereafter, they were stained with 1 μl/ml annexin V/FITC and 0.5 μg/ml propidium iodide for 30 min at room temperature in the dark. Stained cells were analysed using a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson, San Jose, CA, USA). Apoptosis was quantified using MODFIT software (Becton Dickinson, San Jose, CA, USA). To discard necrotic cell death after aspirin challenge, we measured lactate dehydrogenase (LDH) release from cells. LDH leakage was similar (<5%) in both cell transfectants after challenge.

2.4. Measurement of glutathione peroxidase activity

Treated cells were scraped in a medium containing 20 mM Tris–HCl, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, 0.2 g/l Triton X-100 and 0.2 g/l sodium deoxycholate, at pH7.5. Cell extracts were further homogenised using an ultrasound probe (Branson, Danbury, CT, USA) for 6 min at 0 °C. The enzymes were partially purified by ultracentrifugation at 10,000 $\times g$, 4 °C. Protein content in cell lysates was determined using the Bio-Rad assay. Glutathione peroxidase activity was assayed spectrophotometrically at 340 nm using the Cellular Glutathione Peroxidase Assay Kit (Calbiochem, Darmstadt, Germany).

2.5. Determination of glutathione content

Cell cultures were homogenized directly in a cold medium containing 20 mM HCl, 5 mM diethylenetriaminepentaacetic acid (DTPA), 10 mM ascorbic acid and 5% trichloroacetic acid. Suspensions were centrifuged at 14,000 $\times g$ and the resulting supernatants containing GSH were collected and stored at −70 °C. The pellets were washed twice, neutralized in 0.1 M NaOH and stored at −20°C for protein determination. Levels of GSH were determined fluorometrically using the fluorescent probe o-phthalaldehyde, as described elsewhere (Senft et al., 2000).

2.6. Luciferase and β-galactosidase assays

pNF-κB-Luc, which contains two NF-κB sites (5′ AGGGGACTTTCCGAGAGG 3′) in front of the minimal c-*fos* promoter, was transfected using Fugene-6 (Roche, Penzberg, Germany). Cells were split 24 h after transfection, cultured in DMEM supplemented with 0.5% fetal bovine serum and treated with the corresponding amounts of aspirin for 24 h. Luciferase activities were quantified using the Luciferase Assay System (Promega, Madison, WI, USA). Luciferase values were normalized using β-Galactosidase activity assessed using the Luminescent beta-Galactosidase Detection Kit II (Clontech Laboratories, Inc., Palo Alto, CA, USA).

2.7. NF-κB p65 binding assays

To prepare nuclear extracts, treated cells were scraped in 1 ml PBS and centrifuged at 180 $\times g$ for 5 min. The resulting pellet was

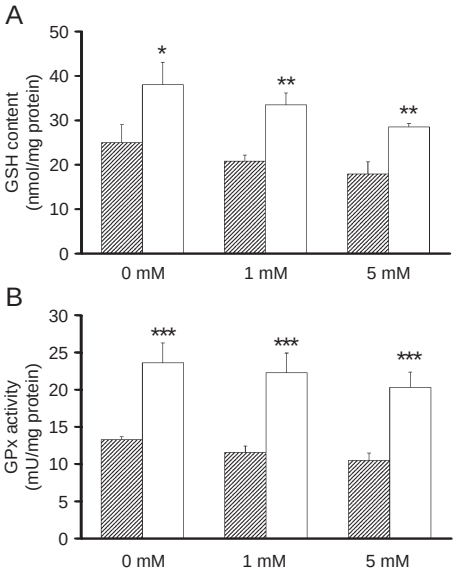

Fig. 1. Effect of aspirin on GSH content (A) and glutathione peroxidase activity (GPx) (B) after 24 h in the presence of the indicated concentrations. Bars represent the mean±S.E.M. of at least five independent experiments performed with pcDNA3 cells (shaded bars) and pFBPase-2 cells (empty bars). Significant differences $P<0.05$, $P<0.01$ and $P<0.001$ vs. the same untreated transfectant are indicated by *, ** and ***.

