Protein phosphatase 2A is required for mesalazine-dependent inhibition of Wnt/β-catenin pathway activity

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The rising incidence and poor prognosis of colorectal cancer have aroused substantial interest in novel chemopreventive strategies. Interestingly, treatment of ulcerative colitis with mesalazine, which displays few side effects during long-term treatment, is associated with a reduced incidence of colorectal cancer, but its molecular mechanism is not known. The effect of mesalazine on the Wnt/β-catenin pathway was studied in colorectal cancer cell lines to find a molecular basis underlying its chemopreventive features. Mesalazine affects the Wnt/β-catenin pathway in adenomatous polyposis coli mutated cells with intact β-catenin, as judged by luciferase reporter assays. Furthermore, mesalazine treatment reduced expression of nuclear β-catenin and Wnt/β-catenin target genes, and increased β-catenin phosphorylation. This effect on the Wnt/β-catenin pathway is mediated via protein phosphatase 2A (PP2A): increased phosphorylation of PP2A after mesalazine treatment is observed, which coincides with decreased PP2A enzymatic activity. The inhibition of PP2A enzymatic activity by mesalazine is essential for its effect on the Wnt/β-catenin pathway, as shown by transient transfection with siPP2A and mutant PP2A. This study shows, using concentrations of mesalazine identical to concentrations seen in patients with inflammatory bowel disease, that mesalazine inhibits the Wnt/β-catenin pathway via inhibition of PP2A.

Introduction

In Western societies, chemoprevention of colorectal cancer is becoming an important issue because the incidence of colorectal cancer is rising. Prevention is important because there is no cure for colorectal cancer when complete surgical resection cannot be performed. Non-steroidal anti-inflammatory drugs (NSAIDs) have attracted substantial interest as chemopreventive agents in colon cancer and have been shown to have a potent inhibitory effect on the growth of colorectal cancer both in animal studies and in human studies [(1) and references therein]. However, the gastrointestinal and renal side effects associated with the long-term use of most classical NSAIDs has limits their usefulness for the purpose of cancer chemoprevention (2-4). Hence, interest has focused on related compounds with fewer side effects such as selective COX-2 inhibitors or mesalazine (5). Mesalazine [5-aminosalicylic acid (5-ASA)], for which there is long-term clinical experience in the treatment of patients with inflammatory bowel disease (IBD), is well tolerated, has limited systemic side effects and has no gastrointestinal toxicity (6,7). In a rodent model of colorectal cancer, mesalazine inhibits tumor growth and reduces the number of aberrant crypt foci, whereas in patients with sporadic polyps or cancer of the large bowel mesalazine induces apoptosis and decreases proliferation in the colorectal mucosa (8-10). Epidemiological data strongly support a chemopreventive role for mesalazine in ulcerative colitis-associated colorectal cancer, and especially the data published by Eaden et al show compelling evidence for a protective effect of long-term mesalazine use in patients with ulcerative colitis (11-14). Accordingly, mesalazine treatment as a chemopreventive strategy for ulcerative colitis-associated colorectal cancer is now standard accepted clinical practice (15). Together, these data suggest a chemopreventive role for mesalazine in colorectal cancer development. However, the molecular mechanisms mediating this chemopreventive effect of mesalazine are not known.

The effects of mesalazine on the incidence of ulcerative colitis-associated colorectal cancer suggest that it acts on a pathway that is both early and common in colorectal carcinogenesis. The most obvious candidate for such a pathway is the Wnt/β-catenin pathway (16-18). In this pathway, Wnt binds to the transmembrane Frizzled receptor, which leads to activation of the cytoplasmic disheveled (Dsh) protein. Dsh forms a complex with the β-catenin degradation complex, which consists of the adenomatous polyposis coli (APC) gene product, glycogen synthase kinase-3β (GSK-3β), axin and β-catenin. In the absence of Wnt signaling, β-catenin within this complex is phosphorylated by GSK-3β, and this leads to its rapid degradation via the ubiquitin pathway (19). In response to Wnt signals, β-catenin is no longer targeted for degradation and accumulates to high levels in the cytoplasm (reviewed in ref. 20). This stabilized β-catenin translocates to the nucleus where it binds with members of the T-cell factor (Tcf)/lymphoid enhancer factor (Lef) family of transcription factors and activates the transcription of Wnt target gene expression (21). Constitutive activation of this pathway is seen in almost all colorectal cancers, mostly owing to a mutation in the Apc gene (reviewed in refs 17,18,22). Hence, it is generally accepted that deregulation of the Wnt/β-catenin pathway is essential for early colorectal tumorigenesis. However, it should be noted that not all colorectal tumors develop through APC or β-catenin mutations; a smaller group is thought to develop...
through the alternative or serrated pathway of cancer where gene silencing through promoter methylation of mismatch repair genes plays an important role (23).

