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# Biphasic effects of luteolin on interleukin-1β-induced cyclooxygenase-2 expression in glioblastoma cells

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ABSTRACT

Success in developing therapeutic approaches to target brain tumor-associated inflammation in patients has been limited. Given that the inflammatory microenvironment is a hallmark signature of solid tumor development, anti-inflammatory targeting strategies have been envisioned as preventing glioblastoma initiation or progression. Consumption of foods from plant origin is associated with reduced risk of developing cancers, a chemopreventive effect that is, in part, attributed to their high content of phytochemicals with potent anti-inflammatory properties. We explored whether luteolin, a common flavonoid in many types of plants, may inhibit interleukin (IL)-1β function induction of the inflammation biomarker cyclooxygenase (COX)-2. We found that IL-1β triggered COX-2 expression in U-87 glioblastoma cells and synergized with luteolin to potentiate or inhibit that induction in a biphasic manner. Luteolin pretreatment of cells inhibited IL-1β-mediated phosphorylation of inhibitor of κB, nuclear transcription factor-κB (NF-κB) p65, extracellular signal-regulated kinase-1/2, and c-Jun amino-terminal kinase in a concentration-dependent manner. Luteolin also inhibited AKT phosphorylation and survivin expression, while it triggered both caspase-3 cleavage and expression of glucose-regulated protein 78. These effects were all potentiated by IL-1β, in part through increased nuclear translocation of NF-κB p65. Finally, luteolin was able to reduce IL-1 receptor gene expression, and treatment with IL-1 receptor antagonist or gene silencing of IL-1 receptor prevented IL-1β/luteolin-induced COX-2 expression. Our results document a novel adaptive cellular response to luteolin, which triggers anti-survival and anti-inflammatory mechanisms that contribute to the chemopreventive properties of this diet-derived molecule.



## 1. Introduction

Glioblastoma multiforme is the most common glioma and is a highly malignant primary brain tumor, associated with a poor survival rate [1]. This glioma is known for its invasiveness and high resistance to standard treatments of chemotherapy and radiotherapy [2,3]. The tumor microenvironment regulates glioblastoma development and progression and, among the pool of inflammatory cytokines, elevated levels of interleukin (IL)-1β are believed to contribute to glioblastoma cell proliferation and invasion [4–6]. Thus, blocking the pro-inflammatory activity of IL-1β would be useful in controlling the invasiveness of glioblastoma cells.

Cyclooxygenases (COXs) catalyze the synthesis of prostaglandins from arachidonic acid and include the constitutive isoform COX-1, the inducible isoform COX-2 and a splice variant COX-3 [7]. COX-2, which is regulated by various growth factors and cytokines such as IL-1β [8], is a crucial target for the control of tumors associated with chronic inflammation [9]. Increased COX-2 has been detected in a variety of human malignant tumors including glioblastomas, in which its expression correlated with the histopathological grade of gliomas [10]. High levels of COX-2 have been correlated with higher proliferation rates of tumors and shorter patient survival times [11]. There is also clear evidence of the positive effects of COX-2 inhibitors, including nonsteroidal anti-inflammatory drugs, against a variety of tumors, which exert chemopreventive activities [12].

The nuclear transcription factor-κB (NF-κB) is one of the major transcription factors associated with cancer development [13]. In response to different stimuli, mainly cytokines, glioblastoma cells exhibit elevated levels of NF-κB activity which contribute significantly to tumor progression [14]. Moreover, it was reported that NF-κB

*Abbreviations:* BBB, blood–brain barrier; CNS, central nervous system; COX-2, cyclooxygenase-2; IκB, inhibitor of κB; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GRP78, glucose-regulated protein 78; IL-1β, interleukin-1β; IL-1Ra, interleukin-1 receptor antagonist; IL-1R1, interleukin-1 receptor type I; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear transcription factor-κB; PMA, phorbol 12-myristate 13-acetate

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activation is a crucial step in the induction of COX-2 in the brain in response to IL-1β [15]. Binding of IL-1β to its type 1 receptor (IL-1R1) activates a cascade of phosphorylation that results in NF-κB activation [16]. NF-κB is a protein complex consisting of a 65-kDa DNA binding subunit (*Rel* A) and an associated 50-kDa protein which, in the classical NF-κB pathway, is maintained in the cytoplasm in an inactive state by a protein complex called inhibitor of κB (IκB) [17]. Once phosphorylated by the IκB kinases (IKKs), IκB then allows the p65 and p50 subunits of NF-κB to translocate to the nucleus and to bind to the κB recognition sites located in the promoter regions of various NF-κB-regulated genes such as COX-2 [18]. Deregulation of the NF-κB signaling pathway contributes to enhanced glioblastoma cell survival, proliferation, cell cycle progression and chemoresistance; it therefore represents an attractive therapeutic target [19–21].

Numerous studies have linked abundant consumption of foods from plant origins with decreased risk of developing various cancers [22], a chemopreventive effect that is related to the high content of several phytochemicals with potent anticancer and anti-inflammatory properties [23]. Among these, luteolin (3′,4′,5,7-tetrahydroxyflavone), a flavonoid found at high levels in common fruits, vegetables and herbs such as green peppers, olive oil, parsley, celery, thyme, broccoli, cabbages, and chamomile tea [24,25], possesses a variety of neuroprotective [26], anticancer [24] and anti-inflammatory [27] properties. For instance, luteolin was demonstrated to antagonize phorbol 12-myristate 13-acetate (PMA)-induced COX-2 in human brain endothelial cells [28] and to inhibit tumor necrosis factor (TNF)-α-induced COX-2 expression by down-regulating the transactivation of NF-κB and activator protein-1 (AP-1) in JB6 mouse epidermis cells [29]. However, the effect of luteolin on the pro-inflammatory cytokine IL-1β-mediated induction of COX-2 has never been investigated. Here, we report for the first time anti-IL-1β effects of luteolin on the NF-κB-mediated transcriptional regulation of COX-2 expression in U-87 glioblastoma cells.

## 2. Materials and methods

### 2.1. Materials

Luteolin (purity ≥ 99%) was purchased from Extrasynthese (Lyon, France). Human recombinant IL-1β and human recombinant IL-1Ra were obtained from R&D Systems (Minneapolis, MN). The MEK inhibitor U0126 and the JNK inhibitor SP60012 were from Calbiochem (La Jolla, CA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The anti-ERK (extracellular signal-regulated kinase 1 and 2) (K-23) polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). The monoclonal antibody against GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was from Advanced Immunochemical Inc. (Long Beach, CA). The Alexa Fluor® 488 donkey anti-rabbit IgG antibody was purchased from Invitrogen (Carlsbad, NM). Antibodies for AKT, Bip/GRP78 (glucose-regulated protein 78), caspase-3, cleaved-caspase-3, IκB, lamin A/C, NF-κB p65, SAPK/JNK (c-Jun amino-terminal kinase), survivin, phospho-AKT, phospho-NF-κB p65, phospho-SAPK/JNK polyclonal antibodies and phospho-ERK and phospho-IκB monoclonal antibodies were from Cell Signaling Technology (Beverly, MA). The anti-COX-2 monoclonal antibody was from BD Transduction Laboratories™ (Franklin Lakes, NJ). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from Denville Scientific Inc. (Metuchen, NJ). Micro bicinchoninic acid protein assay reagents were from Thermo Scientific (Rockford, IL). All other reagents were from Sigma-Aldrich (Oakville, ON).

