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Activation of p53 signalling in acetylsalicylic acid-induced apoptosis in OC2 human oral cancer cells

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Abstract

Background Nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetylsalicylic acid (ASA, aspirin) are well known chemotherapeutic agents of cancers; however, the signalling molecules involved remain unclear. The aim of this study was to investigate the possible existence of a putative p53-dependent pathway underlying the ASA-induced apoptosis in OC2 cells, a human oral cancer cell line.

Materials and methods The methyl tetrazolium (MTT) assay was employed to quantify differences in cell viability. DNA ladder formation on agarose electrophoresis was used as apoptosis assay. The expression levels of several master regulatory molecules controlling various signal pathways were monitored using the immunoblotting techniques. Flow cytometry was used to confirm the effect of ASA on cell cycle. Patterns of changes in expression were scanned and analyzed using the NIH image 1·56 software (NIH, Bethesda, MD, USA). All the data were analyzed by ANOVA.

Results Acetylsalicylic acid reduced cell viability and presence of internucleosomal DNA fragmentation. In the meanwhile, phosphorylation of p53 at serine 15, accumulation of p53 and increased the expression of its downstream target genes, p21 and Bax induced by ASA. The expression of cyclooxygenase-2 was suppressed. Disruption of p53-murine double minute-2 (MDM2) complex formation resulted in increasing the expression of MDM2 60-kDa cleavage fragment. Inhibited the activation of p42/p44 mitogen-activated protein kinase (MAPK) by PD98059, a specific inhibitor of extracellular regulatory kinase (ERK), significantly decreased cell viability and enhanced the expression of p53 induced by ASA. The result of the cell-cycle analysis showed that ASA and PD98059 induced the cell cycle arrested at the G0/G1 phase and resulted in apoptosis.

Conclusion Nonsteroidal anti-inflammatory drug-inhibited cyclooxygenase is not the only or even the most important mechanism of inhibition. Our study presents evidences that activation of p53 signalling involved in apoptosis induced by ASA. Furthermore, the apoptotic effect was enhanced by blocking the activation of p42/p44 MAPK in response to treatment with ASA, thus indicating a negative role for p42/p44 MAPK.

Keywords Acetylsalicylic acid, apoptosis, cell cycle, COX-2, p53, PD98059.
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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA, aspirin), have been used as chemotherapeutic agents of cancers to induce apoptosis or reduce the incidence, to name a few, like colon [1], lung [2], stomach [3] and colorectum [4]. They are known to act by directly suppressing cyclooxygenases 1 and 2 (COX-1 and COX-2), the rate-limiting enzyme catalyzing the biosynthesis of prostaglandins, thereby blocking the production of proinflammatory prostaglandins. The current prevailing theory is that the mechanism for the suppressor effect of NSAIDs on carcinogenesis is attributed mainly to the inhibition

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of COX-2. However, ASA and selective COX-2 inhibitor NS-398 suppressed the proliferation and stimulated apoptosis in the COX-2 negative colon cancer cell line (SW480) [5,6]. It implied a COX-2-independent mechanism of NSIADs. Increased amounts of COX-2 mRNA and protein are commonly found in premalignant and malignant conditions, including head and neck squamous cell carcinoma (HNSCC) [7–10]. Moreover, mutation of p53 is detected in both precancerous and cancerous lesions of the head and neck [11,12]. Subbaramaiah and coworkers indicated that levels of COX-2 protein and mRNA were markedly suppressed by wild-type p53 but not by mutant p53 [13]; indicating that p53 status could possibly be a determinant of COX-2 expression in HNSCC.

It well known that p53 is the most frequently mutated gene in human cancers and its functions have been described, including induction of G1 arrest or apoptosis following DNA damage or other cellular insults, moreover, in the maintenance of genomic stability and inhibition of angiogenesis. When the cell receives an appropriate stress signal, p53 will be phosphorylated and the binding of MDM2 to p53 would be blocked or modified, which results in increasing p53 stability and accumulation. This in turn leads to upregulation of p53-dependent genes required for cell growth arrest ($p21^{WAF1/CIP1}$) and activation of apoptosis (Bax), respectively [14,15].