resuspended in a buffer containing 10 mM Hepes, 10 mM KCl and 1.5 mM $MgCl_2$ and supplemented with phosphatase and protease inhibitors. After centrifugation at 13,000 $\times g$ for 10 s pellets were resuspended in a buffer containing 20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, supplemented with phosphatase and protease inhibitors. After further centrifugation at 13,000 $\times g$ for 2 min, supernatants were collected for final analysis. NF-κB binding to DNA was measured by EZ-Detect™ NFκB p65 Transcription Factor Kit (Pierce Biotechnology, Rockford, IL, USA) (Pizzi et al., 2005).

2.8. Western blot analysis

Cell extracts for Western blot analysis were prepared as described in glutathione peroxidase activity determinations. The membrane was probed with a specific polyclonal antibody against NF-κB p65 subunit (Santa Cruz biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution and visualized with horseradish peroxidase-linked whole antibody from donkey anti-rabbit antibody (Amersham Biosciencies, Little Chalfont Buckinghamshire, UK). α-tubulin was detected using monoclonal anti-α-tubulin (Sigma Chemical, St. Louis, MO, USA) at 1:250 dilution and visualized with horseradish peroxidase linked F(ab′)2 from sheep anti-mouse antibody (Amersham Biosciencies, Little Chalfont Buckinghamshire, UK).

2.9. Data analysis

Results are shown as mean±S.E.M. of the values obtained from the indicated number of experiments. The differences between groups were tested by analysis of variance (ANOVA) and the appropriate a priori contrast methods, and were considered significant at $P<0.05$.

3. Results

3.1. Effect of aspirin on antioxidant defences

The antioxidant capacity of the cell, deduced from its GSH content and GSH/GSSG ratio, modulates the effect of apoptotic stimuli by reducing free radical production and by regulating transcription factors and protease activities (Haddad et al., 2000; Davis et al., 1997; Obrador et al., 2001; Piwocka et al., 2001; Schmidt et al., 1995; Traenckner et al., 1995). Therefore, antioxidant defences are suitable targets to increase cell sensitivity to apoptotic stimuli (Cantin et al., 2000).

The content of reduced glutathione in untreated conditions was 34% higher in pFBPase-2 cells than in pcDNA3 cells (Boada et al., 2002). Following the addition of aspirin at 1 mM and 5 mM, intracellular GSH content and the maximal specific activity of glutathione peroxidase declined to a similar extent in both transfectants (Fig. 1A and B, respectively). Accordingly, both transfectants displayed similar dose rate of aspirin-induced apoptosis, as shown in Fig. 2.

3.2. Effect of aspirin on NF-κB activity

The transcription factor NF-κB regulates the transcription of genes involved in inflammation, stress responses, cell death and transformation (Dudek et al., 2001). NF-κB binding to DNA is regulated by a redox mechanism. Modification of free sulfhydryl