### 2.2. Cell culture

A human glioblastoma cell line (U-87 MG) was purchased from the American Tissue Culture Collection (HTB-14™) and maintained in modified Eagle's Minimum Essential Medium (Wisent, 320-036-CL) containing 10% calf serum (HyClone Laboratories, SH30541.03), 1 mM sodium pyruvate (Sigma-Aldrich, P2256), 2 mM L-glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (Wisent, 450-202-EL). Cells were cultured at 37 °C under a humidified 95%–5% (v/v) mixture of air and $CO_2$. Cells were treated with vehicle (0.1% DMSO) or with luteolin and stimulated with IL-1β. All cellular assays were conducted at 85% confluence.

### 2.3. Western blot analysis

U-87 MG cells were serum-starved in the presence of luteolin, U0126 (10 μM) or SP60012 (10 μM) for 24 h. To study the effects of these molecules on protein expression of COX-2, caspase-3, cleaved caspase-3 or survivin, cells were co-treated with one of these molecules and 50 ng/mL IL-1β or 1 μM PMA for 24 h. To study the phosphorylation status of AKT, IκB, NF-κB p65, ERK and JNK, IL-1β was added to the cells for 5 min after the treatment with luteolin for 24 h. Cells were then washed once with ice-cold phosphate-buffered saline (PBS) containing 1 mM each of NaF and $Na_3VO_4$ and were incubated in the same buffer solution for 30 min at 4 °C. The cells were solubilized on ice in lysis buffer [150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM ethyleneglycol-O, O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100]. To study the translocation of NF-κB p65 into the nucleus, nuclear protein extracts from U-87 MG cells were isolated using the NE-PER Nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). The resulting lysates or nuclear extracts (25 μg protein) were solubilized in Laemmli sample buffer [125 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.00125% bromophenol blue], boiled for 4 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes which were then blocked 1 h at 4 °C with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 147 mM NaCl, 20 mM Tris–HCl, pH 7.5, and 0.1% Tween 20). Membranes were further washed in TBS-T and incubated overnight with the primary antibody in TBS-T containing 3% bovine serum albumin (BSA) and 0.01% sodium azide ($NaN_3$), followed by a 1 h incubation with HRP-conjugated anti-mouse or anti-rabbit antibodies in TBS-T containing 5% nonfat dry milk. Immunoreactive material was visualized with an ECL detection system. The immunoreactive bands were quantified with ImageJ software (NIH).

### 2.4. Total RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted from U-87 MG monolayers using TRIzol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at −80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (BIO-RAD, Hercules, CA). DNA amplification was carried out using an Icycler iQ5 (BIO-RAD, Hercules, CA) and product detection was performed by measuring the binding of the fluorescent dye SYBR Green I to double-stranded DNA. The following primer sets were provided by QIAGEN (Valencia, CA): COX-2 (QT00040586), IL-1R1 (QT00081263), NF-κB p50 (QT00154091), NF-κB p65 (QT00149415), β-Actin (QT01680476), GAPDH (QT00079247), and PPIA (peptidylpropyl isomerase A; QT01866137). The relative quantities of target gene mRNA against an internal control, β-Actin/GAPDH/PPIA RNA, were measured by following a ΔCt method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔCt) between the mean values in the triplicate samples of target gene and those of β-Actin/GAPDH/PPIA RNA was calculated by iQ5 Optical System Software version 2.0 (BIO-RAD, Hercules, CA) and the relative quantified value (RQV) was expressed as $2^{-\Delta Ct}$. Semi-quantitative PCR was performed to examine

amplification products and amplicons resolved on 1.8% agarose gels containing 1 mg/mL ethidium bromide.

### 2.5. Cytotoxicity assays

The sensitivity of U-87 MG cells to luteolin was determined in vitro by using the WST-1 assay (Roche Diagnostics, Montreal, QC). Briefly, after co-treatment of cells with IL-1β and luteolin for 24 h, U-87 MG cells were exposed to 10 μL of the tetrazolium salt WST-1 reagent. The soluble formazan dye produced by metabolically active cells was monitored for 60 min at 37 °C. The absorbance at 450 nm was measured using a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA).

### 2.6. Fluorimetric caspase-3 assay

U-87 MG cells were treated for 24 h with luteolin in the presence or absence of 50 ng/mL IL-1β. Cells were collected, washed in cold PBS and lysed in Apo-alert lysis buffer (Clontech, Palo Alto, CA) for 20 min at 4 °C and the lysates were clarified by centrifugation at 16,000 ×*g* for 20 min. Caspase-3 activity was determined by incubation with 50 mM of the caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0,1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates. The release of AFC was monitored for at least 30 min at 37 °C on a fluorescence plate reader (Molecular Dynamics) (λex = 400 nm, λem = 505 nm).

### 2.7. Transfection method and RNA interference

U-87 MG cells were transiently transfected with 20 nM siRNA against IL-1R1 (SI00017584), NF-κB p50 (SI01326990), NF-κB p65 (SI01399622) or scrambled sequences (AllStar Negative Control siRNA, 1027281) using Lipofectamine 2000 transfection reagent (Invitrogen, CA). Cells were treated for 24 h with luteolin in the presence or absence of 50 ng/mL IL-1β. Small interfering RNA and mismatch siRNA were synthesized by QIAGEN and annealed to form duplexes.

### 2.8. Immunofluorescence

U-87 MG cells were grown on coverslips to 60% confluence, treated for 24 h with 15 μM luteolin and then incubated with 50 ng/mL IL-1β for 10 min. Following this, cells were incubated in 10% formalin phosphate buffer for 20 min, followed by incubation in permeabilization buffer (0.5% Triton X-100 in PBS) for 5 min and then blocked in 1% BSA and 0.1% $NaN_3$ in PBS for 1 h. The coverslips were incubated 1 h with anti-NF-κB p65 (1:500) in 1% BSA, 0.1% $NaN_3$ in PBS. After three PBS washes, the cells were incubated for 1 h with Alexa Fluor® 488 donkey anti-rabbit secondary antibody (1:200) in 1% BSA and 0.1% $NaN_3$. The coverslips were then washed 3 times in PBS and laid down on microscope slides using ProLong Gold Antifade Reagent from Invitrogen (Carlsbad, NM). The samples were viewed under a confocal microscope using NIS Elements and Viewer and analyzed with ImageJ software.

### 2.9. Statistical analysis

Statistical analyses were assessed with Student's t-test when one group was compared with the control group. To compare two or more groups with the control group, one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used. To investigate a significant interaction between two curves (control group versus treated group), a Tukey's post hoc test and a trend analysis were performed. Differences with $p < 0.05$ were considered significant. All statistical analyses and graphs were performed using GraphPad Prism version 5.0b for Macintosh (GraphPad Software, San Diego, CA, USA; www.graphpad.com). The nonlinear regression analysis was used for fitting curves to data.

