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Multiple defects in negative regulation of the PKB/Akt pathway sensitise human cancer cells to the antiproliferative effect of non-steroidal anti-inflammatory drugs

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ABSTRACT

Antitumorigenic effects of non-steroidal anti-inflammatory drugs (NSAIDs) are well established in several types of cancer disease. However, the mechanisms driving these processes are not understood in all details. In our study, we observed significant differences in sensitivity of cancer epithelial cell lines to COX-independent antiproliferative effects of NSAIDs. The prostate cancer cell line LNCaP, lacking both critical enzymes in the negative control of PKB/Akt activation, PTEN and SHIP2, was the most sensitive to these effects, as assessed by analysing the cell cycle profile and expression of cell cycle regulating proteins. We found that p53 protein and its signalling pathway is not involved in early antiproliferative action of the selected NSAID—indomethacin. RNAi provided evidence for the involvement of $p21^{Cip1/Waf1}$, but not GDF-15, in antiproliferative effects of indomethacin in LNCaP cells. Interestingly, we also found that indomethacin activated PKB/Akt and induced nuclear localisation of $p21^{Cip1/Waf1}$ and Akt2 isoform. Our results are in agreement with other studies and suggest that maintaining of the $p21^{Cip1/Waf1}$ level and its intracellular localisation might be influenced by Akt2. Knock-down of SHIP2 by RNAi in PTEN negative prostate and colon cancer cell lines resulted in higher sensitivity to antiproliferative effects of indomethacin. Our data suggest novel mechanisms of NSAIDs antiproliferative action in cancer epithelial cells, which depends on the status of negative regulation of the PKB/Akt pathway and the isoform-specific action of Akt2. Thus, unexpectedly, multiple defects in negative regulation of the PKB/Akt pathway may contribute to increased sensitivity to chemopreventive effects of these widely used drugs.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) represent commonly used drugs for the treatment of pain and inflammation. Interestingly, effective antiproliferative, proapoptotic and anticancer actions of long-term usage of both selective cyclooxygenase-2 (COX-2) inhibitors and non-specific NSAIDs were established by numerous population studies for several types of cancer including colon and prostate (for review see [1]). Most NSAIDs block the activity of cyclooxygenase-1 (COX-1), COX-2 or both, and it is believed that dominant effects of NSAIDs result from inhibition of biosynthesis of their products—prostaglandins. However, it was demonstrated that the chemopreventive effects of NSAIDs also include COX-independent actions (for review see [2]). COX-independent effects of NSAIDs are mediated through modulation of activity of various intracellular kinases, e.g. ERK, JNK, p38 MAPK, Akt [3–6], which can lead to a change in expression and activity of certain transcription factors (e.g. NF-κB, AP-1, Egr-1, p53, PPARγ) [7–10]. The described variety of signalling pathways affected by NSAIDs suggests that the particular mechanism of COX-

Abbreviations: Cdk2, cyclin-dependent kinase-2; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; Egr-1, early growth response-1; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDF-15, growth/differentiation factor-15; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; NSAIDs, non-steroidal anti-inflammatory drugs; PDK1, phosphoinositide-dependent kinase-1; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol-3-kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate; PKB, protein kinase B; POLR2A, polymerase (RNA) II (DNA directed) polypeptide A; PTEN, phosphatase and tensin homolog; SHIP, SH2-containing 5′-inositol phosphatase.

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independent effects of NSAIDs might depend on the drug and type of cells used.

The growth/differentiation factor-15 (GDF-15), a transforming growth factor-β superfamily cytokine, has been shown to be one of the important downstream target proteins of Egr-1 and p53 transcription factors activated by NSAIDs [9]. Nevertheless, the early induction of expression of this cytokine is not unique just for NSAIDs action, and it has been observed after the treatment with a variety of drugs [11]. Moreover, the role of GDF-15 in the NSAIDs action has not been fully revealed yet.

As mentioned above, one of the described prostaglandin-independent mechanisms of NSAIDs action is modulation of the phosphatidylinositol-3-kinase/PKB/Akt (PI3K/PKB/Akt) pathway. This signalling pathway drives cell proliferation, motility and survival, but also regulates important metabolic processes such as glycogen synthesis [12,13]. All these processes are well-known targets for cancer chemoprevention and chemotherapy [14]. PI3K mediated generation of phosphatidylinositol-3,4,5-triphosphate (PIP3), an important second messenger and activator of PDK1 and Akt, can be buffered by phosphatase and tensin homolog (PTEN). However, PTEN is not the exclusive phosphatase reducing PIP3 pool. The SHIP phosphatases SHIP1 and SHIP2 dephosphorylate PIP3 to phosphatidylinositol-3,4-bisphosphate (PI(3,4)P2), differently from PTEN, which degrades PIP3 to phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) [15]. Loss of PTEN expression leads to the accumulation of PIP3 and constitutive activation of Akt, which is associated with progression of various types of cancer diseases [12]. SHIP expression is crucial for negative regulation of insulin-activated mitogenic signalling pathways [16]. Interestingly, disruption of the SHIP gene leads to the myeloproliferative syndrome [17] and significantly increases the number of haemopoietic stem cells presented in bone marrow [18]. It has been shown that NSAIDs can induce activation of PTEN and other phosphatases [19], or inhibit Akt kinase activity [20,21].

Although PI3K/PKB/Akt activity is often deregulated in various types of cancer, very little is known about how it affects the responsiveness of cancer cells to NSAIDs. Therefore, in the present study we have attempted to investigate the role of defects in PTEN and SHIP2 expression in the sensitivity of cells to the antiproliferative effects of NSAIDs. Elucidation of the role of PI3K/PKB/Akt signalling pathway in this process can contribute to our understanding of the molecular events determining the results of non-selective NSAIDs action and might be helpful in finding more effective usages of NSAIDs as chemopreventive drugs.

2. Material and methods

2.1. Reagents

Indomethacin (CAS# 53-86-1, Sigma–Aldrich Corp.; St. Louis, MO, USA), piroxicam (CAS# 36322-90-4, Sigma–Aldrich Corp.; St. Louis, MO, USA), and diclofenac (CAS# 15307-86-5, ICN Biochemicals, Inc.; Aurora, OH, USA) were diluted in 96% ethanol. Sulindac sulphide (CAS# 32004-67-4, Sigma–Aldrich Corp.; St. Louis, MO, USA), prostaglandin E_2 (PGE_2) (CAS# 363-24-6, Cayman Chemical Company; Ann Arbor, MI, USA) and the phosphatidylinositol 3-kinase (PI3K) inhibitor—wortmannin (# 350-020, AXXORA; San Diego, CA, USA) were dissolved in DMSO to 0.2 mM concentration and stored in aliquots.

2.2. Cell culture

LNCaP and PC3 cells were obtained from the American Type Culture Collection (ATCC). HCT-116 $PTEN^{+/+}$ and $PTEN^{-/-}$ cells [22] were kindly provided by T. Waldman (Georgetown University School of Medicine, Washington). LNCaP, PC3 and HCT-116 cells were cultivated in RPMI-1640, Ham's F12 or McCoy's media respectively (Gibco™ Invitrogen Corporation; Carlsbad, CA, USA) with 2 mM L-glutamine, streptomycin (0.1 mg/ml), penicillin (100 U/ml), and supplemented with 10% fetal bovine serum (PAA, Pasching, Austria). All cell lines were cultivated in Nunc (Thermo Fisher Scientific; Roskilde, Denmark) and/or TPP (Trasadingen, Switzerland) cultivation dishes, flasks and plates in a humidified incubator at 37 °C in an atmosphere of 5% CO_2.

2.3. Cell cycle analysis

The cells were fixed in 70% ethanol and stored at 4 °C before analysis. DNA was stained by incubation (37 °C, 30 min) with Vindelov's solution (10 mM Tris–HCl, pH 8; 0.7 mg/ml RNase; 50 μg/ml propidium iodide (both Sigma–Aldrich Corp.; St. Louis, MO, USA); 0.1% Triton X-100; 10 mM NaCl). The DNA content was analysed using flow cytometry (FACSCalibur, Becton Dickinson; San Jose, CA, USA, 488 nm argon laser for excitation). More than 1.5×10^4 cells were acquired per sample, and percentages of cells in the individual cell cycle phases were analysed using ModFit 3.0 software (Verity Software House; Topsham, CA, USA). Single cells were identified and gated by pulse-code processing of the area and the width of the fluorescence signal. Cell debris was excluded from analysis.

