Ibuprofen Inhibits Activation of Nuclear β-Catenin in Human Colon Adenomas and Induces the Phosphorylation of GSK-3β

Emily J. Greenspan¹, James P. Madigan¹, Lisa A. Boardman², and Daniel W. Rosenberg¹

Abstract

Nonselective cyclooxygenase (COX) inhibitors target many of the same cancer-associated molecular pathways as COX-2-specific inhibitors. Although these nonsteroidal anti-inflammatory drugs (NSAIDs) are often associated with gastrointestinal toxicity, there is renewed interest in their use as colorectal cancer (CRC) chemopreventive agents due to the adverse side effects associated with long-term use of selective COX-2 inhibitors. In this study, we investigated the effects of long-term use (up to 25 years) of NSAIDs (ibuprofen or aspirin) on adenoma pathology and β-catenin-mediated signaling in sporadic human colon adenomas. Although NSAID use did not impact overall adenoma size or degree of dysplasia, it did cause a significant inhibition of nuclear β-catenin localization, which correlated with suppression of cyclin D1 expression. To further elucidate the effect of these agents in regulating β-catenin, we treated SW480 colon cancer cells with a panel of NSAIDs and determined their effects on β-catenin levels and cellular localization. In agreement with our in vivo results, both S-ibuprofen and aspirin were found to decrease total levels of β-catenin while increasing its phosphorylation. In addition, S-ibuprofen induced both degradation of IκBα and nuclear localization of NF-κB. Despite its nuclear localization, however, the activation of the NF-κB target genes, Bcl-2, survivin, and cyclin D1, was suppressed. This reduction in NF-κB transcriptional activity may be due to increased phosphorylation of GSK-3β following S-ibuprofen treatment. These data suggest that ibuprofen can effectively target both the Wnt/β-catenin and NF-κB pathways, and potentially uncovers a novel mechanism through which NSAIDS may exert their chemopreventive efficacy. Cancer Prev Res. 4(1): 161–71. ©2011 AACR.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the cyclooxygenase (COX)-1 and COX-2 enzymes, have an impressive history as effective chemopreventive agents for colorectal cancer (CRC; refs. 1, 2). These effects are largely dependent on their ability to inhibit the production of proliferative and inflammatory prostaglandins (PG), most notably prostaglandin E2 (PGE2; ref. 3). More recently, specific COX-2 inhibitors were developed as a means to reduce the gastrointestinal toxicity associated with inhibiting COX-1. However, treatment with this drug class is associated with increased incidence of adverse cardiovascular events, even after short-term exposures (4, 5). Consequently, these negative side effects have limited their clinical application in chemoprevention (6–8). Therefore, there is renewed interest in developing nonselective NSAIDs for clinical use in chemoprevention, especially those chemically modified to circumvent gastrointestinal side effects.

Several double-blinded, placebo-controlled, and case-controlled studies have reported that both selective COX-2 inhibitors, such as celecoxib and rofecoxib, and nonselective inhibitors, such as sulindac and aspirin, are effective in reducing adenoma recurrence, as well as the risk for CRC (9–16). Apart from their abilities to inhibit COX activity and prostaglandin production, both selective and nonselective COX-2 inhibitors can target β-catenin, a key mediator of colon tumorigenesis. NSAIDs, including celecoxib, sulindac, and aspirin, can increase the phosphorylation of β-catenin, thus decreasing its nuclear accumulation and transcription of Wnt/β-catenin target genes, such as cyclin D1, c-myc, and PPARγ (17–22). Sulindac has also been shown to inhibit β-catenin expression in familial adenomatous polyposis (FAP)-related adenomas (21, 23).

In addition to their effects on Wnt/β-catenin signaling, a number of NSAIDs, including aspirin, diclofenac, sulindac, sulindac sulfone, indomethacin, celecoxib, and NS-398, have been shown to induce the degradation of the NF-κB.
inhibitor IκBα, resulting in the direct nuclear translocation of the p65 subunit of NF-κB in the absence of additional NF-κB activators (24–29). In fact, additional studies in human CRC cell lines have shown that aspirin and non-aspirin NSAIDs induce the translocation of NF-κB-p65 into the nucleolus, resulting in inhibition of NF-κB-related gene expression (25, 29, 30). It has thus been proposed that NSAID-mediated suppression of NF-κB target gene expression may contribute to the COX-2–independent growth inhibition and apoptosis induced by these drugs (26, 31, 32). Thus, the identification of additional chemopreventive agents that target both β-catenin- and NF-κB–mediated signaling in human tumors may lead to more effective therapeutic approaches.