Extensive studies of the mechanism of NSAID-induced apoptosis have focused on physiopathological changes of cyclooxygenases and their regulatory pathways for decades. Compared with studies of the cyclooxygenase-centred mechanism, our understanding of the p53-dependent apoptotic NSAID-induced pathway is far behind. We demonstrated that activation of p53 signalling may play an important role in cells undergoing aspirin-induced apoptosis.

Materials and methods

Cell culture

Human oral cancer cell line (OC2) [16] was cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, streptomycin (10 000 U mL^{-1}) and penicillin (10 000 U mL^{-1}). The medium was buffered with sodium bicarbonate (2·0 g L^{-1}) as the manufacturer recommended. Before exposing the cell to aspirin, they were washed with PBS and cultured in serum-free medium. Acetylsalicylic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO as a 1-M stock solution and stored at –20 °C. The volume of DMSO was equalized to 0·3% in all culture dishes.

Methyl tetrazolium reduction assay

To examine the cytotoxic effect, cell viability was measured via the methyl tetrazolium (MTT) bromide mitochondrial activity assay described previously [17]. In short, a total of 5000 cells in 100 μL of medium was seeded per well in a 96-well plate for 24 h before treatment with drugs. At the end of incubation, 20 μL of 5 mg mL^{-1} MTT (Sigma) solution in PBS was added to each well for 4 h. After incubation, the medium was discarded and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was determined at 540 nm.

DNA fragmentation analysis

The method of apoptotic DNA fragment analysis developed by Herrmann and colleagues has been described in detail previously [18]. Briefly, cells were harvested, washed and pelleted by centrifugation. The cell pellets were then treated with lysis buffer (1% NP-40, 20 mM of EDTA, in 50 mM of Tris-HCl pH 7·5) for 10 s. After centrifugation, the supernatants were brought to 1% SDS and treated with RNase A (final concentration at 5 μg mL^{-1}, Sigma) at 56 °C for 2 h followed by digestion with proteinase K (final concentration at 2·5 μg mL^{-1}, Sigma) at 37 °C for at least 2 h. Apoptotic DNA fragments were separated by electrophoresis in 2% agarose gels.

Western blot

The expression level of p53, p53-pSer392 (Sigma), p53-pSer15 (Calbiochem, San Diego, CA) and a small set of its regulatory proteins, including COX-2, MDM2, Bax, p21, the active form of caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and MAPKs (Cell Signalling Technology, Beverly, MA), were analyzed by Western blot. The cells were washed twice with cold PBS before being extracted with 2·5 C Triton X-100. Samples were heated at 95 °C for 5 min in Laemmli buffer and then chilled on ice. Subsequently, after electrophoresis (50 μg $lane^{-1}$), the proteins were electro-blotted to ECL nitrocellular membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The proteins of interest were detected with an ECL Western blotting detection reagent (Amersham). Briefly, nonspecific binding on the nitrocellular membrane was blocked with 5% nonfat dry milk in 20 mM of Tris and 150 mM of NaCl before incubating with primary antibodies against specific antigens. After incubation with the conjugated second antibody, the blotted nitrocellular membrane was exposed to X-ray films, and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD). Blots were routinely re-probed with antiactin to ensure equivalence of loading. If necessary, membranes were stripped by incubation at 50 °C for 30 min in a solution of 62·5 mM of Tris-HCl, pH 6·7, 2% SDS and 100 mM of 2-mercaptoethanol.

Flow cytometry

Cells were trypsinized, pelleted, fixed and propiduim iodide (PI)-stained as previous described [6]. After being

trypsinized and collected, the cells were fixed with ice-cold 75% ethanol in PBS, washed twice with PBS and stored at –20 °C. Before analysis, the cells were incubated at 37 °C for 30 min with 5 μL of RNase A (10 mg mL^{-1}, Sigma) and 1 mL of PI (50 μg mL^{-1}, Sigma). The analysis of samples was performed by flow cytometry (Beckman Coulter Ltd, Buckinghamshire, UK). Windows multiple document interface (WinMDI) software was used to calculate the cell-cycle phase distribution from the resultant DNA histogram, and expressed as a percentage of cells in the G0/G1, S and G2/M phases. The apoptotic cells were observed on a DNA histogram as a subdiploid peak [19].

Statistical analysis

All data were analyzed by ANOVA (analysis of variance) and expressed as mean ± standard deviation. A *P*-value of less than 0·05 was considered statistically significant.