2.4. Preparation of nuclear and cytosolic fractions

The cells were washed with ice-cold PBS, scraped to extraction buffer (Tris 10 mM, KCl 60 mM, EDTA 1.2 mM, (D,L)-1,4-dithiothreitol [DTT] 1 mM; pH 8.0), with 0.1% Nonidet P-40 (NP-40) and protease inhibitor cocktail (P2714, Sigma–Aldrich Corp.; St. Louis, MO, USA), incubated for 10 min on ice, and centrifuged (750 × *g*, 5 min, 4 °C). Supernatants – cytosolic fractions – were collected and stored at −80 °C. The pellets were rinsed with extraction buffer without NP-40, centrifuged, and incubated for 25 min in 50 μl of nuclear extraction buffer (Tris 20 mM, NaCl 420 mM, $MgCl_2$ 0.7 mM, EDTA 0.25 mM and glycerol 25%, v/v) on a rotator at 4 °C. After final centrifugation (12 000 × *g*, 10 min, 4 °C), the supernatants were collected and stored at −80 °C.

2.5. Electrophoresis and Western blotting

Subconfluent cells were harvested in RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, protease inhibitor cocktail and phosphatase inhibitor cocktail set II (Merck, Darmstadt, Germany). Protein concentration was determined using detergent-compatible protein assay (Bio-Rad Laboratories, Inc.; Hercules, CA, USA). The cell lysates were sonicated (5 s, Sonifier® B-12, Branson Ultrasonics Corp.; Danbury, CT, USA), spun and mixed with 3× SDS loading buffer (240 mM Tris–HCl pH 6.8, 6% SDS, 0.02% bromphenol blue, 30% glycerol, 3% β-mercaptoethanol). Equivalent quantities of protein (20 μg) were separated by SDS-PAGE electrophoresis and transferred onto PVDF membranes (Millipore Corp.; Bedford, MA, USA) using established procedures. The membranes were blocked in TBS (20 mM Tris–HCl pH 7.2, 140 mM NaCl) containing 0.1% Tween 20 and 5% non-fat milk. Primary antibodies against Akt1 (sc-1618-R), Akt1/2/3 (sc-8312), Akt2 (sc-5270), Cdk2 (sc-163), GAPDH (sc-20358), lamin B (sc-6217), MDM2 (sc-965), $p21^{Cip1/Waf1}$ (sc-397), p53 (sc-126), PTEN (sc-7974) and SHIP2 (sc-14502) were obtained from Santa Cruz (Santa Cruz, CA, USA); phospho-Akt1/2/3 (S473) (#9271), Bax (#2772) and phospho-Rb (S807/S811) (#9308) antibodies were from Cell Signaling (Danvers, MA, USA); GDF-15 antibody (NAG-1/PTGF-β #07-217) was from Millipore Upstate (Billerica, MA, USA); Rb (#554136) was from BD Pharmingen (San Diego, CA, USA); β-actin (A5441), α-tubulin (T9026) and horseradish peroxidase-conjugated anti-

goat IgG (#A4174) were from Sigma–Aldrich Corp. (St. Louis, MO, USA); horseradish peroxidase-conjugated anti-mouse IgG (#NA931) and anti-rabbit IgG (#NA934) were from GE Healthcare (Buckinghamshire, UK). Detection of antibody reactivity was performed using Immobilon Western HRP Substrate (Millipore, Billerica, MA, USA). Densitometric measurements were performed using ImageJ software (NIH, Bethesda, MD) and normalised to the expression of β-actin and/or α-tubulin.

2.6. RNA isolation and real-time RT-PCR

Total RNA was isolated using a High Pure RNA Isolation Kit (Roche Applied Science, Prague, Czech Republic), according to the manufacturer's instructions. The sequences of primers are listed in Table 1. The amplifications of the samples were carried out in a final volume of 20 μl in a reaction mixture containing 10 μl of One Step SYBR RT-PCR buffer, 0.4 μl of TaKaRa Ex Taq HS and 0.4 μl of PrimeScript RT Enzyme Mix (TaKaRa Bio, Japan), 0.8 μl of a solution of primers (VBC Biotech, Vienna, Austria), 0.4 μl of ROX reference dye, 6 μl of water, and 2 μl of RNA sample. The final concentration of each primer was 0.2 μM. The amplifications were run on the RotorGene 6000 with RotorGene Real-Time Analysis Software (Corbett Research; Sydney, Australia). Detections of selected genes were carried out in a final volume of 20 μl in a reaction mixture containing 10 μl of QuantiTect Probe RT-PCR Master Mix, 0.2 μl of QuantiTect RT Mix (Qiagen; Valencia, CA, USA), 2 μl of a solution of primers and probe (Generi-Biotech; Hradec Králové, Czech Republic), 5.8 μl of water, and 2 μl of RNA sample. The final concentration of each primer was 0.4 μM and for the probe it was 0.1 μM. The amplifications were run on the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). All PCR reactions were performed in triplicates and changes in gene expression were calculated using the comparative threshold cycle method with POLR2A as a normalising gene [23].

2.7. Cdk2 kinase assay

The cells were washed twice in ice-cold PBS and scraped on ice to the RIPA buffer with protease and phosphatase inhibitor cocktail. One hundred micrograms of cell extracts was precleared using RIPA-washed G-protein beads (Sigma–Aldrich Corp.; St. Louis, MO, USA). The supernatants were incubated with 5 μl of anti-Cdk2 (sc-163, Santa Cruz) antibody at 4 °C for 1 h. Then, 25 μl of RIPA-washed G-protein beads were added and incubated overnight at 4 °C. The beads were washed four times in RIPA buffer and twice in kinase buffer (50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 10 mM glycerol phosphate). The reaction was initiated by mixing 25 μl of the sample with 25 μl of the kinase buffer solution containing histone H1 (Sigma–Aldrich Corp.; St. Louis, MO, USA) (2.5 μg) and 0.1 μl [^{32}P] ATP (100 μCi/ml, Institute of Isotopes Co., Ltd., Hungary). The mixture was incubated at 37 °C for 30 min. Then, the reaction was stopped by the addition of 50 μl of 2× Laemmli buffer. Each sample was loaded on 10% SDS-polyacrylamide gel and analysed by SDS-PAGE. The gel was fixed in a mixture of 50% methanol, 40% H_2O and 10% acetic acid, dried, and exposed to the film for autoradiography [26].

2.8. Cell transfection and RNA interference

After overnight incubation in Optimem™ medium (Invitrogen; Carlsbad, CA, USA), the cells were transfected with 10–40 nM siRNA duplexes (Santa Cruz) directed against GDF-15 (sc-39798), $p21^{Waf1/Cip1}$ (sc-29427), SHIP2 (sc-39077) and PTEN (sc-44272) expression or scramble siRNA (sc-37007) using X-treme GENE siRNA transfection reagent (Roche Applied Science, Prague, Czech Republic), according to the manufacturer's recommendation. The transfection mix was removed 4–6 h later and the cells were treated for another 24 h with indomethacin in a normal cultivation medium. General efficiency of the transfection protocol for each cell line was analysed using flow cytometry after transfection of cells with Silencer FAM–GAPDH siRNA (#AM4650, Ambion/Applied Biosystems, Austin, TX, USA). In all cell lines used, the number of FAM–GAPDH siRNA positive cells reached at least 30%.

2.9. Immunofluorescence microscopy

The cells were cultured on Lab-Tek multichamber slides (Thermo Fisher Scientific; Roskilde, Denmark) coated with bovine skin gelatin until they were just subconfluent. The cells were fixed in methanol for 5 min at −20 °C, rinsed with PBS, permeabilizied in 0.1% Triton X-100, blocked in PBS containing 5% goat serum (1 h at 25 °C), and labelled with primary antibody (overnight at 4 °C) anti-Akt2 (1:50, H00000208-M03, Abnova, Taipei City, Taiwan). Secondary antibody was Alexa488-conjugated donkey anti-mouse (1 h at 25 °C, 1:2000, Molecular Probes, Eugene, OR, USA). Nuclear counterstaining was performed using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; 0.1 μg/ml, 5 min at 25 °C). The samples were mounted in a Mowiol 4-88/DABCO anti-photobleaching medium. A FluoView™ 5000 Confocal Laser Scanning Microscope was used for fluorescence imaging (Olympus, Japan).

2.10. Statistical analysis

The data were analysed in STATISTICA for Windows (StatSoft, Prague, Czech Republic) using One-way Analysis of Variance followed by the Tukey range test. If the data variances were non-homogeneous, the Kruskal–Wallis one-way analysis was used.