In the following study, we evaluated the effects of the nonspecific COX-2 inhibitor ibuprofen on the Wnt/β-catenin pathway in human sporadic colon adenomas. Notably, it was found that ibuprofen treatment inhibited the accumulation of nuclear β-catenin expression, an outcome that correlated with reduced levels of cyclin D1. In colon cancer cells in vitro, S-ibuprofen, the active form in cell culture, along with a panel of other NSAIDs, increased phosphorylation-dependent inactivation of β-catenin. In addition, S-ibuprofen induced degradation of IκBα together with phosphorylation and nuclear localization of NF-κB-p63. Despite its nuclear localization, however, the activation of NF-κB target genes was suppressed, possibly related to the observed increase in phosphorylation of GSK-3β. These data suggest that ibuprofen may provide an effective alternative, or useful adjunct, to selective COX-2 inhibitors for use in chemoprevention.

Materials and Methods

Human subjects

Formalin-fixed, paraffin-embedded (FFPE) sections of adenomas from 37 patients taking daily NSAIDs (ibuprofen or aspirin) for up to 25 years and from 39 patients not taking daily NSAIDs were obtained from the Mayo Clinic. In total, there were 76 samples composed of 32 tubular adenomas and 5 tubulovillous adenomas from patients taking NSAIDs as well as 27 tubular adenomas and 12 tubulovillous adenomas from patients not taking NSAIDs. There was also a separate group of 17 adenomas from FAP patients given sulindac for 1 year. Samples were obtained in accordance with University of Connecticut Health Center Institutional Review Board (IRB) guidelines and use of these samples was approved by the IRB at the Mayo Clinic.

Immunohistochemistry

Staining was performed as described in our previous study (33). Briefly, FFPE tissues were de-paraffinized and incubated with 1% hydrogen peroxide for 20 minutes at room temperature. Tissues were subjected to antigen retrieval and blocked with 10% normal goat serum (Vector Laboratories) in PBS-Brij solution. Tissues were then incubated overnight at 4°C with antibodies (1:2,000; Sigma-Aldrich), anti-p21 (1:100; Neomarkers) antibodies. Tissues were washed and incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies for 30 minutes at room temperature, followed by incubation with avidin–biotin complex reagent (Vector Laboratories) for 30 minutes at room temperature. Signal was detected with DAB solution (3,3′-diaminobenzidine; Vector Laboratories), and tissues were counterstained with hematoxylin. For scoring of β-catenin and cyclin D1 nuclear immunostaining, the following scale was used: grade 1, 1%–10% positive nuclei; grade 2, 10%–20% positive nuclei; grade 3, 20%–50% positive nuclei; grade 4, >50% positive nuclei. Samples were considered negative if there were no positively staining nuclei.

Cell culture

SW480 and DLD-1 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin. Cells were treated with 1 mmol/L S-ibuprofen, 5 mmol/L aspirin, 100 μmol/L sulindac sulfone, 600 μmol/L sulindac sulfone, 600 μmol/L indomethacin (all Sigma-Aldrich), or 1 μmol/L PGE2 (Cayman Chemical) for 24 hours. Control cells were treated with vehicle (DMSO or 100% ethanol) alone.

PGE2 quantification

The amount of PGE2 present in cells was quantified using the PGE2 Monoclonal EIA Kit (Cayman Chemical) according to the manufacturer’s instructions. Briefly, cells were grown in 24-well plates to 80% confluence. Following drug treatments (as above, for 24 hours), cell culture supernatants were collected and immediately incubated for 18 hours at 4°C with a PGE2–acyetylcholinesterase conjugate (PGE2 tracer) and a PGE2 monoclonal antibody in plates precoated with goat anti-mouse IgG. Following washing to remove unbound reagents, the plates were developed with Ellman’s reagent, which contains the substrate for acetylcholinesterase, and read at 405 nm. The absorbance readings were proportional to the amount of bound PGE2 tracer and inversely proportional to the amount of PGE2 in the samples. A standard curve was also measured using known concentrations of PGE2 to calculate the absolute amounts of PGE2 in the samples.