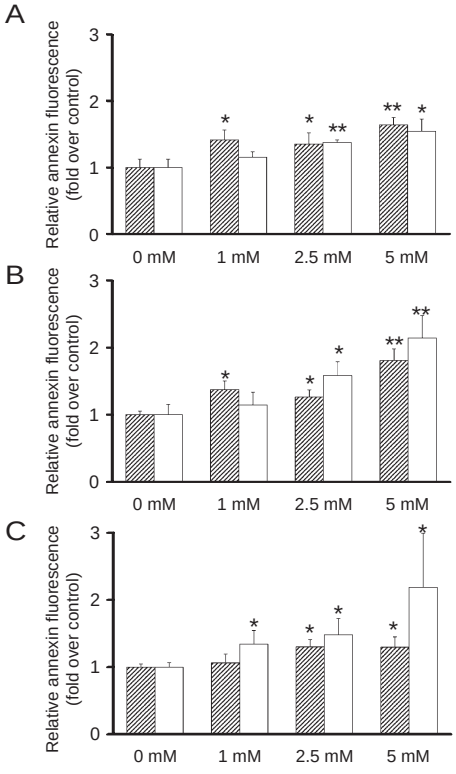

Fig. 2. Fold induced apoptosis in pcDNA3 cells (shaded bars) and pFBPase-2 cells (empty bars) incubated for 24 h (A), 48 h (B) and 72 h (C) in the presence of the indicated concentrations of aspirin. Bars represent the mean±S.E.M. of at least five independent experiments. Significant differences with $P<0.05$ and $P<0.01$ vs. the same untreated transfectant are indicated by * and **, respectively.

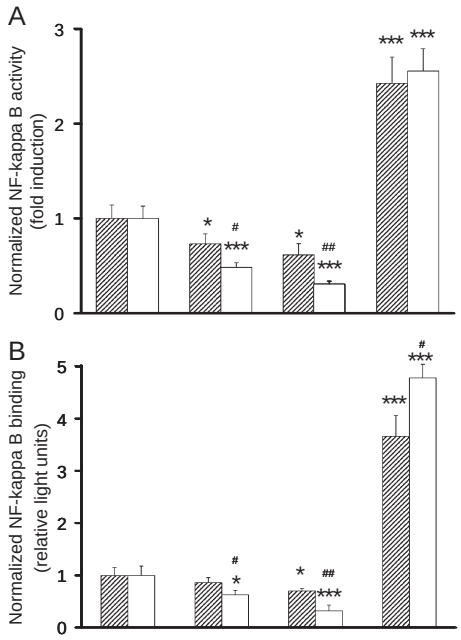

Fig. 3. Inhibitory effect of aspirin (ASA) on NF-κB activity (A) and binding (B) in pcDNA3 cells (shaded bars) and pFBPase-2 cells (empty bars) following 24 h of the indicated concentrations of aspirin (ASA) and PDB. Bars represent the mean±S.E.M. of at least five independent experiments. Significant differences with $P<0.05$ and $P<0.001$ vs. the same untreated transfectant are indicated by * and ***, respectively. Significant differences with $P<0.05$ and $P<0.01$ between pFBPase-2 cells and pcDNA3 cells under the same treatment are indicated by # and ##, respectively.

groups by oxidants and reductants inhibit and enhance, respectively, NF-κB binding to DNA (Okamoto et al., 1997; Daily et al., 2001).

To elucidate the effect of aspirin on NF-κB activity in glycolysis-depleted pFBPase-2 cells, we compared NF-κB transcriptional activity and binding in the presence of aspirin in pFBPase-2 and pcDNA3 cells transiently transfected with the reporter plasmid pNF-κB-Luc. Under untreated conditions, NF-κB activity was three times higher in pFBPase-2 than in pcDNA3 cells (Boada et al., 2002). Aspirin diminished NF-κB activity in both cell types, but pFBPase-2 cells displayed a higher sensitivity to aspirin than pcDNA3 cells (Fig. 3A). These results were confirmed by measurements of NF-κB binding to DNA (Fig. 3B). Indeed, 5 mM aspirin inhibited NF-κB activity and binding by 30% and 70% in pcDNA3 and in pFBPase-2 cells, respectively.

To ascertain whether aspirin-induced reduction of NF-κB activity and binding were associated with a decrease of NF-κB content, the total content of subunit p65 (Pizzi et al., 2005) was evaluated in both transfectants following 24 h of aspirin challenge. Aspirin did not affect the content of NF-κB in either cell type (Fig. 4).