## 3. Results

### 3.1. Biphasic effects of luteolin on IL-1β-induced COX-2 gene and protein expression in human glioblastoma cells

Since modulations in COX-2 expression control brain tumor development within chronic inflammation [9,10], we first examined the effect of luteolin on IL-1β-induced COX-2 expression in U-87 MG cell line. We choose this model because Taniura et al. [6] demonstrated that COX-2 expression was remarkably increased in U-87 MG cell line compared to other glioma cell lines tested (A172, T98G). U-87 MG cells were serum-starved in the presence of 50 ng/mL IL-1β and various concentrations of luteolin for 24 h. Under these conditions, IL-1β alone caused a marked increase in COX-2 protein expression in U-87 MG (Fig. 1A). This induction was potentiated by luteolin treatment at concentrations ranging between 1–15 μM and decreased at higher concentrations (20–50 μM) (Fig. 1A). These results show that the peak of synergistic IL-1β/luteolin stimulation of COX-2 expression was reached at 15 μM, but was completely inhibited at 25 μM (Fig. 1B). A biphasic effect of luteolin on IL-1β-induced COX-2 expression was also observed at the transcriptional level (Fig. 1C). Such biphasic effects of luteolin were not observed when COX-2 expression was induced with the tumor-promoting agent PMA, where a concentration-dependent inhibition of COX-2 protein expression by luteolin was observed (Fig. 1D). This result thus suggests that luteolin affects downstream IL-1β receptor-mediated signaling.

### 3.2. Luteolin inhibits IL-1β-induced downstream signaling events in human glioblastoma cells

We next evaluated the effect of luteolin treatment on IL-1β-induced activation of NF-κB and MAPK signaling pathways known to up-regulate COX-2 expression [15,30]. U-87 MG cells were pre-treated for 24 h with various concentrations of luteolin in serum-free medium, and then stimulated with 50 ng/mL IL-1β for 5 min. Protein expression and phosphorylation status of downstream signaling intermediates targeted by luteolin were assessed by immunoblotting using specific antibodies. Pretreatment with luteolin resulted in a concentration-dependent inhibition of IL-1β-induced phosphorylation of IκB, NF-κB p65, ERK and JNK with half-maximal inhibition concentrations ($IC_{50}$) of 21 μM, 18 μM, 11.1 μM and 0.7 μM, respectively (Fig. 2A–D). Interestingly, the $IC_{50}$ values obtained for the inhibition of MAPK signaling pathways by luteolin were low compared to those obtained for NF-κB, suggesting that the MAPK cascade may be predominantly involved in the synergistic IL-1β/luteolin effects on COX-2 expression, while NF-κB signaling may possibly be involved in the diminished IL-1β-induction of COX-2 at higher luteolin concentrations. Moreover, the fact that low concentrations of luteolin appeared to increase the phosphorylation of NF-κB indicates a possible translocation of p65 subunit to the nucleus, which correlated with the induction of COX-2.

### 3.3. Effect of luteolin on human glioblastoma cell viability

To determine whether the anti-IL-1β inhibitory effects of luteolin observed at higher concentrations (>20 μM) were due to cytotoxicity, we next assessed the effect of this compound on IL-1β-induced death/survival signaling pathways. U-87 MG cells were treated with various concentrations of luteolin in serum-free medium for 24 h, and then stimulated with 50 ng/mL IL-1β for 5 min. Luteolin caused a concentration-dependent decrease in phosphorylated AKT, while the amount of unphosphorylated AKT was unaltered (Fig. 3A). These effects were observed in the basal conditions and were significantly potentiated in the presence of IL-1β with the $IC_{50}$ value for luteolin decreasing from 1.8 μM to 0.6 μM. To assess whether luteolin affects GRP78, survivin or caspase-3 protein levels, U-87 MG cells were serum-starved in the presence of 50 ng/mL IL-1β and various concentrations

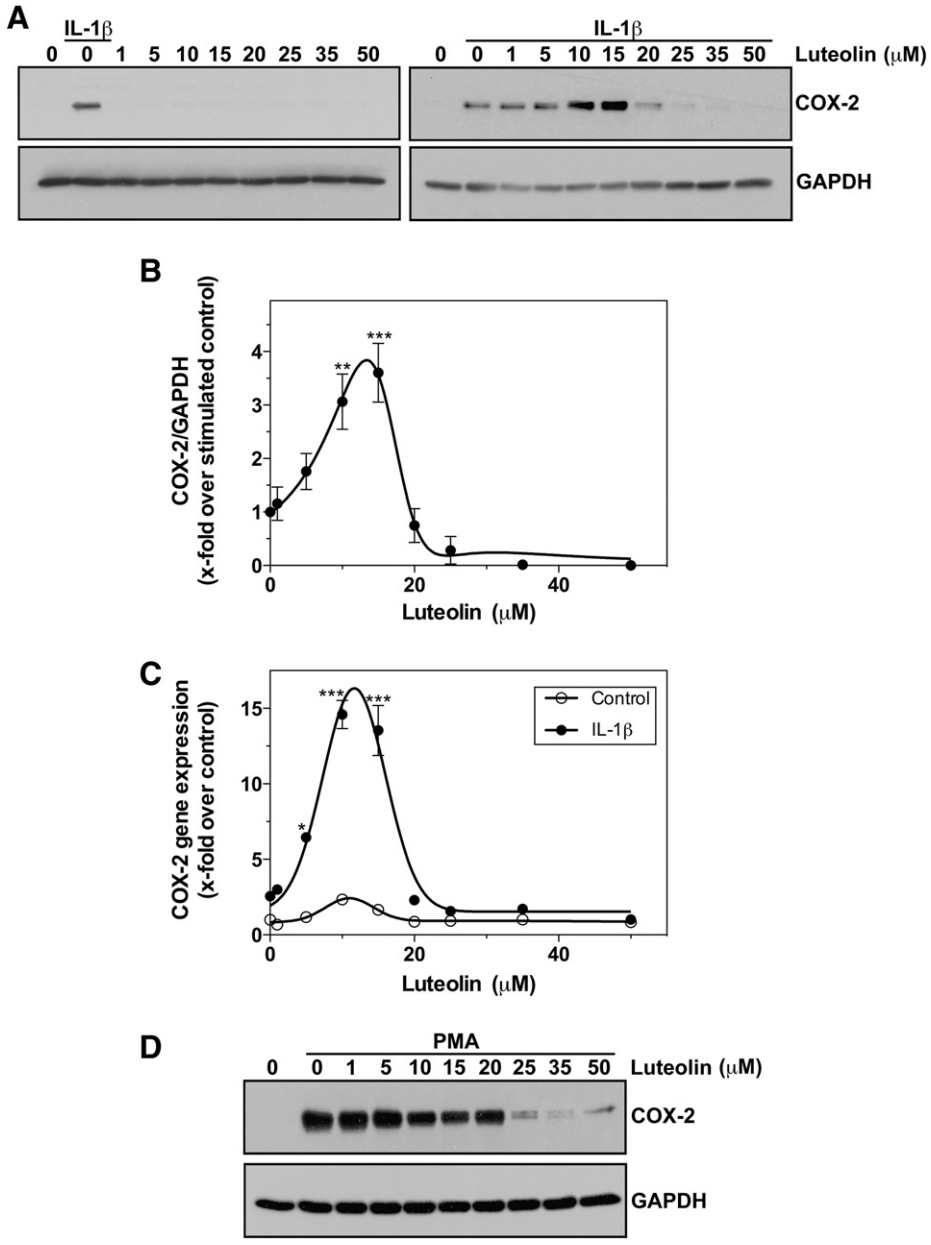


**Fig. 1.** Effect of luteolin on IL-1β-induced COX-2 gene and protein expression in human glioblastoma cells. U-87 MG cells were serum-starved in the presence of various concentrations of luteolin containing (or lacking) 50 ng/mL IL-1β for 24 h. (A) Cells were lysed and the levels of COX-2 protein expression were monitored by immunoblotting. Immunodetections obtained from representative experiments are shown. (B) The band intensities were analyzed by scanning densitometry using ImageJ software and the quantification of three independent experiments is shown. Values are means ± SEM (**$p < 0.01$ and ***$p < 0.001$ versus IL-1β alone). For each sample, the COX-2 level was normalized for GAPDH. (C) Total RNA was isolated from conditions described above, cDNA synthesis, and qPCR performed to assess COX-2 gene expression. Values are means ± SEM of three independent experiments (*$p < 0.05$ and ***$p < 0.001$ versus basal control alone). (D) U-87 MG cells were serum-starved in the presence of various concentrations of luteolin containing (or lacking) 1 μM PMA for 24 h. A representative Western blot depicting COX-2 levels, and GAPDH as a loading control, is shown.