Immunoblotting

Following drug treatment (as above, for 24 hours), cells were lysed in a buffer containing 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1% Triton X-100 supplemented with protease and phosphatase inhibitors (50 mmol/L NaF, 10 mmol/L sodium β-glycerophosphate, 5 mmol/L sodium pyrophosphate, 1 mmol/L sodium vanadate, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride). Following centrifugation at 14,000 rpm for 15 minutes at 4°C, the supernatant was removed and quantified for total protein. Thirty micrograms of protein was incubated at 95°C for 5 minutes and 4× sample loading buffer containing 8% β-mercaptoethanol. Tissues were then incubated overnight at 4°C with antibodies (1:2,000; Sigma-Aldrich), anti-cyclin D1 (1:100; Immuno-Biological Laboratories, a kind gift from Dr. A. Arnold), antiproliferating cell nuclear antigen (anti-PCNA; 1:150; Novacastra Laboratories), or antip21 (1:100; Neomarkers) antibodies. Tissues were washed and incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies for 30 minutes at room temperature, followed by incubation with avidin–biotin complex reagent (Vector Laboratories) for 30 minutes at room temperature. Signal was detected with DAB solution (3,3′-diaminobenzidine; Vector Laboratories), and tissues were counterstained with hematoxylin.
tostained with 4 secondary antibody (goat anti-mouse IgG Alexa 568; 
Aldrich) in 1% BSA in PBS for 1 hour at room tempera-
tion in 0.5% Triton X-100 in PBS for 5 minutes. Cells were 
then blocked in 3% bovine serum albumin (BSA; 10 mg/
PBS and then fixed in 4% para-
slides using Prolong Gold Antifade Reagent (Molecular 
1:10,000). Cells on coverslips were mounted onto glass 
Probes) and visualized using an Olympus IX50 fluores-
Long-term NSAID use does not significantly affect adenoma size or morphology
The sample set of human adenomas was composed of 76 sporadic adenomas, 37 from patients taking daily ibuprofen or aspirin for 1 year or more and 39 from patients with no history of NSAID use. In addition, there were 17 adenomas from FAP patients given sulindac for 1 year. To evaluate the effects of 1 year or more of ibu-
profen or aspirin use on adenoma phenotype, we per-
formed statistical analyses comparing dysplasia or size with NSAID use. As shown in Table 1, there was no 
significant correlation found between degree of dysplasia 
and NSAID use (P = 0.12, 2-tailed Fisher’s exact test) or 
between size and NSAID use (P = 0.21, 2-tailed Fisher’s 
exact test). However, given the limited number of dysplas-
tic adenomas observed in the present study (5 of a total of 
76), a conclusive determination of the effects of NSAID 
use on histologic tumor grade is not possible. This indi-
cates that in this sample set, NSAID use did not signifi-
cantly impact the pathologic features of the adenomas.

Immunofluorescence
Cells were grown on 22 × 22-mm glass coverslips in 6-
well plates to approximately 50% confluence and then 
treated with drugs (as above, for 24 hours). Cells were 
washed with cold 1× PBS and then fixed in 4% parafo-
maldehyde for 10 minutes, followed by permeabiliza-
ton 0.5% Triton X-100 in PBS for 5 minutes. Cells were 
then blocked in 3% bovine serum albumin (BSA; 10 mg/
PBS for 1 hour at room temperature, followed by 
incubation with anti-β-catenin antibody (1:100; Sigma-
Aldrich) or β-actin (1:6,000; Santa Cruz Biotechnology) 
antibodies. Blots were then washed multiple times and 
incubated with goat anti-mouse IgG HRP (horseradish per-
oxidase; 1:10,000; Upstate Biotechnology) or donkey anti-
rabbit IgG HRP (1:4,000; Santa Cruz Biotechnology) for 45 
minutes at room temperature. HRP was visualized with 
enhanced luminal reagent (Millipore Corp.).