Results

Aspirin inhibits cell proliferation and induces apoptosis in human OC2 cells

It was noted that by increasing concentrations of ASA (0, 0·5, 1, 2, and 4 mM) for 24 h, drastic changes in morphology, e.g. cell rounding, blebbing, and detachment leading to cell death, took place (data not shown). In the MTT assay, the effect of ASA on cell viability was dose-dependent (Fig. 1a). To verify, the coincided morphological changes and MTT results were caused by ASA-induced apoptosis, and DNA agarose electrophoresis of genomic DNA extracted from ASA-treated cells were conducted. As shown in Fig. 1(b), DNA ladders were formed by typical internucleosomal fragments characteristic of apoptosis. This result agreed with previous study that showed a ladder pattern on agarose gel in human gastric cancer cells (AGS) treated with ASA (1, 10 mM) for 24 h [5]. In contrast, no DNA fragmentation of HT-29 cells in response to ASA occurred (3 mM, 72 h) [6].

In addition, a distinct active form of caspase-3 expression was induced early at 3 h and 12 h (Fig. 1c). Janicke and colleagues reported that introduction of the CASP-3 gene into the MCF-7 breast carcinoma cell line resulted in DNA fragmentation [20]. It indicated that activated caspase-3 is required for DNA fragmentation of cells undergoing apoptosis. Markedly increasing the expression of activated caspase-3 protein may help to explain ASA-induced formation of the DNA ladder in this experiment.

Up-regulation of p53 signalling by ASA

To investigate the kinetics of the p53 protein induced by ASA, we detected the expression of the p53 protein in time- (3, 12, and 24 h) and dose- (0, 0·5, 1, 2, 4 mM) dependent designs. Results of an immunoblotting analysis of the p53 protein showed that cells treated with ASA increased the accumulation of the p53 protein (Fig. 2a). In response to most stressors, the wild-type p53 protein level increases rapidly within 1–12 h of treatment with UV or ionizing radiation [21]. Moreover, the expression of phospho-p53 (p-p53), which phosphorylated at serine 15 ($pSer^{15}$), raised as expected (Fig. 2b), it in turn up-regulates its downstream target genes; a key inhibitor for cell cycle progression, $p21^{WAF1/CIP1}$ and a pro-apoptotic member of the Bcl-2 family, Bax (Fig. 2b).

In an independent experiment, we decreased the concentration of ASA used to avoid the acidic effect of ASA. The concentration range (10^{-1}–10^{-4} mM) is consistent with the plasma salicylate concentrations in individuals taking therapeutic doses of aspirin or sodium salicylate [22]. Cells were treated with various concentrations (0, 0·01, 0·1 and 1 mM) for 24 h. The expressions of p53, p21 and Bax also increased (Fig. 2c).