Table 1
Sequences of primers used in quantitative RT-PCR.

Gene	Oligonucleotide	Accession no.	Reference
GDF-15 (growth/differentiation factor-15)	F: 5′-CCCGGGACCCTCAGAGTT-3′, R: 5′-CCGCAGCCTGGTTAGCA-3′	NM_004864	RTPrimerDB ID: 3475 [24]
CDKN1A (cyclin-dependent kinase inhibitor 1A)	F: 5′-CGCTAATGGCGGGCTG-3′, R: 5′-CGGTGACAAAGTCGAAGTTCC-3′	NM_000389, NM_078467	RTPrimerDB ID: 1695 [24]
BAX (Bcl-2 associated X protein)	F: 5′-GATGATTGCCGCCGTGGACA-3′, R: 5′-GCCCCAGTTGAAGTTGCCGT-3′, P: 5′-CCCCCCGAGAGGTCTTTTTCCGAGT-3′	NM_004324	Generi Biotech
MDM2 (Mdm2, transformed 3T3 cell double minute 2, p53 binding protein)	F: 5′-ACCCAAGACAAAGAAGAGAGTG-3′, R: 5′-TAAGATGTCCTGTTTTGCCA-3′, P: 5′-CCCCTTAATGCCATTGAACC TGTTG-3′	NM_002392	Generi Biotech
TP53 (p53) (tumor protein 53)	F: 5′-GAGCACTAAGCGAGCACTG-3′, R: 5′-CTGGGCATCCTTGAGTTCC-3′ P: 5′-CCAACAACACCAGCTCCTCTCCCC-3′	NM_000546	Generi Biotech
POLR2A (polymerase (RNA) II (DNA directed) polypeptide A)	F: 5′-ATCTCTCCTGCCATGACACC-3′, R: 5′-AGACCAGGCAGGGGAGTAAC-3′	NM_000937	[25]

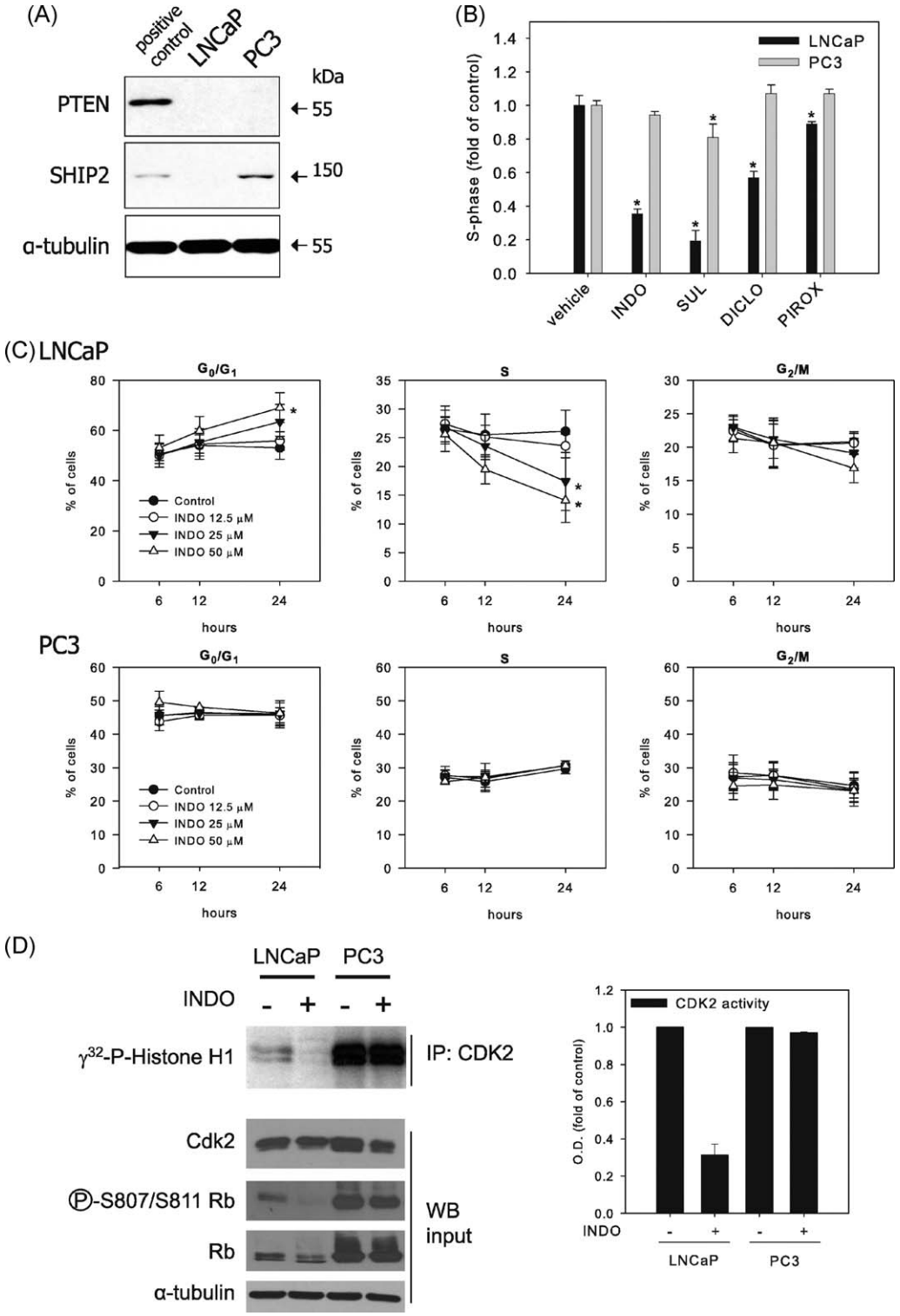

Fig. 1. NSAIDs induce accumulation of cells in the G_0/G_1 phase, inhibition of the S-phase of the cell cycle, inhibition of Cdk2 activity, and inhibition of phosphorylation of Rb protein at S807/S811 in LNCaP but not in PC3 cells. (A) Expression of PTEN and SHIP2 proteins was analysed using Western blot in LNCaP and PC3 cell lines. (B) The cells were treated by 50 μM of indomethacin (INDO), sulindac sulphide (SUL), diclofenac (DICLO), and piroxicam (PIROX) for 24 h. The percentage of cells in the S-phase of the cell cycle was analysed using flow cytometry. (C) Cell cycle analysis was performed in LNCaP and PC3 cells after the treatment by vehicle or various concentrations of indomethacin (12.5–50 μM) for 6, 12, and 24 h. The bars and points represent means ± standard deviations (S.D.) of at least three independent experiments. The symbol "*" denotes significant difference ($p < 0.05$) between vehicle-treated control and indomethacin-treated cells. (D) Cdk2 protein was immunoprecipitated using anti-Cdk2 antibody from PC3 and LNCaP cells treated by 50 μM of indomethacin for 24 h. The activity of Cdk2 was determined using kinase assay as described in Section 2.7. To control Cdk2, Rb and phospho-Rb (S807/811) protein expressions, SDS-PAGE and Western blot were performed. The bar graph represents the average optical density ± S.D. of phosphohistone H1 levels normalised to the untreated control of each particular cell line.

3. Results

3.1. Antiproliferative effects of NSAIDs in prostate cancer epithelial cells

It has been shown that prostate cancer cell lines display distinct expression of negative regulators of PKB/Akt activity, PTEN and SHIP2 proteins, which is reflected in different regulation of Akt kinase activity [27]. Here we show that androgen-sensitive prostate cancer cells – LNCaP – are negative for both PTEN and SHIP2 protein; however, androgen-independent prostate cancer cells – PC3 – are negative for PTEN, but express SHIP2 protein (Fig. 1A). We reasoned that a comparison of the effects of subtoxic doses of NSAIDs in these cell lines would provide information about the role of deregulation of the PKB/Akt pathway in the sensitivity to the antiproliferative effects of NSAIDs. To start addressing this issue, we first compared antiproliferative effects of various NSAIDs—indomethacin, sulindac sulphide, diclofenac, and piroxicam, using cell cycle analysis (Fig. 1B). Our data demonstrate a clear difference between the effects of NSAIDs in PC3 and LNCaP cells. While in LNCaP cells all tested NSAIDs reduced the number of cells in the S-phase of the cell cycle at 24 h, PC3 cells were resistant to the antiproliferative effects of all tested NSAIDs, except for a slight response to the effect of sulindac sulphide in PC3 cells.