Results
Long-term NSAID use does not significantly affect adenoma size or morphology
The sample set of human adenomas was composed of 76 sporadic adenomas, 37 from patients taking daily ibuprofen or aspirin for 1 year or more and 39 from patients with no history of NSAID use. In addition, there were 17 adenomas from FAP patients given sulindac for 1 year. To evaluate the effects of 1 year or more of ibu-
profen or aspirin use on adenoma phenotype, we per-
formed statistical analyses comparing dysplasia or size with NSAID use. As shown in Table 1, there was no 
significant correlation found between degree of dysplasia 
and NSAID use (P = 0.12, 2-tailed Fisher’s exact test) or 
between size and NSAID use (P = 0.21, 2-tailed Fisher’s 
exact test). However, given the limited number of dysplas-
tic adenomas observed in the present study (5 of a total of 
76), a conclusive determination of the effects of NSAID 
use on histologic tumor grade is not possible. This indi-
cates that in this sample set, NSAID use did not signifi-
cantly impact the pathologic features of the adenomas.

Long-term NSAID use affects β-catenin cellular localization
NSAIDs, including aspirin, sulindac, and indomethacin, 
have been shown to decrease nuclear levels of β-catenin in 
colon cancer cells (17–19). To determine whether long-
term NSAID use in this adenoma sample set could affect 
cellular localization of β-catenin, immunohistochemical 
analysis of β-catenin was performed in the 76 sporadic 
adenomas and 17 FAP adenomas. As shown in the repre-
sentative examples in Figure 1A, in adenomas from non-
NSAID patients, there was strong nuclear staining of β-cate-

nuclei (a, b) whereas NSAID use significantly decreased 
nuclear β-catenin expression (c, d). Nuclear β-catenin was quan-
tified and these data are shown in Table 2. Over-
all, 14 of 36 (39%) adenomas in the non-NSAID group had 
nuclear β-catenin staining compared with only 3 of 34 
(9%) adenomas from patients with a history of NSAID 
itake (P = 0.0047). In addition, the 3 adenomas from the 
NSAID group with positive nuclear β-catenin staining were 
all from patients taking daily aspirin, indicating that daily 
ibuprofen use completely inhibited nuclear β-catenin. 
Neither ibuprofen nor aspirin use had an effect on cell 
proliferation, as assessed by PCNA immunostaining, or 
p21 expression. In the adenomas from FAP patients treated 
with sulindac, 9 of 17 (53%) had nuclear β-catenin stain-
ing, indicating that sulindac was only variably effective 
in inhibiting translocation of β-catenin in these patients. 
However, it was not possible to determine whether the 
percentage of positive nuclear β-catenin cells in the ade-
nomas was suppressed by sulindac because there was no 
nontreated group for comparison.

Nuclear β-catenin correlates with nuclear cyclin D1 expression in human adenomas
When β-catenin translocates to the nucleus and binds to 
TGF/LEF (T-cell factor/hypothion enhancer factor) tran-
scription factors, it drives the transcription of a panel of 
Wnt target genes including cyclin D1, c-myc, COX-2, and 
PPARγ (34–36). Thus, we wanted to determine whether 
nuclear β-catenin localization in the adenoma samples 
might have downstream effects on the expression of cyclin 
D1. As shown in the representative photomicrographs 
(Fig. 1B), immunohistochemical analysis revealed strong 
nuclear expression of cyclin D1 in non-NSAID adenomas
that had nuclear β-catenin staining (a, b); however, in adenomas from patients in the NSAID exposure group, the absence of nuclear β-catenin was associated with diminished nuclear cyclin D1 immunostaining (c, d). As shown in Table 2, however, there was no significant correlation between nuclear cyclin D1 expression and NSAID use (P > 0.05); however 12 of 16 (75%) adenomas with nuclear β-catenin expression also had nuclear expression of cyclin D1 (P = 0.022). This finding is illustrated in greater detail in Figure 1C, which shows a representative example of β-catenin (a–c) and cyclin D1 (d–f) colocalization in serial sections of a representative non-NSAID adenoma. This indicates that nuclear β-catenin does affect Wnt target gene expression.

**Ibuprofen affects β-catenin localization in human colon cancer cells**

To further investigate the effect of ibuprofen on β-catenin cellular localization, we treated human colon cancer cells with S-ibuprofen and a panel of other NSAIDs for comparison. Treatment of SW480 or DLD-1 cells with 1 mmol/L S-ibuprofen, 5 mmol/L aspirin, 100 μmol/L sulindac sulfide, 600 μmol/L sulindac sulfone, or 600 μmol/L indomethacin for 24 hours significantly inhibited PGE2 production compared with vehicle alone, indicating that COX-1/2 enzymes were being effectively targeted by drug treatment (Fig. 2A and B).