of luteolin for 24 h. Under these conditions, luteolin inhibited survivin expression while it triggered both caspase-3 cleavage and GRP78 expression. Again, these effects were potentiated by the presence of IL-1β, indicating that all of these responses were possibly triggered through IL-1R1-mediated signaling. In order to strengthen these results, we measured the enzymatic activation of caspase-3 under the same conditions. Luteolin induced caspase-3 activity in a concentration-dependent manner, indicating that apoptosis signaling was involved (Fig. 3B). Furthermore, statistical analysis confirmed that the two curves at lower concentrations had similar effects on the induction of caspase-3 activity; however, at luteolin concentrations above 15 μM, the IL-1β-treated cells began to behave differently than IL-1β-untreated cells suggesting that, above this concentration, IL-1β-mediated signaling potentiated the action of luteolin towards the apoptosis pathway. Consistent with this result, the highly sensitive WST-1 assay showed that the viability of the cells treated with ≥15 μM luteolin significantly decreased in a concentration-dependent manner with an $IC_{50}$ of 19.2 μM (Fig. 3B) indicating a mode of cell death by apoptosis. Since MAPK signaling pathway mediates growth factor-dependent cell survival [31], we examined whether ERK and JNK were involved in survival of U-87 MG cells. As shown in Fig. 3C, the treatment of cells with the MEK inhibitor U0126 or the JNK inhibitor SP60012 inhibited both AKT

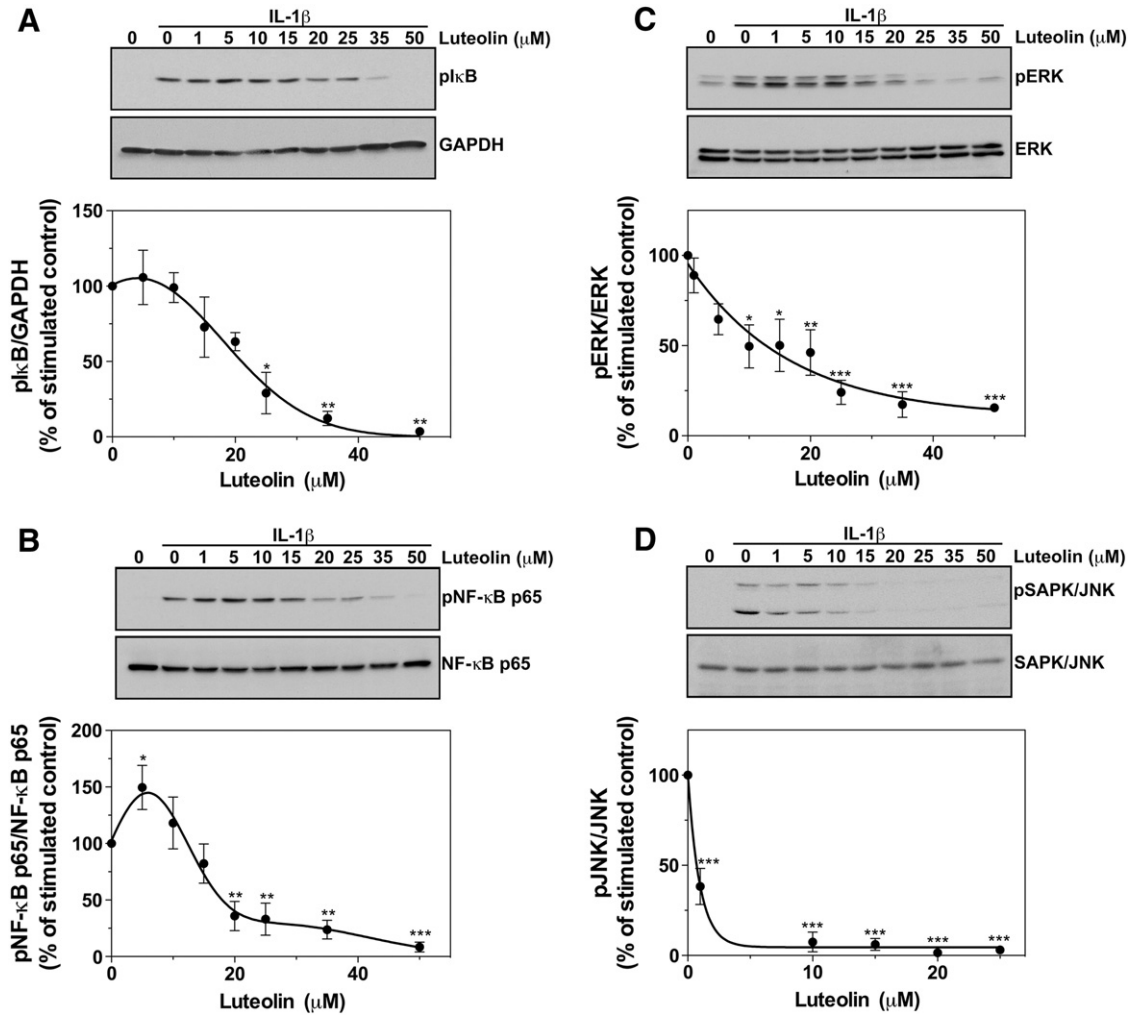


**Fig. 2.** Luteolin inhibits IL-1β-induced downstream signaling events in human glioblastoma cells. U-87 MG cells were serum-starved in the presence of various concentrations of luteolin for 24 h. Then cells were stimulated with 50 ng/mL IL-1β for 5 min. After these treatments, the phosphorylated forms of (A) IκB, (B) NF-κB, (C) ERK, or (D) JNK, along with their total protein levels, were monitored by immunoblotting. Immunodetection obtained from representative experiments is shown (*top panels*). The band intensities were analyzed by densitometry using ImageJ software and expressed in arbitrary units as a ratio of levels of phosphorylated protein to those of the total protein to correct for variation in the amount of protein (*bottom panel*). For pIκB, each band was normalized for GAPDH. The relative levels of phosphorylated protein were also normalized to those seen in IL-1β control (value = 100). Values are means ± SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus IL-1 alone).

phosphorylation and survivin protein expression and triggered caspase-3 activation. These data supported the implication of ERK and JNK activated by IL-1β as survival signals in U-87 MG cells.