For further studies, indomethacin was selected as a representative NSAID due to its chemopreventive, COX-independent effects [28]. We tested the kinetics (6–24 h) of the effect of various concentrations of indomethacin (12.5, 25, and 50 μM) on cell cycle distribution. The trend of the antiproliferative effect of indomethacin was already distinguishable after 12 h of treatment using 25 and 50 μM concentrations in LNCaP cells (Fig. 1C). LNCaP cells were arrested in the G_0/G_1 phase of the cell cycle and the amount of cells in the S-phase was significantly decreased. Again, we were unable to detect any significant changes in cell cycle distribution after indomethacin treatment in PC3 cells. We found that indomethacin induced a significant decrease of Cdk2 activity in LNCaP cells, but not in PC3 cells (Fig. 1D). Inhibition of Cdk2 activity induced by indomethacin was paralleled with a decrease of the

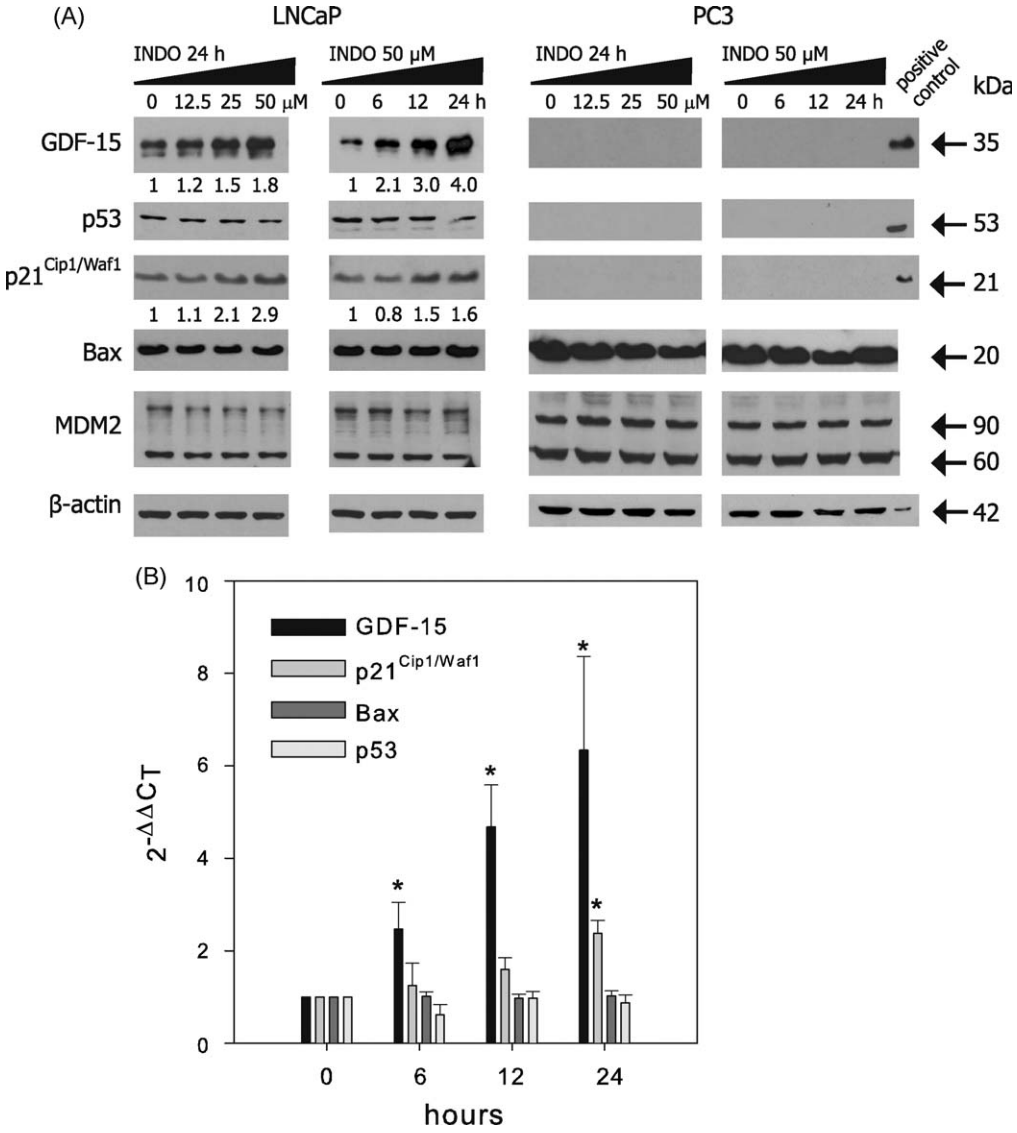

Fig. 2. Indomethacin treatment is associated with the induction of GDF-15 and $p21^{Cip1/Waf1}$ protein in LNCaP but not in PC3 cells. (A) The cells were treated by vehicle or different concentrations of indomethacin (12.5, 25, 50 μM) for 24 h, or by 50 μM of indomethacin for different time intervals (6–24 h). The harvested cells were extracted in RIPA buffer and SDS-PAGE followed by immunoblotting detection of GDF-15, p53, $p21^{Cip1/Waf1}$, Bax, MDM2, and β-actin was performed as described in Section 2.5. The results represent an example of three separate experiments. (B) qRT-PCR for detection of GDF-15, p21, Bax, and p53 mRNA levels was performed in samples prepared from LNCaP cells treated by 50 μM indomethacin for 0–24 h. The data were normalised to the POLR2A housekeeping gene expression. The results represent means ± S.D. of three independent experiments. The symbol "*" denotes statistically significant difference between vehicle-treated control and indomethacin-treated samples ($p < 0.05$).

phosphorylation level of Rb protein at Ser 807/811 (Fig. 1D). Taken together, these data showed that LNCaP cells that are negative for both PTEN and SHIP2 display a higher sensitivity to the antiproliferative effects of NSAIDs.

3.2. Effects of indomethacin on expression of p53 target genes

It has been previously shown that NSAIDs may exert their effects by mechanisms other than inhibition of prostaglandin synthesis. Thus, the proliferation-associated effects of NSAIDs observed here prompted us to analyse concentration-dependent (12.5–50 μM) and time-dependent (6–24 h) effects of indomethacin on the expression of p53, GDF-15 (NSAIDs-induced gene-1, Nag-1) and other p53-controlled targets such as $p21^{Cip1/Waf1}$, Bax, and MDM2. Indomethacin increased the expression of both GDF-15 and $p21^{Cip1/Waf1}$ protein in a time- and concentration-dependent manner, but did not affect the expression of p53, Bax and MDM2 proteins in LNCaP cells (p53 wild-type). On the other hand, indomethacin did not affect the expression of any analysed protein in PC3 cells (p53 null) (Fig. 2A). Analysis of mRNA expression in LNCaP cells revealed differences between GDF-15 and $p21^{Cip1/Waf1}$ in the kinetics and intensity of their induction (Fig. 2B). While GDF-15 was significantly induced already after 6 h of treatment, no significant increase of the $p21^{Cip1/Waf1}$ mRNA level was detectable until after 24 h. Expression of p53, Bax, and Mdm2 mRNA was not affected in indomethacin-treated LNCaP cells (Fig. 2B). These data, together with no changes in phosphorylation of p53 at Ser15 and Ser33 (data not shown), suggest that induction of $p21^{Cip1/Waf1}$ and GDF-15 proteins in LNCaP cells is independent of p53 activation.

3.3. Induction of GDF-15 and $p21^{Cip1/Waf1}$ and cell cycle arrest by indomethacin in LNCaP cells are not dependent on inhibition of PGE_2 synthesis

It was demonstrated by several groups that many effects of NSAIDs are not due to the inhibition of the activity of COX(s) [2]. For these reasons our next step was to test if external addition of one of the main COX(s)' pathway metabolite – prostaglandin E_2 (PGE_2) – can abolish effects of indomethacin observed in LNCaP cells. Our data shown in Fig. 3 demonstrate that pretreatment of LNCaP cells with various concentrations of PGE_2 (1, 10, 50 μM) was unable to reverse the indomethacin-induced increase of GDF-15 and $p21^{Cip1/Waf1}$ protein levels (Fig. 3A) or inhibition of the S-phase of the cell cycle (Fig. 3B). These observations support the hypothesis that antiproliferative effects of indomethacin are independent of inhibition of prostaglandin synthesis.