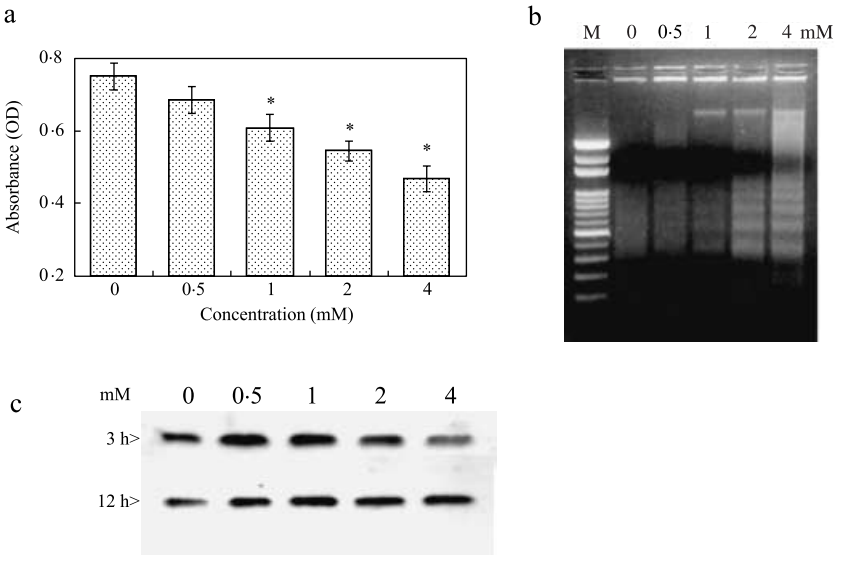

Figure 1 Effect of aspirin on cell proliferation and apoptosis. OC2 cells were treated with various concentrations (0, 0·5, 1, 2, 4 mM) of aspirin for 24 h in serum-free medium. (a) Cell viability was measured by methyl tetrazolium assay as described in Materials and methods. The values are expressed as means ± SD from four independent experiments ($P < 0·05$). (b) Formation of oligonucleosomal fragments was determined by 2% agarose gel electrophoresis. Lane M: 100-bp DNA ladder as the size marker. (c) OC2 cells were exposed to aspirin for 3 and 12 h, and the expressions of activated caspase-3 protein were determined by Western blotting. Experiments were performed three times and a representative blot is shown.

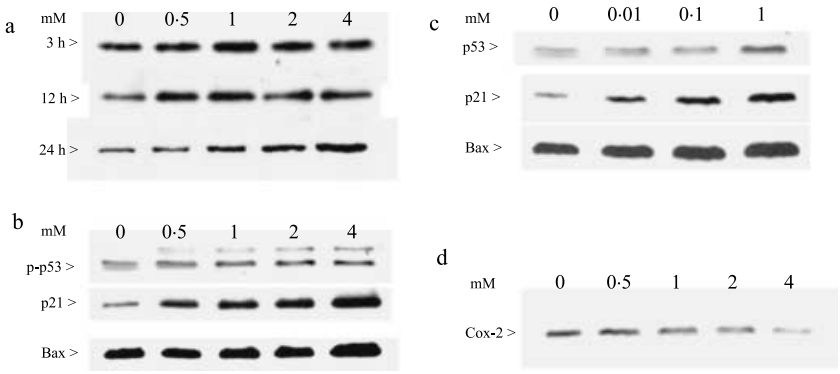

Figure 2 Effect of aspirin on activation of p53 signalling and expression of cyclooxygenase (COX)-2). OC2 cells were treated with aspirin for 3, 12 and 24 h in serum-free medium. (a) Kinetics of p53 in response to aspirin. The expression of p53 was induced as early as 3 h. (b) Expression of phospho-p53 (p-p53) was determined by an antibody reacted with p53, which phosphorylated at serine 15. (c) Decreased concentrations of aspirin were used to avoid the acidic effect. Expression of p53 and its downstream genes, p21 and Bax, were induced by aspirin. (d) Expression of COX-2 was suppressed by the aspirin. Experiments were performed three times and a representative blot is shown.

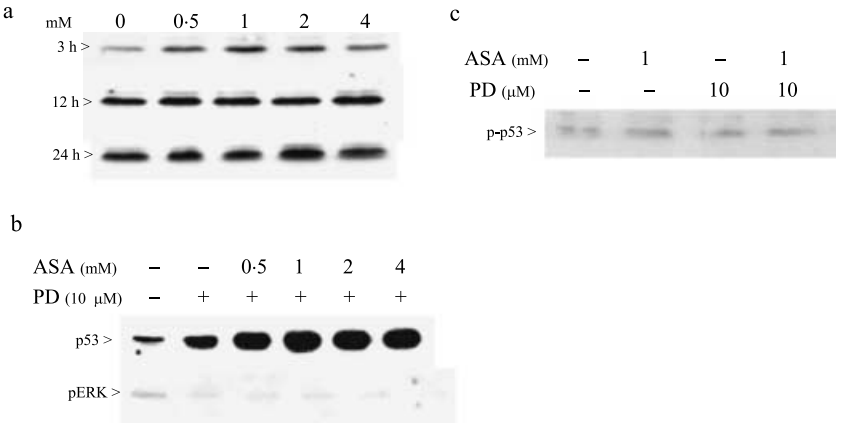

Figure 3 Activation of p42/p44 mitogen-activated protein kinase (MAPK) in p53-signalling induced by aspirin. OC2 cells were treated with acetylsalicylic acid (ASA) in serum-free medium. (a) Aspirin induced the activation of extracellular regulatory kinase 1/2 early as 3 h, then gradually to less than the levels of control at 24 h. (b,c) PD98059, a specific inhibitor of ERK kinase, was used in this study to investigate the role of p42/p44 MAPK played in the p53 signalling induced by the aspirin. Cells were exposed to aspirin and/or PD98059 for (b) 3 and (c) 24 h. Experiments were performed three times and a representative blot is shown.