### 3.4. IL-1 receptor-mediated events are required in the luteolin inhibition of IL-1β-induced COX-2 expression

In order to elucidate the mechanisms underlying the synergistic IL-1β/luteolin-induction of COX-2, we next explored whether IL-1R1-mediated events were involved. IL-1R1 blockade was performed with the use of another member of the IL-1 family, the antagonist IL-1Ra. U-87 MG cells were serum-starved in the presence of 50 ng/mL IL-1β containing (or lacking) 15 μM of luteolin or 500 ng/mL IL-1Ra for 24 h. While IL-1Ra itself had no effect on COX-2 under these conditions, it significantly reduced the IL-1β/luteolin-induction of COX-2 gene expression (Fig. 4A). The requirement for IL-1R1 was confirmed through gene silencing strategies showing that the effects of IL-1β alone or combined IL-1β/luteolin on COX-2 protein expression were greatly attenuated by the gene silencing (Fig. 4B). Interestingly, while effective silencing of IL-1R1 also significantly reduced COX-2 transcription in IL-1β/luteolin-treated cells (Fig. 4C, *bottom panel*), IL-1β/luteolin was able to reduce IL-1R1 transcription in siScrambled control cells (Fig. 4C, *top panel*). This suggests that part of the synergistic IL-1β/luteolin-mediated effects on COX-2 transcription involves IL-1R1 and part involves IL-1R1-induced downstream signaling. However, even if IL-1R1 gene silencing completely abrogated IL-1β-induced COX-2, luteolin was still able to trigger some COX-2 gene and protein expressions (panels B–C), although to reduced levels in IL-1R-depleted cells, indicating the requirement of IL-1R-independent mechanisms. The fact that IL-1Ra did not reduce IL-1β signal except if luteolin was present (Fig. 4A) supports this observation.

### 3.5. Gene silencing of NF-κB p65, but not of NF-κB p50, abrogates IL-1β-mediated induction of COX-2 expression

Among the nuclear factors that regulate COX-2 transcription, the NF-κB p50 and NF-κB p65 transcription factors were examined to determine if they were involved in the synergistic IL-1β/luteolin-induction of COX-2. Gene silencing was performed in U-87 MG cells and specificity of knockdown was validated (Fig. 5A). While IL1-β or IL-1β/luteolin triggered COX-2 protein expression under control (siScrambled) conditions, COX-2 expression was completely abrogated only in those cells in which NF-κB p65 gene expression was specifically downregulated (Fig. 5B). The involvement of NF-κB p65 was further correlated at the transcriptional level control of COX-2, and this confirms that its absence

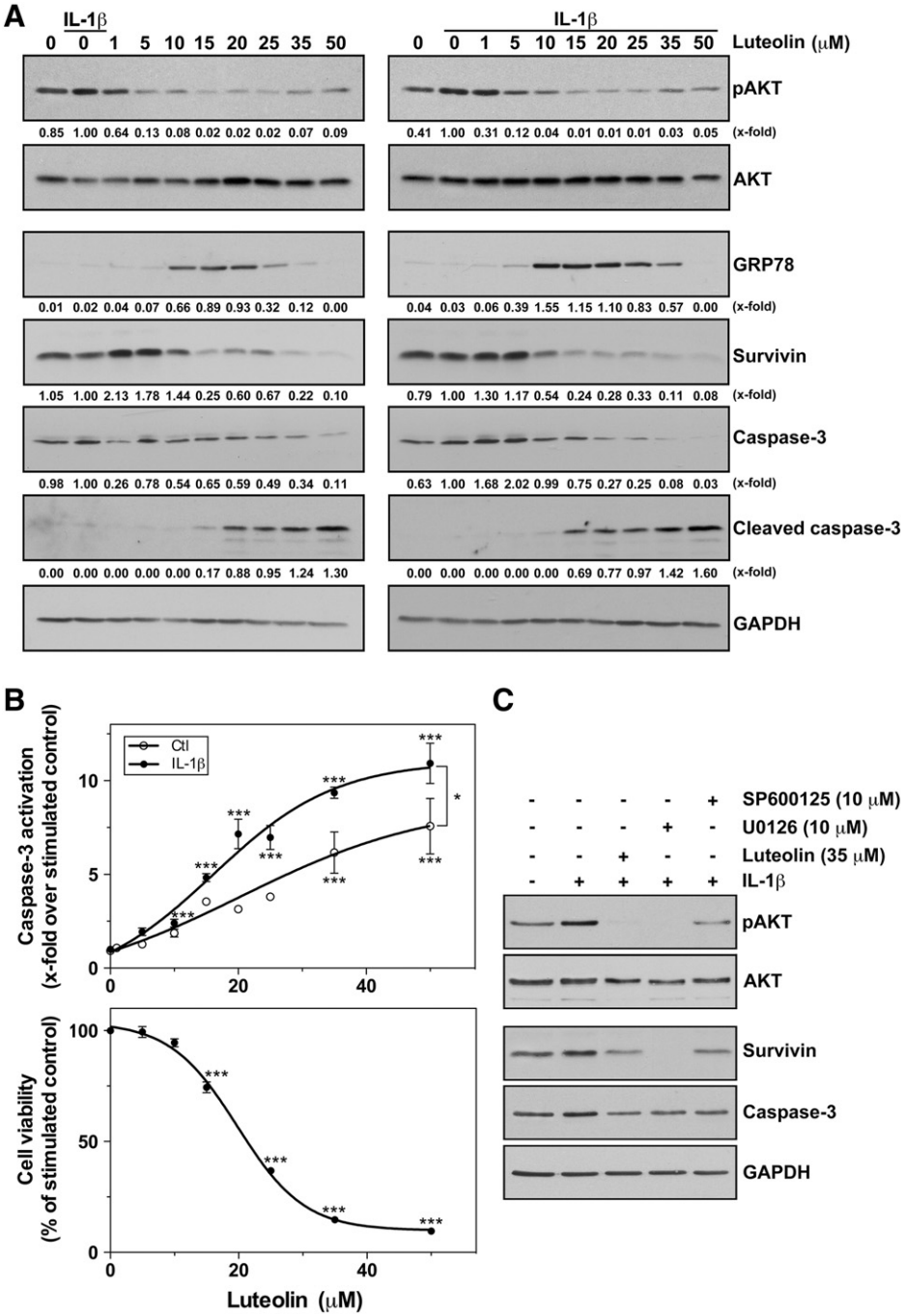


**Fig. 3.** Luteolin modulates IL-1β-induced apoptotic and survival signaling pathways in human glioblastoma cells. (A) U-87 MG cells were serum-starved in the presence of various concentrations of luteolin containing (or lacking) 50 ng/mL IL-1β for 24 h except for AKT phosphorylation where IL-1β was added to the cells for 5 min after cell treatment with luteolin. Representative Western blots (from three independent experiments) are shown for the expression of pAKT, AKT, GRP78, survivin, caspase-3, cleaved caspase-3 and GAPDH. Western blots were quantified and bands were corrected for their specific protein loading control, AKT for pAKT, GAPDH for the others. The relative levels of phosphorylated AKT were also normalized to those seen in IL-1β control (value = 1). (B) Extracts from control and treated cells were used to determine DEVDase (caspase-3) activity (*top panel*). Cell viability was assessed by WST-1 assay (*bottom panel*), as described in the Materials and methods section. Values are means ± SEM of three independent experiments performed in triplicate (***$p < 0.001$ versus IL-1β alone). Statistical significance of differences between control and IL-1β was calculated by one-way analysis of variance followed by Tukey's test and a trend analysis (*$p < 0.05$). (C) U-87 MG cells were serum-starved in the presence or not of 35 μM of luteolin, 10 μM of U0126, or 10 μM of SP60012 for 24 h with 50 ng/mL IL-1β or before adding IL-1β for 5 min. Representative Western blots (from two independent experiments) are shown.

completely prevents the synergistic action of IL-1β/luteolin on COX-2 (Fig. 5C).