To determine the effect of this panel of NSAIDs on β-catenin expression, immunofluorescence microscopy was carried out on SW480 cells, which have mutant APC and consequent high levels of nuclear β-catenin. As shown in Figure 3A, strong membrane, cytoplasmic, and nuclear staining of β-catenin was present in untreated cells. However, treatment with NSAIDs greatly decreased the overall intensity of β-catenin staining, most notably with S-ibuprofen, sulindac sulfide, and indomethacin treatment. These data indicate that ibuprofen can inhibit the accumulation of nuclear β-catenin, as well as suppress the levels of total cellular β-catenin, despite the loss of APC protein. Figure 3B shows that in cytoplasmic extracts from SW480 cells, S-ibuprofen, aspirin, sulindac sulfide, and indomethacin could increase the accumulation of the phosphorylated form of β-catenin, suggesting that these drugs decrease nuclear levels of β-catenin by increasing its cytoplasmic degradation.

**Ibuprofen treatment results in NF-κB-p65 phosphorylation and nuclear localization**

To explore the possibility that ibuprofen may affect other signaling pathways in addition to the Wnt/β-catenin cascade, NF-κB signaling was examined in human SW480 colon cancer cells. NSAIDs, including aspirin, sulindac, and diclofenac, have been shown to activate NF-κB in a variety of CRC cells (24, 26). As shown in Figure 4A, 24-hour treatment of SW480 cells with

---

**Table 1. Patient demographics and adenoma classification**

<table>
<thead>
<tr>
<th>Group</th>
<th>Overall</th>
<th>-NSAID</th>
<th>+NSAIDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>25 (50%)</td>
<td>25 (50%)</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>14 (54%)</td>
<td>12 (46%)</td>
</tr>
<tr>
<td>B. Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>2</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>40–65</td>
<td>43</td>
<td>26 (60%)</td>
<td>17 (40%)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>31</td>
<td>11 (35%)</td>
<td>20 (65%)</td>
</tr>
<tr>
<td>C. Polyp type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular adenoma</td>
<td>61</td>
<td>29 (48%)</td>
<td>32 (52%)</td>
</tr>
<tr>
<td>Tubulovillous adenoma</td>
<td>15</td>
<td>10 (67%)</td>
<td>5 (33%)</td>
</tr>
<tr>
<td>FAP</td>
<td>17</td>
<td>0 (0%)</td>
<td>17 (100%)</td>
</tr>
<tr>
<td>D. Dysplasia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>71</td>
<td>35 (49%)</td>
<td>36 (51%)</td>
</tr>
<tr>
<td>High grade</td>
<td>4</td>
<td>4 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Invasive</td>
<td>1</td>
<td>0 (0%)</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>E. Size, mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>23</td>
<td>9 (39%)</td>
<td>14 (61%)</td>
</tr>
<tr>
<td>10–30</td>
<td>46</td>
<td>26 (57%)</td>
<td>20 (43%)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>7</td>
<td>4 (57%)</td>
<td>3 (43%)</td>
</tr>
</tbody>
</table>

aNSAID treatment is aspirin or ibuprofen for sporadic patients and sulindac for FAP patients, both for 1 year.
bSporadic patients only.
cFAP patients, no patient demographic or adenoma classification data available.
dP = 0.12 between groups (2-tailed Fisher’s exact test).
eP = 0.21 between groups (2-tailed Fisher’s exact test).
increasing concentrations of S-ibuprofen resulted in a dose-dependent decrease in the levels of the NF-kB inhibitor protein IκBα. Furthermore, a dose-dependent increase in the phosphorylated form (serine-536) of the p65 subunit of the NF-kB transcription factor complex was observed (Fig. 4A). Although phosphorylation of NF-kB-p65 at this serine residue has been shown to be important in the kinetics of its nuclear import (37), further direct evidence of NF-kB-p65 nuclear localization was required. As shown in Figure 4B, 24-hour treatment with S-ibuprofen resulted in nuclear translocation and accumulation of NF-kB-p65 in SW480 cells, as assessed by immunofluorescence staining.