3.4. $p21^{Cip1/Waf1}$ but not GDF-15 plays a functional role in the antiproliferative effects of indomethacin

The cytokine GDF-15 has been postulated as a mediator of COX(s)-independent effects of NSAIDs in several experimental models. $p21^{Cip1/Waf1}$ is a well-known negative regulator of cell cycle progression. To reveal the role of GDF-15 and $p21^{Cip1/Waf1}$ expression in the antiproliferative effects of indomethacin observed in LNCaP cells, we performed RNA interference (RNAi) experiments. LNCaP cells were transfected with scramble siRNA or specific siRNA against GDF-15 and $p21^{Cip1/Waf1}$ followed by indomethacin treatment. Transfection using various concentrations of siRNA specific to GDF-15 led to a partial but significant decrease of its basal and also indomethacin-induced levels (Fig. 4A). However, this decrease of GDF-15 expression had no effect on indomethacin-induced decrease of the number of cells in the S-phase of the cell cycle (Fig. 4B). On the other hand, decreasing the basal and induced level of $p21^{Cip1/Waf1}$ by using various

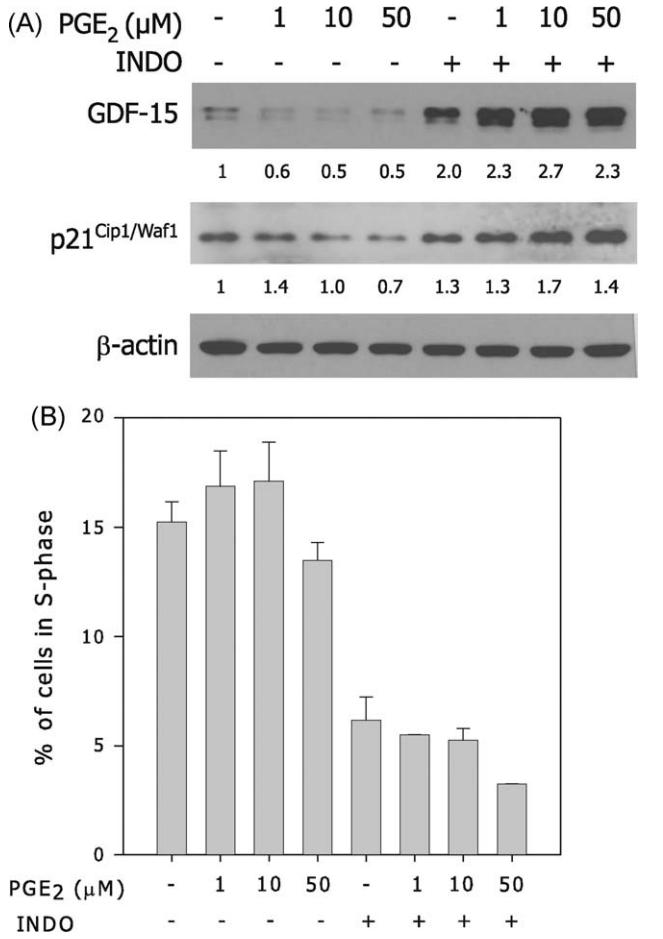

Fig. 3. Indomethacin-induced GDF-15 and $p21^{Cip1/Waf1}$ expression and antiproliferative effect is not dependent on inhibition of prostaglandin E_2 synthesis. LNCaP cells were pretreated with various concentrations of PGE_2 (1, 10, 50 μM) or vehicle (0.05% DMSO) for 1 h followed by treatment with indomethacin (50 μM) for another 24 h. (A) Cells were lysed using RIPA buffer, SDS-PAGE and immunoblotting detection of GDF-15 and $p21^{Cip1/Waf1}$ was performed. The results represent an example of three separate experiments. The numbers below the blots represent normalised optical densities. (B) Numbers of cells in the S-phase of the cell cycle were analysed using flow cytometry. The bars represent means ± S.D. of three separate experiments.

concentrations of specific siRNA (Fig. 4C) significantly reduced the effects of indomethacin and led to an increase of the number of cells in the S-phase of the cell cycle (Fig. 4D). These data demonstrate an involment of $p21^{Cip1/Waf1}$, but not GDF-15, in antiproliferative effects of indomethacin in LNCaP cells.

3.5. Indomethacin treatment leads to activation of Akt kinase

Accumulation of $p21^{Cip1/Waf1}$ protein preceded up-regulation of its mRNA in indomethacin-treated LNCaP cells (Fig. 2A and B). It has been proposed that the $p21^{Cip1/Waf1}$ protein can be stabilised by phosphorylation induced by Akt kinase [29]. Akt can be activated by PI3K induced localisation in lipid membrane followed by phosphorylation at Thr308 by PDK1 and by phosphorylation within the carboxy terminus at Ser473. We tested if the increased level of $p21^{Cip1/Waf1}$ induced by indomethacin was paralleled with activation and/or increased level of Akt. We observed that the time-dependent increase of $p21^{Cip1/Waf1}$ protein expression positively correlated with the induction of Akt phosphorylation at Ser473 (Fig. 5A). The levels of total Akt remained without any significant change. To obtain further evidence that Akt activation induces stabilisation and increased level of $p21^{Cip1/Waf1}$, we used

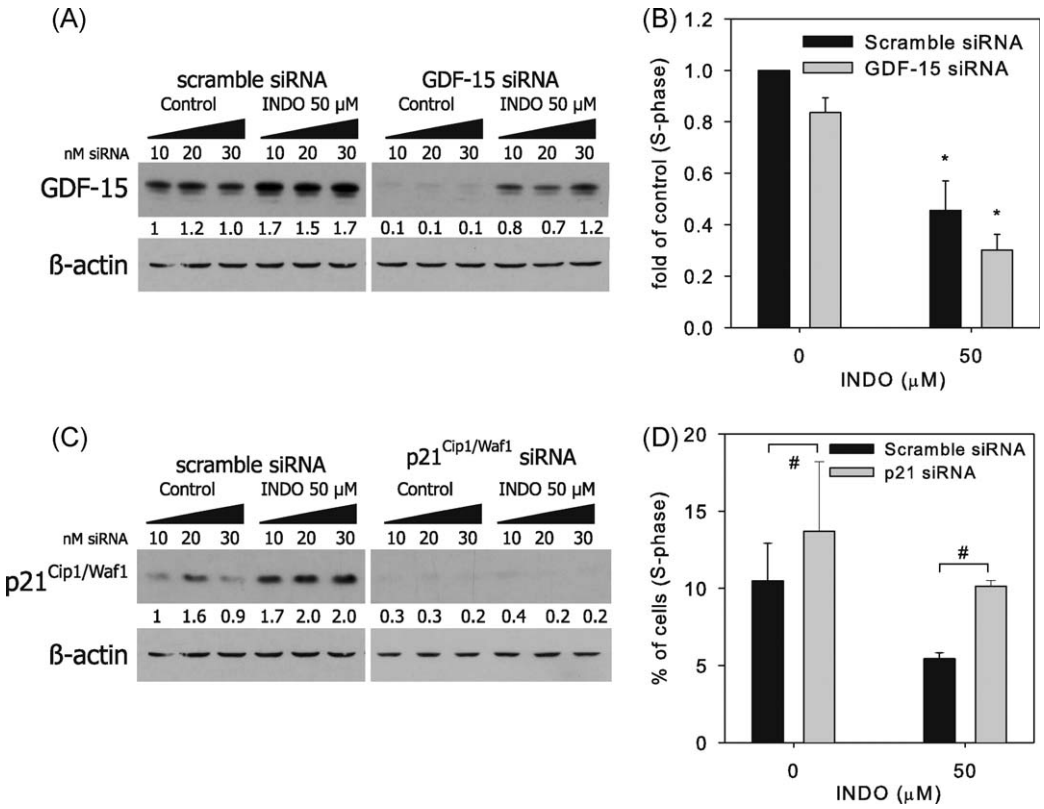

Fig. 4. siRNA-mediated down-regulation of $p21^{Cip1/Waf1}$, but not GDF-15, abolishes indomethacin-induced cell cycle arrest in LNCaP cells. The cells were transfected using either GDF-15 and $p21^{Cip1/Waf1}$ specific or control siRNAs as described in Section 2.8. The transfection mix was removed and the cells were treated with 50 μM of indomethacin for another 24 h. The harvested cells were extracted using RIPA buffer, SDS-PAGE and immunoblotting detections of GDF-15 (A), $p21^{Cip1/Waf1}$ (C) and β-actin (loading control) were performed. The results are representative of three independent experiments. The cell cycle was analysed using flow cytometry on cells transfected by control or specific siRNA for GDF-15 (20 nM) (B) and $p21^{Cip1/Waf1}$ (20 nM) (D). The bars represent mean ± S.D. of three independent experiments. The symbol "*" denotes statistically significant difference between vehicle-treated control and indomethacin-treated samples ($p < 0.05$). The symbol "#" denotes significant difference between the effects of scramble and specific siRNA ($p < 0.05$).

wortmannin, a pharmacological inhibitor of PI3K. As shown in Fig. 5B, both concentrations of wortmannin, 100 and 300 nM, completely inhibited phosphorylation of Akt at Ser473 and induced decrease of the $p21^{Cip1/Waf1}$ protein level in LNCaP cells. When the cells were pretreated with indomethacin followed by wortmannin treatment, the level of $p21^{Cip1/Waf1}$ remained increased. Indomethacin also partially reduced the effects of 100 nM concentration of wortmannin on inhibition of Akt phosphorylation at Ser473. Similar effects were observed using another PI3K inhibitor—LY294002 (data not shown). These data are consistent with previous reports, which show that the level of $p21^{Cip1/Waf1}$ can be controlled by PI3K/PKB/Akt activity [30]. Taken together, our data indicate that indomethacin-induced Akt activation results in an increased level of $p21^{Cip1/Waf1}$ protein and leads to a relatively rapid induction of G_0/G_1 cell cycle arrest and inhibition of the S-phase.