### *3.6. Luteolin potentiates IL-1β-mediated NF-κB p65 translocation to the nucleus*

In order to further investigate the role of NF-κB p65 and its role in the transcriptional control of COX-2 in the nucleus, we pre-treated U-87 MG cells with or without 15 μM luteolin, stimulated the cells with IL-1β for up to 15 min, and monitored the protein expression of NF-κB p65 and of lamin A/C protein in the nuclear fractions (Fig. 6A, *left panel*). While the expression of lamin A/C remained constant under both control and luteolin-treated conditions, NF-κB p65 expression increased in IL-1β-treated cells, and this effect was potentiated at 10–15 min when the cells were pre-incubated with luteolin (Fig. 6A, *right panel*). Confocal microscopic images of immunofluorescence staining

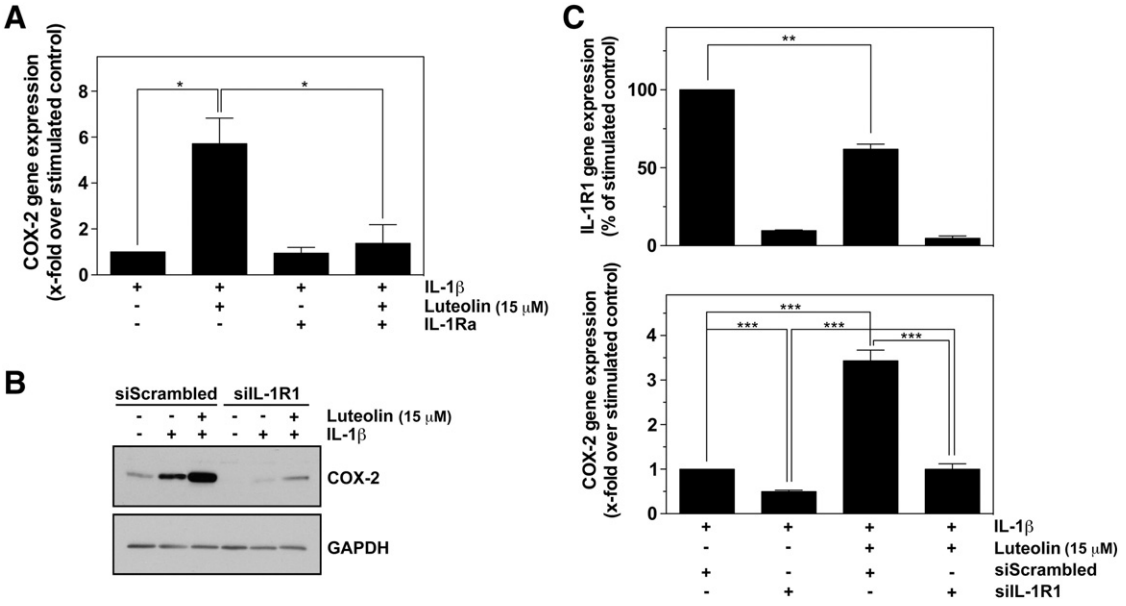


**Fig. 4.** IL-1 receptor antagonist and IL-1 receptor gene silencing affect IL-1β/luteolin-induced COX-2 gene and protein expression. (A) U-87 MG cells were serum-starved in the presence of 50 ng/mL IL-1β containing (or lacking) 15 μM of luteolin or 500 ng/mL IL-1Ra for 24 h. Total RNA was isolated, cDNA synthesis, and qPCR performed to assess COX-2 gene expression. Values are means ± SEM of three independent experiments (*$p < 0.05$ versus IL-1β alone). (B-C) U-87 MG cells were transiently transfected with siRNA against IL-1R (siIL-1R1) or with a scrambled sequence (siScrambled) as described in the Materials and methods section. (B) Representative Western blots are shown for the expression of COX-2, and GAPDH as a loading control. (C) Total RNA was extracted and qRT-PCR was used to assess IL-1R1 and COX-2 gene expression upon treatment with 50 ng/mL IL-1β or 15 μM luteolin for 24 h. Values are means ± SEM of three independent experiments (**$p < 0.01$ and ***$p < 0.001$ versus IL-1β alone).

of NF-κB (Fig. 6B) confirmed the significantly increased nuclear localization of NF-κB in IL-1β/luteolin-treated cells (Fig. 6C). Altogether, this is the first molecular evidence that explains how increased COX-2 expression is triggered by low concentrations of luteolin in the presence of IL-1β.

## 4. Discussion

Our current study sheds further light on the anti-inflammatory and potential chemopreventive properties of luteolin [24]. We document for the first time that, within a pathological inflammatory environment such as that mimicked by the action of PMA or IL-1β, luteolin concentrations higher than 15 μM can exert significant inhibition of IL-1β-induced COX-2 expression in U-87 glioblastoma cells. This observation is relevant to the physiological plasma level of 10–20 μM [32,33] and of 14 μM after oral gavage with 50 μmol/kg of luteolin administration in rats [34].

Chemopreventive assessment of luteolin has also established this class of diet-derived molecule as a potent signal transduction inhibitor [35], and this is supported by our current data which demonstrate that luteolin inhibited IL-1β-induced phosphorylation of JNK, ERK, IκB, and NF-κB. Interestingly, the $IC_{50}$ values for luteolin inhibition against IL-1β signaling ranged from 0.7–11.1 μM for the inhibition of ERK and JNK pathways, while $IC_{50}$ values ranged from 18–21 μM for IκB and NF-κB signaling. Our study is therefore the first to document the differential effect of luteolin at sub-physiological concentrations on MAPK versus NF-κB signaling pathways triggered by IL-1β. Intriguingly, we also observed biphasic effects of luteolin on IL-1β-induced COX-2 expression whereas this effect was not observed in the inhibition of pAKT, survivin, or caspase-3 cleavage. More specifically, low concentrations of luteolin (≤15 μM) clearly potentiated IL-1β-induction of COX-2 expression, while higher concentrations (>15 μM) of luteolin exerted anti-IL-1β effects. The synergistic IL-1β/luteolin effects at low luteolin concentrations could be consequent to a rapid (less than 10 min) increase in NF-κB p65 translocation processes from the cytosol to the nucleus which leads to elevated COX-2 mRNA levels. This is correlated with the induction of the phosphorylation of NF-κB p65 by IL-1β/luteolin observed in our study indicating an accumulation of the p65 subunit. Since qRT-PCR analysis of the p50 and p65 genes confirmed their presence in U-87 MG cells, it is possible that not only p65/p50 heterodimers were localized in the cytoplasm, but also p65/p65 homodimers [36]. Western blotting analysis of the expression of these subunits showed also that the gene silencing of NF-κB p65, but not of NF-κB p50, abrogated IL-1β-mediated induction of COX-2 expression. These results indicate that p50 is not involved in COX-2 upregulation by IL-1β in U-87 MG cells. Gu et al. [37] reported differential alterations of NF-κB to oxidative stress in basal forebrain primary cultures and that NF-κB activation appeared to mostly affect p65 translocation to the nucleus but not that of the p50 subunit. They observed that there was a distinctive translocation of NF-κB p65 subunits in response to extrinsic hydrogen peroxide, nerve growth factor or TNF-α stimuli, without any corresponding changes in the distribution of p50. Consistent with these data, Liu et al. [38] reported that PIAS1 (protein inhibitor of activated STAT1), an important negative regulator of NF-κB, interacted with p65 but not p50 subunit of NF-κB. The synergistic IL-1β/luteolin effects at low luteolin concentrations could also be explained by the requirement of IL-1R-independent mechanisms in the action of luteolin. Since IL-1R1 gene silencing completely abrogated IL-1β-induced COX-2, while luteolin was still able to trigger some COX-2 protein expression although to a reduced level in IL-1R-depleted cells. This conclusion is finally supported by our own data where >90% gene silencing of IL-1R1 at best reduced by only ~50% IL-1β-mediated COX-2 transcriptional regulation. More importantly, luteolin was able to further reduce COX-2 transcript levels to >70% through either the residual gene expression of IL-1R1 upon its silencing or through IL-1R1-independent mechanisms. Interestingly, effects of IL-1Ra independent of the activation of IL-1R1 have been demonstrated and reported elsewhere [39]. Although speculative, such mechanisms may involve IL-1Ra binding to recently identified members of the IL-1R family, which include TIR8/SIGIRR, IL-1RAcPb, TIGIRR-1 and IL-1RAPL [40].