**Ibuprofen treatment decreases expression of NF-kB target genes, inhibits GSK-3β, and induces apoptosis**

After showing that ibuprofen treatment (in the absence of additional NFκB activation) resulted in nuclear localization of NF-kB-p65, we next examined the possible effects of prolonged ibuprofen treatment on NF-kB target gene expression. As shown in Figure 5A, despite the observed nuclear localization, expression of the NF-kB target genes, Bcl-2, survivin, and cyclin D1, were decreased in a time-dependent manner following treatment of SW480 cells with S-ibuprofen, an effect that occurred through 72 hours. In addition, S-ibuprofen caused a time-dependent increase in apoptosis in SW480 cells treated, assessed by PARP cleavage (Fig. 5A). Recent studies have indicated that the serine/threonine kinase GSK-3β positively regulates NF-kB–mediated gene expression (38, 39). Thus, we investigated the possibility that ibuprofen treatment may inhibit the activity of GSK-3β, which is present constitutively in an active form, and phosphorylation of its serine-9 residue by Akt results in the inhibition of its signaling activity (40). As shown in Figure 5A, there was a time-dependent increase in the inhibitory phosphorylation of GSK-3β at serine-9. In

---

**Figure 1.** β-Catenin and cyclin D1 expression in human sporadic adenomas. A, representative examples of β-catenin immunohistochemical analyses in untreated (a, b) and NSAID-treated (c, d) adenomas at 200× (a, c) and 400× (b, d) magnification, performed as described under Materials and Methods. B, representative examples of cyclin D1 immunohistochemical analysis in untreated (a, b) and NSAID-treated (c, d) adenomas at 200× (a, c) and 400× (b, d) magnification, performed as described under Materials and Methods. C, representative examples of β-catenin (a, b) and cyclin D1 (c, d) colocalized immunostaining in serial sections of an untreated adenoma at 100× (a, c) and 400× (b, d) magnification.
addition, as shown in Figure 5B, we observed a rapid (within minutes) increase in the phosphorylation of GSK-3β at serine-9 in SW480 cells following treatment with S-ibuprofen. Interestingly, there was a concomitant increase in the active, phosphorylated form of Akt (serine-476), consistent with its established role in GSK-3β inhibition (40–42).

Discussion

The adverse cardiovascular effects associated with specific COX-2 inhibitors have limited their application in cancer chemoprevention, especially in high-risk individuals (43–46). Therefore, a renewed interest in the development of less toxic, nonselective NSAIDs has emerged. Our study found significant inhibition of nuclear β-catenin expression in sporadic colon adenomas from patients with a history of daily intake of ibuprofen or aspirin. One important distinguishing feature of our study is related to the patient population, comprised primarily of sporadic adenoma cases. Currently, the only clinical human data showing that NSAIDs can suppress β-catenin expression are in FAP patients (21, 23). Of note, no adenomas from subjects in our study who reported daily ibuprofen use exhibited the presence of nuclear β-catenin, indicating that this drug could completely suppress its nuclear translocation. Surprisingly, adenomas from FAP patients maintained on sulindac for up to 1 year showed only a modest reduction in the levels of nuclear β-catenin. However, pretreatment adenomas were not available from either population group and thus it is difficult to establish a definitive comparison in response between these groups. It remains a possibility that

Table 2. Association of β-catenin/cyclin D1 nuclear localization with NSAID intake

<table>
<thead>
<tr>
<th>Grade</th>
<th>−NSAID</th>
<th>+NSAID</th>
<th>FAP sulindac</th>
<th>+Nuclear β-catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>1</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total positive: 14 (39%) 3 (9%) 9 (53%)
Total: 36 34 17 N/A

Abbreviation. N/A, not available.

a There is a significant association between nuclear β-catenin and NSAID use, \( P = 0.0047 \) (2-tailed Fisher’s exact test).
b There is a significant association between nuclear cyclin D1 and nuclear β-catenin in adenomas, \( P = 0.022 \) (2-tailed Fisher’s exact test) but not with NSAID use, \( P = 0.149 \).