3.6. Indomethacin induces nuclear localisation of Akt2

It has been shown that Akt1 and Akt2 isoforms may play different roles in the regulation of cell proliferation through stabilisation and/or relocalisation of $p21^{Cip1/Waf1}$ [31]. These facts led us to investigate how indomethacin affects intracellular localisation of Akt1 and Akt2 in LNCaP cells. Notably, under indomethacin treatment, the Akt2 expression was increased and predominantly localised in the nuclear fraction; however, most of the Akt1 isoform remained in the cytosol (Fig. 6A). The indomethacin-induced presence of Akt2 in the nuclear fraction was associated with the increased expression of $p21^{Cip1/Waf1}$. Induction of concentration- and time-dependent localisation of Akt2 in the nuclei was also confirmed by immunofluorescence staining (Fig. 6B). To examine a possible dependence between Akt2 expression and nuclear localisation of $p21^{Cip1/Waf1}$, we used RNAi followed by immunofluorescence analysis. Unluckily, we were able to reduce significantly the expression of Akt2 only in the cytosolic fraction but not in the nuclear fraction using RNAi. Therefore we observed only a slight relocalisation of $p21^{Cip1/Waf1}$ from the nucleus to the cytoplasm after silencing of Akt2 expression (data not shown). Silencing of Akt1 had no effect on $p21^{Cip1/Waf1}$ localisation in LNCaP cells (data not shown). Our results are in agreement with other studies and suggest that maintaining the $p21^{Cip1/Waf1}$ level and its intracellular localisation might be influenced by Akt2.

3.7. Reduction of SHIP2 expression leads to sensitisation of cells to the antiproliferative effects of indomethacin

We finally employed RNA interference to determine whether down-regulation of SHIP2 in PTEN deficient cells leads to increased sensitivity of the cells to antiproliferative effects of indomethacin. Due to the fact that the LNCaP cells are negative for both PTEN and SHIP2, the prostate cancer cells PC3 (PTEN negative, SHIP2 positive) and $PTEN^{+/+}$ and $PTEN^{-/-}$ clones derived from HCT-116 colorectal cancer cells (Fig. 7A) were transfected with scramble or SHIP2 specific siRNA. The transfected cells were treated for 24 h with vehicle or indomethacin (50 μM). The efficiency of SHIP2 knock-down was verified using Western blot (Fig. 7B) and the effects on the cell cycle were analysed using flow cytometry

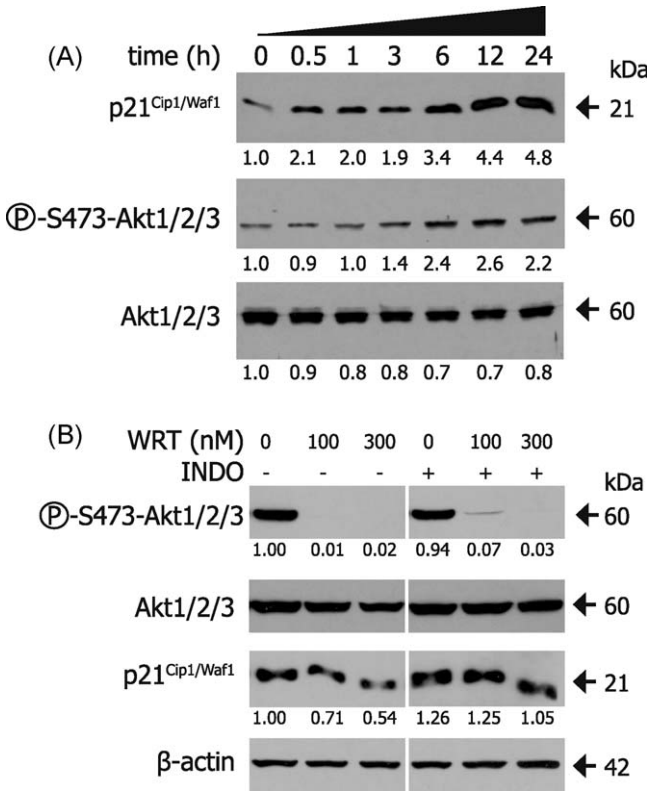

Fig. 5. The indomethacin-induced increased level of $p21^{Cip1/Waf1}$ is paralleled by phosphorylation of Akt1/2/3 at Ser473. Indomethacin abolishes the wortmannin-induced down-regulation of $p21^{Cip1/Waf1}$. (A) LNCaP cells were treated by vehicle or 50 μM of indomethacin for various time intervals (0.5–24 h) and $p21^{Cip1/Waf1}$, total Akt1/2/3, and phospho-Akt1/2/3 (S473) protein levels were analysed using Western blot. (B) LNCaP cells were pretreated with vehicle or indomethacin (50 μM for 24 h) followed by 100 and 300 nM of wortmannin (WRT) treatment for another 2 h. Detections of phospho-Akt1/2/3 (S473), total Akt1/2/3, $p21^{Cip1/Waf1}$, and β-actin (loading control) protein level were performed using Western blot. The results represent an example of three separate experiments. The numbers below the blots represent normalised optical densities.

(Fig. 7C). Interestingly, our results showed that indomethacin induced increase in SHIP2 expression (1.4-fold) in PC3 cells transfected with scramble siRNA. However, transfection of PC3 cells with specific SHIP2 siRNA abolished this indomethacin-induced effect (Fig. 7B). Our cell cycle data demonstrated that indomethacin treatment in combination with the decrease in SHIP2 expression significantly reduced the number of cells in the S-phase of the cell cycle in PC3 and HCT-116 $PTEN^{-/-}$ cells (on average ~40% inhibition). The same trend was also observable in HCT-116 $PTEN^{+/+}$ (~20% decrease), but it was not statistically significant at an estimated value of $p < 0.05$. In order to determine the mechanism of inhibition of the S-phase induced by indomethacin in SHIP2 siRNA transfected cells, we analysed Cdk2 activity and the phosphorylation status of Rb protein in PC3 cells. We found that indomethacin significantly decreased Cdk2 kinase activity and phosphorylation of Rb protein at Ser807 and Ser811 in cells transfected with SHIP2 siRNA, compared to scramble siRNA (Fig. 7D). To prove the role of deregulation of PKB/Akt activity in the effect of indomethacin from the other side, we used DU145 prostate cancer cells (pRb mutant, p53 mutant, $p16^{Ink4}$ mutant), which do not express SHIP2 protein (data not shown), but which express wild-type PTEN. Treatment of DU145 cells with 50 μM of indomethacin for 24 h led to the induction of $p21^{Cip1/Waf1}$ protein (Fig. 7E). Decrease of PTEN expression using specific siRNA was associated with an even higher induction of $p21^{Cip1/Waf1}$ expression (Fig. 7E). Our results proved that disruption of negative regulation of PKB/Akt pathway through down-regulation of both SHIP2 and PTEN sensitised various epithelial cancer cells to the antiproliferative effects of indomethacin.