Interestingly, synergistic induction of GRP78, which plays an essential role in the progression and therapeutic resistance of many cancers [41], was found to be a cellular response to stress and was modulated at luteolin concentrations between 10–20 μM. This increase in GRP78

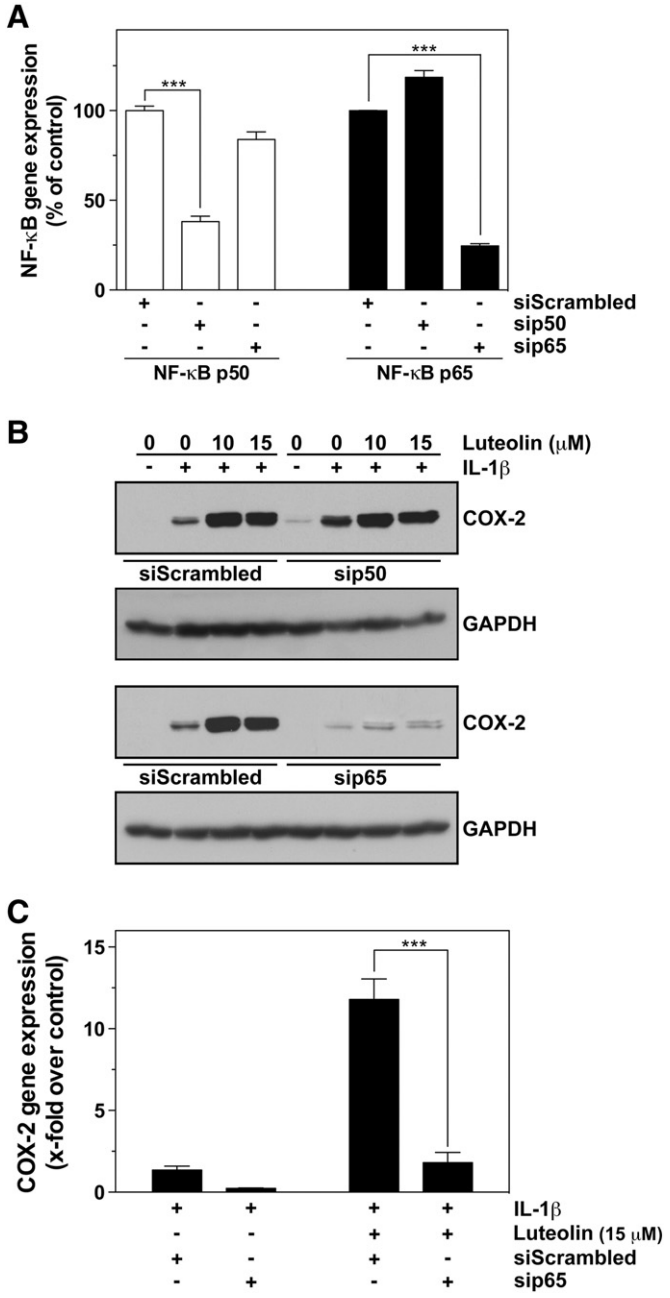


**Fig. 5.** NF-κB p65, but not NF-κB p50, gene silencing reverses the effect of luteolin on IL-1β-mediated induction of COX-2 expression. U-87 MG cells were transiently transfected with siRNA against NF-κB p50 (sip50), NF-κB p65 (sip65) or with a scrambled sequence (siScrambled) as described in the Materials and methods section. (A) Total RNA was extracted and qRT-PCR was used to assess NF-κB p50 and NF-κB p65 gene expression in the transfected U-87 MG cells. Values are means ± SEM of three independent experiments (***$p < 0.001$ versus IL-1β alone). (B) Representative Western blots are shown for the expression of COX-2 with GAPDH as a loading control. (C) Total RNA was extracted and qRT-PCR was used to assess COX-2 gene expression in the siScrambled and sip65-transfected U-87 MG cells upon treatment with 50 ng/mL IL-1β or 15 μM luteolin for 24 h. Values are means ± SEM of three independent experiments (***$p < 0.001$ versus IL-1β alone).

appeared to correlate with lowered survivin expression. These events suggest that low luteolin concentrations are efficient in triggering endoplasmic reticulum (ER) stress in inflammatory settings. Similarly, enhanced ER stress was recently shown to restore GRP78 trafficking to the cell surface, thereby sensitizing cancer cells to apoptosis [42]. At higher concentrations of luteolin, the increase in GRP78 protein was diminished in a concentration-dependent manner and this was correlated with an increase in caspase-3 protein cleavage. In line with this, our data also show that IL-1β alone was inefficient at cleaving and inducing caspase-3, while cleavage and activation of caspase-3 and cell death were significantly potentiated in the presence of luteolin in a concentration-dependent manner (15–50 μM). The fact that luteolin inhibited AKT phosphorylation in both basal conditions and with IL-1β treatment is consistent with a previous report indicating that the inhibition of constitutive AKT activation induced apoptosis in various human pancreatic carcinoma cell lines [43].

We showed that luteolin preferentially inhibited the MAPK pathway, with a lower $IC_{50}$ value than seen with the NF-κB pathway. This differential inhibitory effect could be explained by the involvement of each pathway in the cellular stress response [44,45]. It was reported that signal transduction from the ER to the cell nucleus could be mediated by a signaling cascade similar to the plasma membrane-initiated cell signaling such as MAPK [46,47]. Members of the MAPK family phosphorylate a number of transcription factors with subsequent induction of inflammatory gene expression such as COX-2 [30]. It may be envisioned that the MAPK pathway is involved in the early adaptive cellular stress response during which COX-2 gene and protein expressions were significantly increased, and that simultaneously activates luteolin-mediated NF-κB nuclear translocation, which is required to trigger COX-2 expression. Consequent increases in prostaglandin E2 may, thereafter, contribute to important paracrine biological activities related to the production of immune-regulatory cytokines that allow cancer cells to try to initially escape the immune system [48] or to inhibit tumor cell apoptosis [49]. Since multiple levels of cross-talk exist between mitogenic Ras/MAPK and survival PI3K/AKT pathways [50], both pathways can therefore activate or inhibit each other. It is noteworthy that MAPK pathway inhibition by low concentrations of luteolin could prevent the induction of escape mechanisms to survival pathways (PI3K/AKT) inhibition, and consequently, sustain the downregulation of expression of survivin and AKT phosphorylation, and initiate the cellular apoptotic machinery. The fact that MAPK synthetic inhibitors affected also these proteins in our experimental conditions supports the implication of ERK and JNK pathways in survival of U-87 MG cells.