Expression of COX-2 inhibited by ASA

In this experiment, the expression of COX-2 significantly decreased with increasing ASA concentrations (Fig. 2d). This result agrees with a previous study that suggested salicylate exerts its anti-inflammatory action in part by suppressing COX-2 induction, thereby reducing the synthesis of prostaglandins [22].

ERK 1/2 plays a negative role in p53 regulation

Extracellular regulatory kinases are activated in response to growth factors and play a role in mitosis and differentiation. The effect of NSAIDs on activation of ERK1/2 is controversial. Treatment of HT-29 colorectal carcinoma cells with NS-398 caused activation of ERK1/2 [23]. However, sodium salicylate, the metabolite of aspirin, blocked the phosphorylation of p42/p44 MAPK [24,25]. Interestingly, the expression of phospho-ERK (pERK) was induced after treatment with ASA for 3 h, and then gradually reduced to the level of control at 12 h and to less than the level of control at 24 h in this study (Fig. 3a). To investigate the role of MAP kinase played in p53 signalling induced by ASA, a specific inhibitor of MEK (MAPK or ERK kinase), PD98059 (Calbiochem), was used in this study. The expression of p53 and p53-pSer^{15} up-regulation by ASA was markedly enhanced when ERK1/2 activation was suppressed by 10 μM of PD98059 for 3 h (Fig. 3b) and 24 h (Fig. 3c), thus indicating a negative role for ERK1/2 in p53 signalling induced by ASA. The expression of other components of MAPKs, JNK and p38 could not be detected in this study (data not shown). Overexpression of p53 implied that the cells led to cell-cycle arrest and induction of apoptosis. To confirm the effect of PD98059 on cell viability, the cells were treated with 10 μM of PD98059 on days 1, 2, 3 and 4. PD98059 alone decreased cell viability from ~0·89, ~0·69 and ~0·61, respectively, to ~0·52. In the meanwhile, ASA (1 mM) coordination with PD98059 (10 μM) reduced cell viability from ~0·89, ~0·70 and ~0·59, respectively, to ~0·43 compared with the level of control (data not shown).

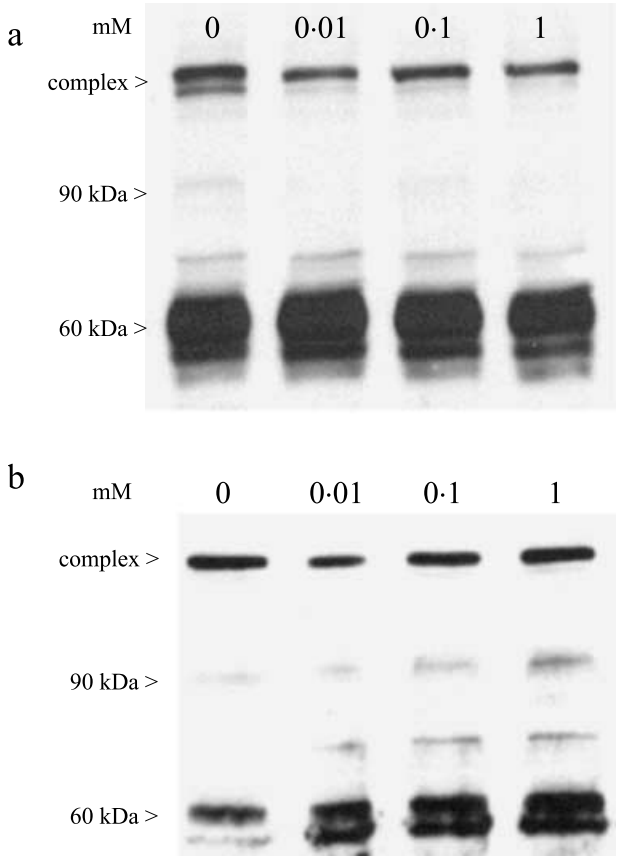

Figure 4 Disruption of p53-p53-murine double minute-2 complex in response to aspirin. OC2 cells were treatment with aspirin for (a) 3 and (b) 24 h in serum-free medium. The antibody used in this experiment can react with the p53-MDM2 complex, 90-kDa intact MDM2 and 60-kDa fragment. Experiments were performed three times and a representative blot is shown.