4. Discussion

Millions of individuals have been taking daily non-specific NSAIDs to control pain and inflammation. Interestingly, not all NSAIDs-induced effects are dependent on inhibition of activity of COX(s) and elimination of synthesis of the crucial mediators of inflammation—prostaglandins. It can be expected that this multi-target effect will probably be beneficial to the chemopreventive effects of NSAIDs [32]. However, it is not fully understood if there exist any specific preconditions which determine the sensitivity of cells to the anticancer actions of NSAIDs. Therefore, in the present study, we have attempted to investigate what role plays the deregulation of the PKB/Akt signalling pathway – often found in various types of cancer – in the sensitivity of cells to the antiproliferative effects of NSAIDs.

NSAIDs have been shown to affect proliferation and induce apoptosis in various cell types in vitro and in vivo [28,33,34]. However, most of these observations are associated with relatively long time intervals of treatment and application of high dosage of the drugs. Interestingly, it has been demonstrated that the serum half-life of several NSAIDs is generally in the range of several hours, particularly for indomethacin in the range from 1 to 16 h [35]. For these reasons we focused on the analysis of NSAIDs' effects within 24 h after treatment and we used concentrations which are accessible in the serum of the patients in case of common oral applications of the drugs.

Our study showed clear differences in the sensitivity of epithelial cancer cell lines to the antiproliferative effects of NSAIDs. Although the cell lines used in this study differed in p53 status, the early effects observed were not dependent on p53 protein and its signalling pathway (Section 3.2). This observation is in agreement with other studies showing that both GDF-15 and $p21^{Cip1/Waf1}$ can be induced independently of p53 function [36,37]. It is known that some effects of NSAIDs are not dependent on inhibition of COX(s) activity (for review see [2]). In accord with other studies, our results in Section 3.3 revealed that early antiproliferative effects of indomethacin in LNCaP cells are not dependent on inhibition of prostaglandin synthesis.

GDF-15, a cytokine belonging to the TGF-β family, has been demonstrated to be an important mediator of proapoptotic antitumorigenic effects of NSAIDs [38]; however, its role in the regulation of cell cycle driving machinery is unknown. On the contrary, cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$ is a well-known regulator of inhibition of proliferation induced by NSAIDs [39]. As shown in Section 3.4, the RNAi experiment pointed to $p21^{Cip1/Waf1}$, but not to GDF-15 as a crucial mediator of early induction of the antiproliferative effect of indomethacin in LNCaP cells. Moreover, our data also showed that the kinetics of induction of $p21^{Cip1/Waf1}$ protein and mRNA after indomethacin treatment do not correlate. While the increase of $p21^{Cip1/Waf1}$ protein is paralleled by induction of G_0/G_1 arrest, the increase of mRNA is delayed compared to the increase in protein level. The results provide evidence supporting the hypothesis that NSAIDs induce early increase of the $p21^{Cip1/Waf1}$ protein level by a mechanism which is not dependent on regulation of gene expression.

Several studies showed that the stability and localisation of $p21^{Cip1/Waf1}$ protein can be regulated by the PKB/Akt signalling pathway [29,31,40]. Interestingly, one of the prostaglandin-independent mechanisms of NSAIDs action could be inhibition of the PI3K/PKB/Akt pathway. It has been shown that higher doses of NSAIDs can induce activation of PTEN and other phosphatases [19], or inhibit Akt kinase activity [20,21]. However, we observed opposite effects of indomethacin in LNCaP cells, since activating

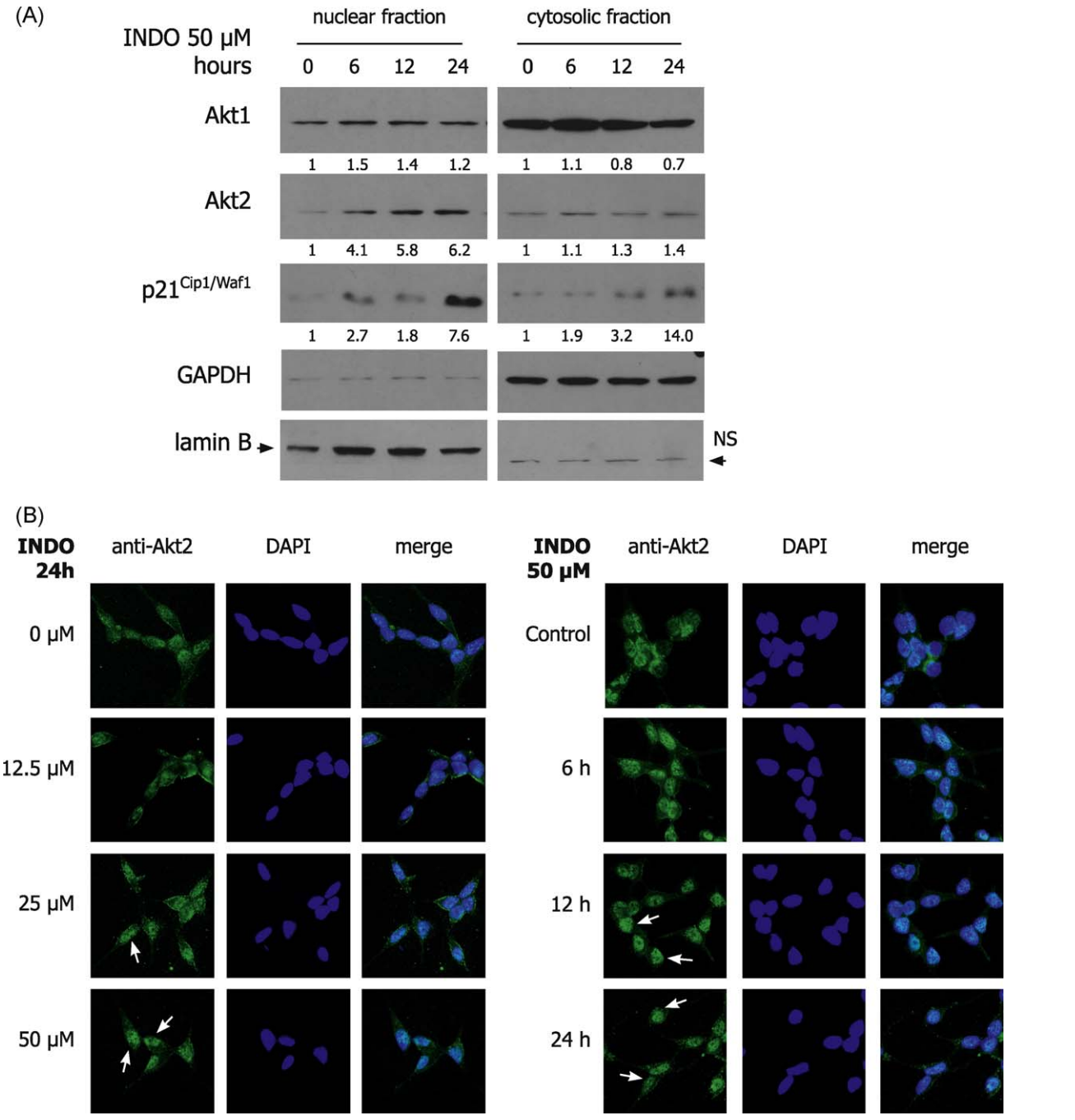

Fig. 6. Localisation of $p21^{Cip1/Waf1}$ and Akt2, but not Akt1, in the nuclear fraction of LNCaP cells is induced by indomethacin. (A) LNCaP cells were treated with 50 μM of indomethacin for various time intervals (6–24 h) and nuclear/cytosolic fractionations were performed as described in Section 2.4. Akt1, Akt2, $p21^{Cip1/Waf1}$, GAPDH (cytosolic fraction loading control), and lamin B (nuclear fraction loading control) protein levels were determined by Western blot. (B) Indirect immunofluorescence and confocal microscopy were used for intracellular visualisation of Akt2 protein. LNCaP cells were grown in Lab-Tek chambers and treated by different concentrations of indomethacin (12.5–50 μM) or vehicle for 24 h (left panel); or the cells were treated by 50 μM indomethacin or vehicle for different time intervals (6–12 h, right panel). White arrows indicate nuclear localisation of Akt2 protein. The results represent an example of three separate experiments.

phosphorylation of Akt at Ser437 was induced time-dependently after the treatment by the drug. Moreover, the effect of the PI3K inhibitor wortmannin on the down-regulation of $p21^{Cip1/Waf1}$ expression was reduced by indomethacin co-treatment. Our results thus paradoxically indicate that indomethacin-induced up-regulation of $p21^{Cip1/Waf1}$ can be a consequence of Akt activation.