The synergistic and biphasic inductions of COX-2 and GRP78 observed in our study seem to be performed through a possible common IL-1β/luteolin responsive mechanism. Such behavior is not unique to luteolin, since Young et al. [51] also reported a biphasic effect for the polyacetylene falcarinol, isolated from carrots, which induced proliferative and apoptotic characteristics in a human colon carcinoma cell line at low (<20 μM) and high (>10 μM) concentrations, respectively. Moreover, phytochemicals such as luteolin, can also function either as antioxidant or as pro-oxidant molecules [24]. Oxidative stress has been involved in various neurological disorders and in the central nervous system (CNS) [52] and there is a positive association between the presence of glioblastoma multiforme and oxidative stress [53]. It can therefore be envisioned that the chemopreventive properties of luteolin, specifically within inflammatory and oxidative stress settings such as those triggered by IL-1β (this study, [54]), take place at low concentrations due to its ability to induce a state of oxidative stress as reflected by subsequent induction of COX-2 and GRP78 expressions. Such mechanisms were previously documented where chemopreventive agents induced COX-2 in human colon and pancreatic cancer cell lines [55]. Induction of oxidative stress by chemopreventive agents in cancer cells was suggested to lead to COX-2 overexpression and to COX-2-independent cell death [55]. The increased COX-2 expression observed in our study is also in good agreement with a previous report indicating a similar COX-2 response by flavonoids to lipopolysaccharide treatment, a condition also known to trigger inflammation in enterocytes [56]. Consistent with our data, they also reported a downregulation of COX-2 under conditions of high stress, indicating differential effects of flavonoids in inflammatory settings. Therefore, understanding whether and how luteolin's intracellular redox state regulation activity is involved in its cellular effects will be essential to evaluating its overall potential as an anticancer agent. Because luteolin exhibits slow

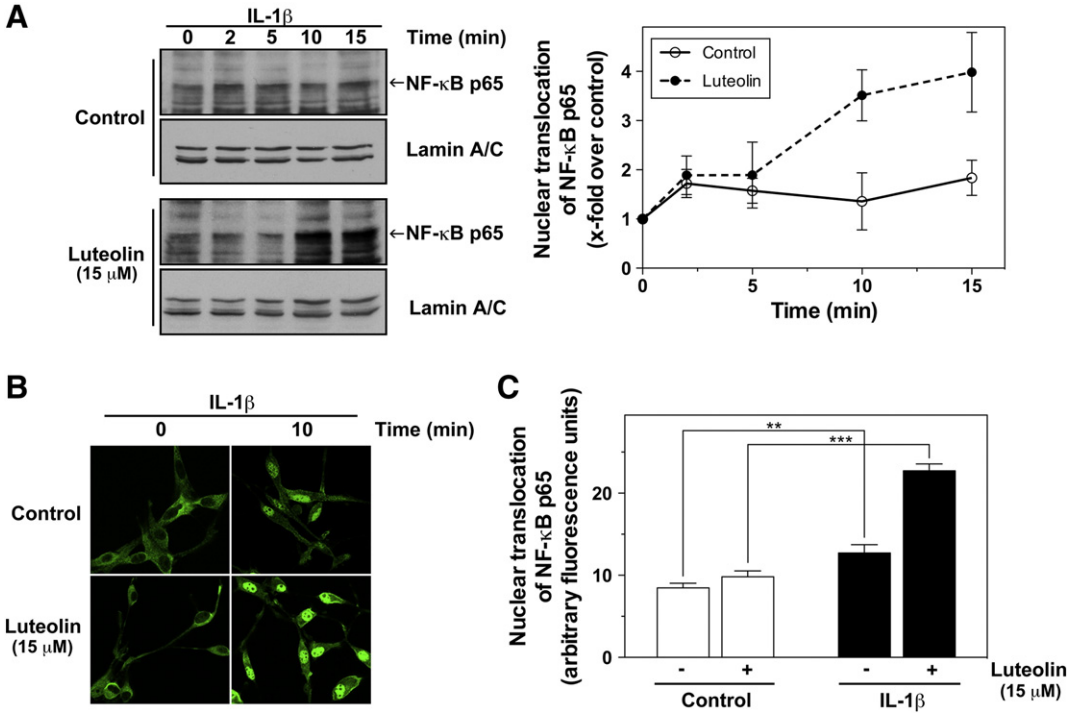


**Fig. 6.** Effect of luteolin on IL-1β-induced NF-κB translocation to the nucleus. (A) U-87 MG cells were serum-starved in the presence (or absence) of 15 μM luteolin for 24 h, followed with 50 ng/mL IL-1β stimulation for the indicated time. Nuclear cell fractions were prepared and submitted to Western blot analysis using antibodies against NF-κB p65, lamin A/C as a loading control. Representative Western blots from at least three independent experiments are shown (*left panel*) and were quantified (*right panel*). (B) U-87 MG cells were seeded on coverslips for immunofluorescence and serum-starved in the presence (or absence) of 15 μM luteolin for 24 h. Then cells were stimulated with 50 ng/mL IL-1β for 10 min. The coverslips were fixed and incubated with anti-NF-κB p65 antibody. NF-κB p65-stained cells were then visualized using a fluorescence microscope. Representative images from two independent experiments are shown. (C) NF-κB p65 localized in the nucleus was quantified. Values are means ± SEM of two independent experiments ($^{**}p < 0.01$ and $^{***}p < 0.001$).

pharmacokinetics, which may enable accumulation of this molecule in tissues [57], it is thus tempting to speculate that low concentrations of luteolin are as beneficial as higher concentrations and may provide a "metronomic-chemoprevention"-like inhibitory effect on COX-2 for preventing cancer development.

Clinically, increased plasma levels of surrogate markers of IL-1β-mediated CNS inflammation were found to correlate with fatigue, sleep disturbance, and cognitive difficulties in cancer patients exposed to cytotoxic chemotherapeutic agents [58]. Interestingly, preclinical work suggests that IL-1β-mediated CNS inflammation may cause these cancer treatment-related symptoms by altering hypothalamic and hippocampal functioning, which implies that luteolin-based pharmacologic targeting will require access to the brain through the vascular endothelium compartment. Evidence suggests that flavonoids generally can indeed penetrate the blood–brain barrier (BBB) [59–62]. It was recently reported that peripherally administered luteolin freely penetrated the BBB and entered the brain in a mouse model of Alzheimer's disease [63]. Thus, it is reasonable to predict that circulating luteolin may have access to the microglial cell compartment and that, although speculative, proper consumption of phytochemicals such as luteolin may help reduce the IL-1β-mediated inflammatory processes and prevent these symptoms from occurring in cancer patients.

The fact that only 5–10% of all cancer cases are due to genetic defects and that the remaining 90–95% are due to environment and lifestyle clearly provides opportunities for preventing cancer [64]. Overall, our results provide evidence of the pleiotropic actions of luteolin as a signal transduction inhibitor, an anti-inflammatory, and proapoptotic agent, and shed light on new mechanisms supporting its physiological benefits. Consequently, our findings could help in the promotion of new diet strategies to prevent brain tumor-associated neuroinflammation.

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