ASA blocks p53-MDM2 complex formation

Murine double minute 2 (MDM2) acts as a master regulator of the p53 tumour suppressor protein. MDM2 and p53 are involved in a negative feedback loop. We found that ASA decreased the expression of the p53-MDM2 complex and increased the expression of the 60-kDa MDM2 fragment as early as 3 h and 24 h (Fig. 4). Previous studies indicated that MDM2 oncoprotein, encode a 90-kDa protein, is cleaved by caspase-3 (CPP-32) during apoptosis, generating a 60-kDa fragment [26,27]. In this aspect, increased the expression of 60 kDa MDM2 fragment may contribute by overexpression of activated caspase-3 in this experiment, at least in part (Fig. 1c).

Effect of ASA on the cell-cycle phase distribution

To identify the effect of ASA on p53 signalling, we studied the cell-cycle phase distribution using FACS analysis. The result showed that ASA increased the proportion of cells in the G0/G1 phase, decreased the proportion of cells in the G2/M phase and induced apoptosis in a dose-dependent manner with a typical subdiploid peak on the histogram (Fig. 5). Furthermore, cells treated with PD98059 (10 CM) for 24 h showed apoptosis and cell-cycle arrest at the G0/G1 phase (Fig. 5). It well known that p53 transduces the DNA damage signals to its downstream genes, p21 and bax, and induces the expression of those genes. This, in turn, arrests the cell cycle at the G1 phase and results in apoptosis.

Discussion

Phosphorylation at several different serine residues in p53 had been shown to occur after cells were exposed to DNA-damage agents. The expression of p53-phosphorylated p53 at serine 15 ($pSer^{15}$) and serine 392 ($pSer^{392}$) was detected. However, no signal could be detected at p53-$pSer^{392}$ in this experiment. Clearly, ASA induces p53 phosphorylation at serine 15 other than serine 392. Recent reports suggest that phosphorylation of serine 392 may be important for p53 oligomerization [28,29]. While, serines 15 and 37 become phosphorylated after DNA damage, this phosphorylation reduces MDM2 biding to p53 [30,31].

The function and stability of the tumour suppressor p53 are tightly controlled by the negative regulator MDM2, which binds to p53, blocking DNA binding and targeting p53 for proteosome-mediated degradation. Pochampally and coworkers reported that human tumour cell lines often express high levels of a 60-kDa MDM2 isoform in the absence of apoptosis [32]. This helps to explain why the OC2 cells used in this study expressed high levels of 60-kDa MDM2 fragment. The 60-kDa fragment is a product of caspase cleavage of full-length MDM2 between residues 361 and 362 [27]. Whether the 60-kDa MDM2 fragment functions in regulation of p53 remains unclear. A previous study indicated that cleavage of MDM2 by CPP32-like proteases may result in losing the ability to promote p53 degradation [26]. On the contrary, others have suggested that the p53 binding and inhibition functions of MDM2 are not affected by the cleavage [27,32].

Cyclooxygenases-2 is an isoform of cyclooxygenase that increased in response to growth factors, cytokines and other mitogenic stimuli. Xu and colleagues suggested that ASA blocked COX-2 mRNA and protein levels [22]. In our study, the expression of COX-2 protein decreased in a dose-dependent manner after treatment with ASA. It seems plausible that salicylate exerts its anti-inflammatory action by suppressing COX-2 induction. However, a variety of research proposed that the effect of NSAIDs on cellular signal transduction pathways other than those involving prostaglandins, like effect on nuclear factor kappaB (NFκB) activity [33], regulation of mitogen-activated protein kinases [24,34] and regulation of p53 signalling [13,35]. Here, we present evidence that ASA can induce apoptosis via a p53-dependent pathway and that inhibited p42/p44 MAPK activity enhances the accumulation of p53.