In mammals, three Akt isoforms are expressed, Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBδ [41]. Although all the three isoforms share a high degree of similarity and are activated through the PI3K pathway, clear isoform-specific effects on cell cycle regulation were recently demonstrated [31,42]. Akt1 has been found to be essential for G_1/S transition; however, nuclear localisation of Akt2 was associated with an increased level of $p21^{Cip1/Waf1}$ and induction of cell cycle arrest [31]. Our observation that Akt2, but not Akt1, paralleled $p21^{Cip1/Waf1}$ nuclear localisation and cell cycle arrest induced by NSAIDs, is consistent with the suggested specific role of Akt2 in cell cycle exit [31]. Silencing of Akt2 expression in the cytosolic fraction using siRNA led to partial relocalisation of $p21^{Cip1/Waf1}$ from the nuclei of LNCaP cells (data

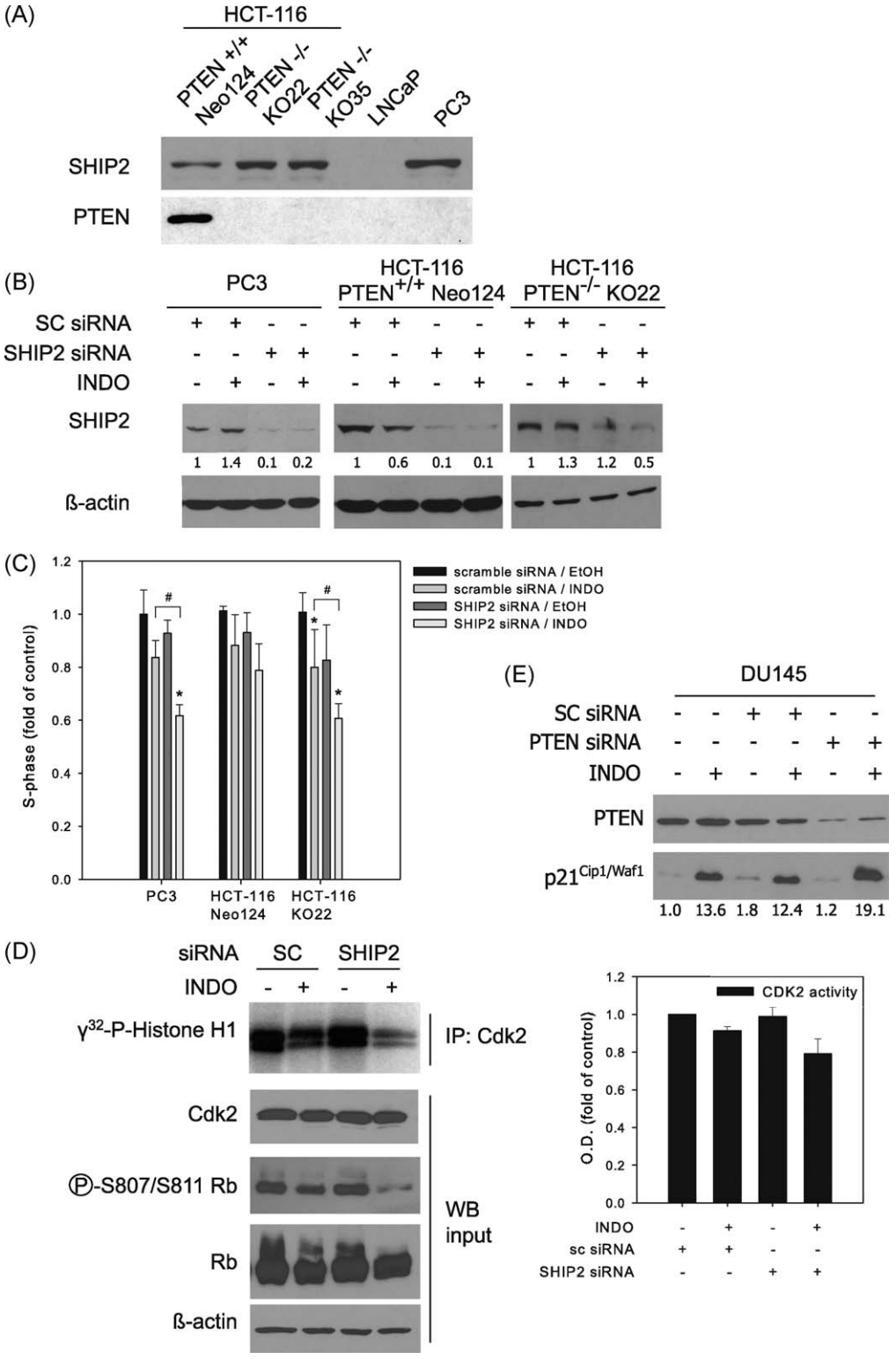

Fig. 7. siRNA specific to SHIP2 sensitises PC3 and HCT-116 PTEN$^{-/-}$ cells to the antiproliferative effect of indomethacin. (A) SHIP2 and PTEN protein levels were analysed using Western blot in HCT-116 PTEN$^{+/+}$, HCT-116 PTEN$^{-/-}$, LNCaP, and PC3 cells in RIPA buffer whole cell extracts. (B) PC3, HCT-116 PTEN$^{+/+}$ and PTEN$^{-/-}$ cells were transfected using either SHIP2 specific or control siRNAs, as described in Section 2.8. The transfection mix was removed and the cells were treated with 50 μM of indomethacin for another 24 h. The harvested cells were extracted using RIPA buffer, SDS-PAGE and immunoblotting detections of SHIP2 and β-actin (loading control) were performed. The results are representative of three independent experiments. (C) The cell cycle was analysed using flow cytometry on the cells transfected by control or specific siRNA for SHIP2. The bars represent mean ± S.D. of three separate experiments. The symbol "*" denotes statistically significant difference between vehicle-treated control and indomethacin-treated samples ($p < 0.05$). The symbol "#" denotes significant difference between the effects of scramble and SHIP2 specific siRNA in indomethacin-treated cells ($p < 0.05$). (D) Cdk2 protein was immunoprecipitated using anti-Cdk2 antibody from PC3 cells transfected either using scramble or SHIP2 specific siRNA and treated by vehicle or 50 μM of indomethacin for another 24 h. Cdk2 activity was determined using kinase assay as described in Section 2.7. Western blot was used to control Cdk2, Rb, and phospho-Rb (S807/811) protein expressions. The bar graph represents the average optical density (O.D.) ± S.D. of phosphohistone levels normalised to the vehicle-treated cells transfected by scramble siRNA. (E) DU145 cells were transfected using either PTEN specific or scramble siRNAs, as described in Section 2.8. The transfection mix was removed and the cells were treated with 50 μM of indomethacin or vehicle for another 24 h. Expression of p21$^{Cip1/Waf1}$ and PTEN was determined using Western blot.

not shown). However, we were unable to reduce significantly the expression of Akt2 using RNA interference in the nuclear fraction (data not shown). These results indicate that Akt2 might be a part of a relatively stable protein complex which can retain the pool of $p21^{Cip1/Waf1}$ in the nucleus. Thus, NSAIDs might modulate the level of the $p21^{Cip1/Waf1}$ pool through regulation of the activity, expression, and localisation of Akt2 in cells with multiple defects in the negative regulation of PKB/Akt activity.

The loss and/or mutation of PTEN tumor suppressor, which leads to the constitutive activation of PKB/Akt, is one of the most frequent phenomena in human cancer [43]. It has been reported that SHIP2 can substitute for PTEN in the negative regulation of PI3K activity [27]. Moreover, SHIP2 phosphatase predominantly influences Akt2 activity, but not Akt1; however, PTEN regulates phosphorylation of both Akt1 and Akt2 [44,45]. In this study we observed that inhibition of SHIP2 expression in PTEN negative cell lines led to sensitisation to the antiproliferative effects of indomethacin. Furthermore, inhibition of PTEN expression in SHIP2 negative DU145 cells was associated with the potentiation of $p21^{Cip1/Waf1}$ induction after indomethacin treatment. These results suggest that multiple defects in negative regulation of PKB/Akt activity associated with isoform-specific deregulation of Akt activity might be a surprising advantage in prostaglandin-independent antiproliferative effects of NSAIDs in cancer cells. At the same time, these results indicate possible pitfalls included in combining NSAIDs with other chemotherapy and/or chemopreventive drugs [46].

Taken together, our data suggest novel mechanisms of NSAIDs antiproliferative action in cancer epithelial cells, which depends on the status of negative regulation of the PKB/Akt pathway and the isoform-specific action of Akt2. Thus, paradoxically, multiple defects in negative regulation of the PKB/Akt pathway may contribute to increased sensitivity to chemopreventive effects of these widely used drugs.

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