Figure 2. NSAIDs inhibit PGE2 production in human colon cancer cells. SW480 (A) and DLD-1 (B) human colon cancer cell lines were treated with a panel of NSAIDs (S-ibuprofen, aspirin, sulindac sulfide, sulindac sulfone, and indomethacin) for 24 hours as described under Materials and Methods. At the end of drug treatment, supernatants were collected and PGE2 levels were determined by ELISA. Columns, mean of 3 samples per group; bars, SE. *, \( P = 0.0008 \) for A and 0.033 for B, ANOVA between groups.
Ibuprofen Blocks Nuclear β-Catenin in Adenomas

Sulindac treatment suppressed nuclear β-catenin translocation if compared with pretreatment adenomas. Studies in human colon cancer cells have found that inhibition of nuclear β-catenin by NSAIDs, such as sulindac and indomethacin, results in a dramatic downregulation of its transcriptional targets, including cyclin D1 and c-myc (22, 47). Consistent with these in vitro findings, our study also found an association between nuclear β-catenin expression and positive cyclin D1 staining in the cytoplasmic fraction of SW480 cells following 24-hour treatment with a panel of NSAIDs (S-ibuprofen, aspirin, sulindac sulfide, sulindac sulfone, and indomethacin). PGE2 was added as a negative control. Total cell lysates from SW480 cells containing both nuclear and cytoplasmic fractions is also included as a negative control. Blots were reprobed with anti-β-actin as a loading control.

Figure 3. S-Ibuprofen inhibits β-catenin expression and nuclear localization in SW480 colon cancer cells. A, immunofluorescence microscopy of β-catenin (Alexa Fluor 568, red) in SW480 cells following treatment with a panel of NSAIDs (1 mmol/L S-ibuprofen, 5 mmol/L aspirin, 100 μmol/L sulindac sulfide, 600 μmol/L sulindac sulfone, and 600 μmol/L indomethacin) for 24 hours as described in Materials and Methods. Nuclei were counterstained with DAPI (blue). Merged images represent the overlay of the image at 603 nm (β-catenin, Alexa Fluor 568) with the image at 488 nm (DAPI). B, Western blot analysis of phospho-β-catenin and total β-catenin in the cytoplasmic fraction of SW480 cells following 24-hour treatment with a panel of NSAIDs (S-ibuprofen, aspirin, sulindac sulfide, sulindac sulfone, and indomethacin). PGE2 was added as a negative control. Total cell lysates from SW480 cells containing both nuclear and cytoplasmic fractions is also included as a negative control. Blots were reprobed with anti-β-actin as a loading control.
differentiation, proliferation, and apoptotic cell death (49). In resting cells, the heterodimeric NF-κB transcription factor complex is sequestered in the cytoplasm by the inhibitor protein IκBα. Cellular stimulation by various agents can result in the phosphorylation and proteosomal degradation of IκBα, causing the phosphorylation, release, and nuclear translocation of the NF-κB complex (49). As shown in Figure 4B, further evaluation of NF-κB-p65 staining, using immunofluorescence analysis, revealed its punctate, nuclear localization after treatment with S-ibuprofen. Previous studies have shown that nucleolar sequestration of the p65 subunit of NF-κB is essential for aspirin-mediated apoptosis (26, 50, 51). Furthermore, nucleolar sequestration of NF-κB-p65 may contribute to the suppression of NF-κB target gene activation by preventing proper binding of the NF-κB complex to the promoters of these genes (51). Future studies will be aimed at determining whether S-ibuprofen treatment results in nucleolar localization of NF-κB-p65.

Consistent with these findings, we also observed that treatment of SW480 cells in vitro with S-ibuprofen (for up

<table>
<thead>
<tr>
<th>μmol/L ibuprofen, 24 h</th>
<th>0</th>
<th>500</th>
<th>750</th>
<th>1,000</th>
<th>2,500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IκBα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-NF-κB-p65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total-NF-κB-p65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Ibuprofen treatment relieves IκBα-mediated repression of NF-κB-p65 and induces its phosphorylation and nuclear localization. A, SW480 cells were treated for 24 hours with increasing doses of S-ibuprofen and protein lysates were collected as described under Materials and Methods. Proteins were resolved by SDS-PAGE and blotted for IκBα, phospho-NF-κB-p65 (serine-536) and β-tubulin (loading control). The phospho-NF-κB-p65 blot was stripped and blotted for total NF-κB-p65. B, SW480 cells were grown overnight on coverslips and then treated for 24 hours with either vehicle control or 2,500 μmol/L S-ibuprofen. Immunofluorescence microscopy was performed to visualize NF-κB-p65 (Alexa Fluor 568, red). Nuclei were counterstained with DAPI (blue). Localization of NF-κB-p65 in the nucleus is represented by magenta staining after merging of the 2 images.