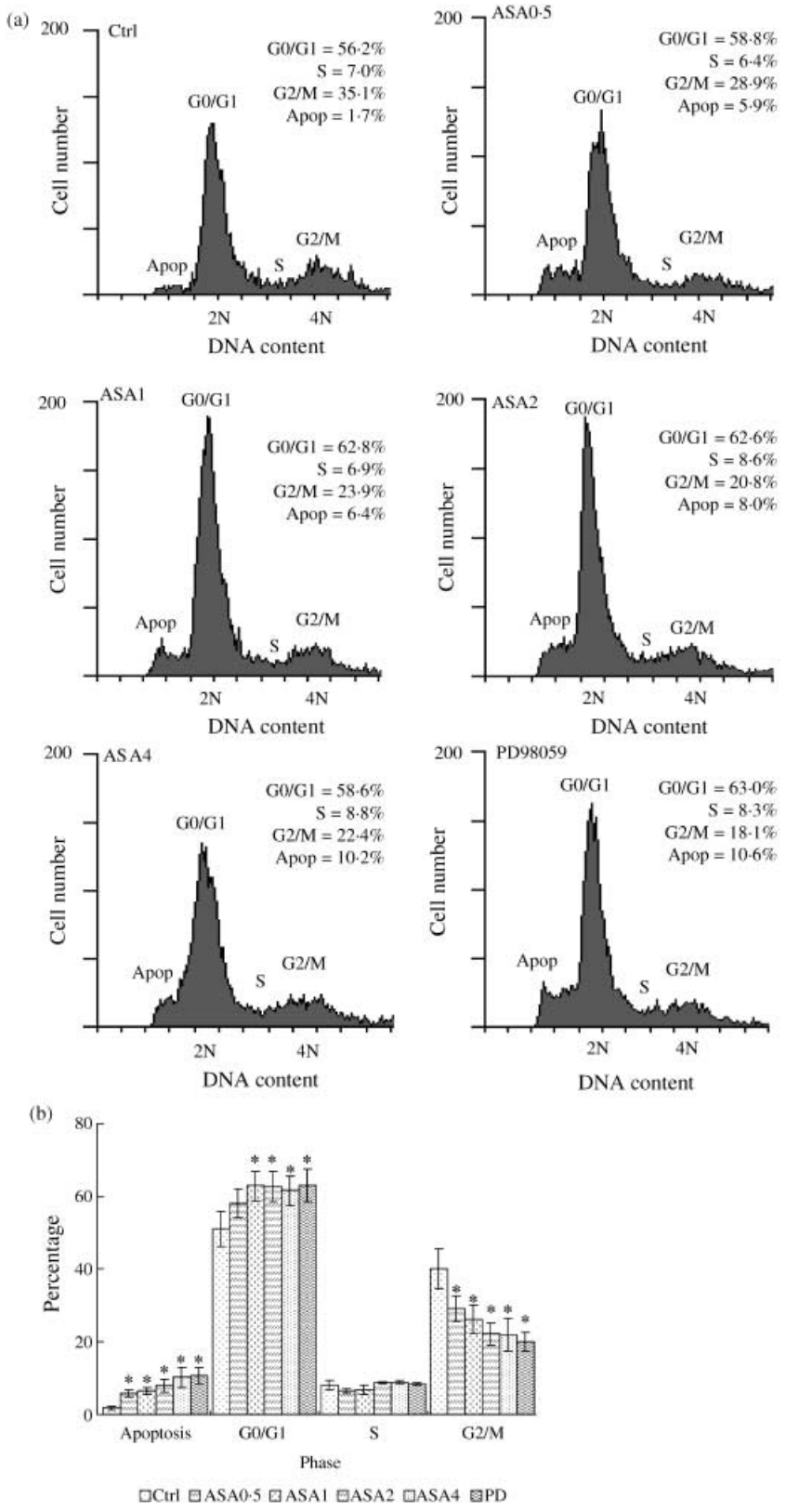

Figure 5 Cell-cycle phase distribution of OC2 cells treated with aspirin. Cells were treated with aspirin for 24 h in serum-free medium and the DNA content was determined by FACS, as described in Materials and methods. (a) DNA histogram. (b) Cell-cycle phase distribution of OC2 cells treated with aspirin and PD98059. The cells distributed in each cycle were determined by flow cytometry. The apoptotic cells were observed on a DNA histogram as a subdiploid peak. The data are expressed as means ± SD from three independent experiments ($P < 0{\cdot}05$).

Conclusion

Nonsteroidal anti-inflammatory drug-inhibited cyclooxygenase may not be the only or even the most important mechanism of inhibition. Our study presents evidence that activation of p53 signalling is involved in apoptosis induced by ASA. Furthermore, the apoptotic effect was enhanced by blocking the activation of p42/p44 MAPK in response to treatment with ASA, thus indicating a negative role for p42/p44 MAPK.

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