4. Discussion

Enhanced levels of GSH are associated with increased resistance to antineoplastic treatments (Lewandowicz et al., 2002). The increased activity of the glutathione peroxidase/reductase cycle in pFBPase-2, as revealed by glutathione peroxidase activity (Fig. 1B), requires a concomitant increase in available NADPH, which may be explained by the enhanced pentose phosphate pathway flux observed in these cells (Boada et al., 2000). The oxidative branch of pentose phosphate pathway is the main source of NADPH in the cell and its activation provides the glutathione reductase/peroxidase cycle with the essential NADPH required for the maintenance of high GSH content and GSH/GSSG ratio in pFBPase-2 cells. GSH is the principal regulator of intracellular redox status that participates in the reduction of hydrogen peroxide, hydroxyl radicals, peroxynitrites and lipid peroxides (Rahman and MacNee, 2000). Thus, the maintenance of high levels of GSH in pFBPase-2 cells explains the enhanced resistance of these glycolysis-depleted clones to hydrogen peroxide (Boada et al., 2000) and tumor necrosis factor-alpha (TNF-α) (Boada et al., 2002). Other studies have confirmed the control of antioxidant capacity in cells by regulating the flux through the oxidative branch of the pentose phosphate pathway (Le Goffe et al., 2002).

Oxidative stress is involved in the cytotoxicity and pathogenesis of aspirin and related compounds (Elliott and Koliwad, 1997; Guppy et al., 1994; Koliwad et al., 1996; Land and Hochachka, 1994). Aspirin impairs antioxidant system and causes peroxidation in human erythrocytes and guinea pig myocardial tissue (Koliwad et al., 1996), gastric mucosa (Gupte et al., 2002; Guppy et al., 1994) and platelets (Rolfe and Brown, 1997). One of the keys to the

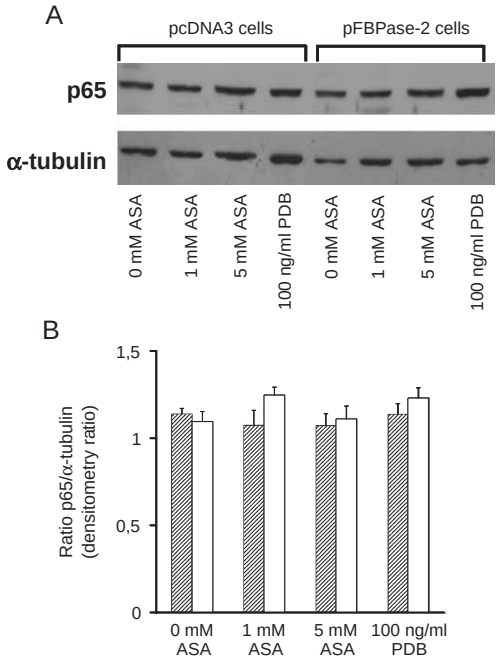

Fig. 4. Effect of aspirin on NF-κB content in pcDNA3 cells (shaded bars) and pFBPase-2 cells (empty bars) as assessed by Western blotting of NF-κB p65 subunit following 24 h of incubation with the indicated concentrations of aspirin (ASA) and PDB. Bars represent the mean±S.E.M. of at least five independent experiments. No significant differences were found.

differential effects of NSAIDs in tumour and normal cells is the redox state of the cells or tissues under investigation, since many tumor cells exhibit a higher sensitivity to oxidative stress. Thus, manipulation of the redox state can affect the outcome or balance of cell proliferation versus cell death (Elliott and Koliwad, 1997; Guppy et al., 1994; Koliwad et al., 1996; Land and Hochachka, 1994; Tian et al., 1998, 1999). Since active oxygen species and lipid peroxidation were reported to play a role in the pathogenesis caused by aspirin, we studied aspirin-induced injury in cells displaying improved antioxidant defences associated with the metabolic restriction induced by transfection of fructose-2,6-bisphosphatase.