<table>
<thead>
<tr>
<th>Hours treatment, 2,500 μmol/L ibuprofen</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin-D1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-GSK-3β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total GSK-3β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Akt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-GSK-3β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total GSK-3β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5. Ibuprofen decreases selective NF-κB target gene expression and induces inhibitory phosphorylation of GSK-3β. A, SW480 cells were treated with 2,500 μmol/L S-ibuprofen for either 24, 48, or 72 hours, and protein lysates were collected. Additional SW480 cells were treated for 24 hours with vehicle only and served as a baseline control. Proteins were resolved by SDS-PAGE and initially blotted for the NF-κB target genes Bcl-2, survivin, and cyclin D1. Protein lysates were also blotted for PARP, phospho-NF-κB-p65 (serine-536), phospho-GSK-3β (serine-9), and β-tubulin. Blots were stripped and blotted for total NF-κB-p65 and total GSK-3β. Arrow indicates the apoptosis-dependent cleavage product of PARP. B, SW480 cells were treated with 2,500 μmol/L S-ibuprofen for various time points up to 60 minutes and protein lysates were collected. Proteins were resolved by SDS-PAGE and initially blotted for phospho-Akt (serine-473), phospho-GSK-3β (serine-9), and β-tubulin. Blots were stripped and rebotted for total Akt and total GSK-3β.
Ibuprofen Blocks Nuclear β-Catenin in Adenomas

The use of modified NSAIDs, including those containing nitric oxide that purportedly maintain mucosal integrity in a manner similar to that of endogenous prostaglandins, may circumvent gastrointestinal toxicity (5). Other approaches include the use of dual COX and 5-lipoxygenase inhibitors, as well as NSAIDs that are capable of releasing hydrogen sulfide (62). Thus, continued development of these novel strategies to circumvent gastrointestinal toxicity while still affording chemopreventive benefit is clearly needed to expand the clinical safety and efficacy of this important drug class.

To our knowledge, our study is the first to show that ibuprofen is effective at inhibiting β-catenin nuclear translocation in both human adenomas and in colon cancer cells in vitro. It is also the first to show that an NSAID can suppress β-catenin in sporadic adenomas following long-term treatment in patients. This indicates that the Wnt pathway in colon adenomas is sensitive to NSAID exposure, regardless of whether the lesions are from FAP patients or are sporadic in origin. In addition, our current data suggest that S-ibuprofen treatment results in the phosphorylation and nuclear translocation of NF-κB. However, this effect is associated with a concomitant loss in transcriptional activity. This reduced transcriptional activity of NF-κB may be due to the novel finding of increased S-ibuprofen–dependent phosphorylation of GSK-3β, leading to inhibition of NF-κB signaling. Future studies will establish the precise role that ibuprofen treatment plays in augmenting the GSK-3β–NF-κB signaling axis. In addition, because the Wnt/β-catenin and NF-κB signaling pathways share overlapping target genes, it will be important to establish the relative contribution of these pathway(s) to the transcriptional changes observed after ibuprofen treatment. In summary, we believe that these findings warrant the development of case-controlled studies for CRC chemoprevention using ibuprofen.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by National Institute of Health grants 5RO1CA125691 and 5RO1CA114635 (to D.W. Rosenberg).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 2, 2010; revised November 11, 2010; accepted November 17, 2010; published online January 4, 2011.

References

4. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by...
Ibuprofen Inhibits Activation of Nuclear $\beta$-Catenin in Human Colon Adenomas and Induces the Phosphorylation of GSK-3 $\beta$

Emily J. Greenspan, James P. Madigan, Lisa A. Boardman, et al.


Updated version  Access the most recent version of this article at: [http://cancerpreventionresearch.aacrjournals.org/content/4/1/161](http://cancerpreventionresearch.aacrjournals.org/content/4/1/161)

Cited articles  This article cites 62 articles, 26 of which you can access for free at: [http://cancerpreventionresearch.aacrjournals.org/content/4/1/161.full.html#ref-list-1](http://cancerpreventionresearch.aacrjournals.org/content/4/1/161.full.html#ref-list-1)

Citing articles  This article has been cited by 9 HighWire-hosted articles. Access the articles at: [http://cancerpreventionresearch.aacrjournals.org/content/4/1/161.full.html#related-urls](http://cancerpreventionresearch.aacrjournals.org/content/4/1/161.full.html#related-urls)

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.