Prevention of GSH decrease protected Jurkat cells against mitochondrial cytochrome *c* release and caspase-3 activation (Piwocka et al., 2001), precisely the mechanism of aspirin-induced apoptosis reported in several cell lines (Pique et al., 2000; Piwocka et al., 2001). Our results show that aspirin reduces GSH content and glutathione peroxidase activity in a similar dose-dependent manner in both transfectants (Fig. 1). The decline in GSH content and glutathione peroxidase activity upon aspirin treatment agrees with results reported elsewhere (Gupte et al., 2002; Elliott and Koliwad, 1997; Guppy et al., 1994; Schwarzbaum et al., 1991). This dose-dependent reduction of GSH content and Glutathione peroxidase activity probably contributed to the similar rate of aspirin-induced apoptosis in both transfectants.

Inhibition of NF-κB activity has been implicated in the dose-dependent induction of apoptosis by aspirin and other NSAIDs in several cell types, including lung epithelial cells (Menshikova and Salama, 2000; Bourne and Cossins, 1984; Elliott and Koliwad, 1997; Guppy et al., 1994; Koliwad et al., 1996; Land and Hochachka, 1994; McCormick and Bern, 1989; Nanda and Grinstein, 1991). NF-κB activation and gene transcription appeared to be modulated by intracellular GSH levels. In contrast to its cytosolic effects, a higher content of GSH in the nucleus facilitates the binding of NF-κB to DNA and promotes target gene transcription (Haddad, 2000). NF-κB subunits, as well as other transcription factors like activator protein-1 (AP-1), contain conserved cysteine residues in their DNA binding domains, which are sensitive to the nuclear redox status, being activated by reducing agents and inhibited by oxidants (Daily et al., 2001; Abate et al., 1990). Therefore, NF-κB activation depends on the balance between cytosolic inhibitory kappa B protein (IκB) degradation and nuclear DNA binding, the former being enhanced by an oxidative environment (Lopez-Rovira et al., 2000) and the latter by reductive conditions.

In the Mv1Lu cell line, as well as in other cell types, activation of NF-κB operates to enhance resistance to apoptotic stimuli (Daily et al., 2001; Boada et al., 2002; Lallemand et al., 2001). Moreover, in many tumour cells, activation of NF-κB induces the expression of anti-apoptotic genes, which contributes to prevention of cell death and compromises the efficiency of antineoplastic therapies (Baichwal and Baeuerle, 1997). Consequently, inhibition of NF-κB is a logical target for adjuvant approaches to cancer therapy (Baldwin, 2001).

Under untreated conditions, fructose-2,6-bisphosphatase-overexpressing cells displayed a three-fold higher NF-κB activity than control cells. TNF-α activated NF-κB in both transfectants, but to a greater extent in pFBPase-2 cells (Boada et al., 2002). Results reported here show that aspirin induced a higher reduction of NF-κB binding and activity in cells overexpressing fructose-2,6-bisphosphatase than in control cells (Fig. 3A and B). The aspirin-induced decrease of NF-κB binding and transcription activity could be caused by a decrease in NF-κB content, as well as of reducing power in the cell nucleus. Results shown in Fig. 4 indicate that NF-κB content was not affected by aspirin treatments, suggesting a higher reduction of the NF-κB binding capacity to DNA as the cause of the greater loss of NF-κB activity in pFBPase-2 cells than in pcDNA3 cells.

These results suggest that overexpression of fructose-2,6-bisphosphatase, to reduced proliferation rate, does not induce significant resistance to aspirin-induced oxidative stress and apoptosis, but displays a higher sensitivity to aspirin-induced NF-κB inhibition. Consequently, aspirin may be a suitable complement to gene therapy strategies based on glycolysis restriction to overcome resistance associated with increased NF-κB activity and oxidative stress.

Acknowledgements

We are grateful to Dr. J. Gil for helpful discussion. We also thank Robin Rycroft for linguistic assistance and Dr. E. Castaño of SCT for technical assistance. E. Cuesta is the recipient of a research fellowship from the University of Barcelona. This study was supported by the Ministerio de Ciencia y Tecnología, Spain (HU02-22, SAF2002-02964, BFI2003-02539).

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