The precise biochemical mechanism by which the β-catenin degradation complex is regulated is not yet known. Alterations in protein phosphorylation states are likely to be central to this regulation, because all the elements of the β-catenin degradation complex are phosphoproteins. The phosphorylation status of a protein is determined by the relative activities of both kinases and phosphatases. A number of protein kinases have been shown to influence Wnt/β-catenin signaling, including GSK3β, protein kinase C and casein kinase I and II (24). The protein phosphatases involved have received less attention. However, in Xenopus laevis axis formation, the catalytic subunit of the serine/threonine protein phosphatase 2A (PP2A) exerts a positive role on Wnt/β-catenin pathway activity, and conversely, the inhibitory regulatory B56 subunit of PP2A exerts a negative influence on Wnt/β-catenin signaling in mammalian cells and Xenopus embryo explants (25,26). Mutations in PP2A subunits in various types of cancer, including colon cancer, have also been described, but their precise role in the cancerous process is as yet not known (reviewed in ref. 27). Thus, evidence exists that implicates PP2A in the control of the Wnt/β-catenin pathway.

For these reasons we decided to study the effect of mesalazine on the Wnt/β-catenin pathway and the role of PP2A in these effects. Our results show that mesalazine inhibits Wnt/β-catenin pathway activity at the level of PP2A enzymatic activity, providing a possible molecular mechanism for mesalazine effects in chemoprevention.

Materials and methods

Cell culture

DLD-1, SW480, HCT116 and SW48 cells were obtained from the ATCC and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Paisley, UK) with 4.5 g/l glucose and L-glutamine, supplemented with antibiotic-antimycotic, 10% fetal bovine serum (FBS) and 10% pyruvate. Cells were grown in a humidified atmosphere at 37°C in a 5% CO2 incubator.

TOPFLASH assay

SW480 cells were seeded in 24-well plates at 1 × 10^5 cells/well and transfected with 0.4 μg of the TCF reporter, pTOPFLASH or pTOPFLASH (28) (both constructs were a gift of Dr H.Clevers), and 0.008 μg of pCMV-Renilla luciferase (Promega, Madison, WI, USA) per well, using LipofectAMINE-Plus reagent according to the manufacturer’s instructions. Cells were then incubated for 30 min with 100 nM okadaic acid or vehicle control and hereafter incubated for 1 h with mesalazine, with or without okadaic acid in culture medium. Samples were further prepared using the dual luciferase reporter kit (Roche) according to the manufacturer’s instructions, and luciferase levels were measured using a dual injector luminometer.

Isolation of nuclear fractions

DLD-1 or SW480 cells were treated with 1 mg/ml mesalazine during a time course: 0, 1, 3, 6, 12 and 24 h. Afterwards, cell lysates were prepared and separated into nuclear and cytosolic fractions, as described before (29). Briefly, cells were washed in phosphate-buffered saline (PBS), scraped into 200 μl 20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM Pefablock (Merck, Darmstadt, Germany), 0.5 μg/ml aprotinin (Roche), 0.5 μg/ml leupeptin (Boehringer-Mannheim, Mannheim, Germany) and passed 10× through a 27 gauge needle. After a 10 min centrifugation at 1000 g at 4°C, the resultant pellets (nuclear fraction) and the supernatants (cytosolic fraction) were further prepared as follows: to the supernatants 100 μl of 3× sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min at 95°C and followed by SDS–PAGE and blotted on PVDF membrane (Millipore). Blots were blocked with either 1% low-fat milk powder [for phospho-β-catenin, β-catenin (both from Cell Signaling Technology), β-actin, phospho-PP2A (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PP2A (BD Transduction Laboratories), α-tubulin, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), glucose-6-phosphate dehydrogenase (G6PDH) and 0.5% low-fat milk powder [for cyclin D1 (Neomarkers, Fremont, CA, USA), c-myc, c-met (both from Santa Cruz Biotechnology)] in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) for 1 h at room temperature and were incubated overnight at 4°C with primary antibody in blocking buffer. Subsequently, blots were washed with TBST and incubated with, depending on primary antibody, a secondary HRP-linked antibody (goat-α-mouse, swine-α-rabbit, goat-α-rabbit, goat-α-rat, all from DAKO, Glostrup, Denmark) or 0.5% low-fat milk powder [for phospho-PP2A, PP2A (both from Santa Cruz Biotechnology™, Beverly, MA, USA)]. Western blot analysis

DLD-1, SW480, HCT116 and SW48 cells were treated with 1 mg/ml mesalazine during a time course: 0, 5, 10, 15, 30 and 60 min. Afterwards, cells were washed in PBS and scraped into 200 μl cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Trition-X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM Pefablock). Subsequently, lysates were mixed with 3× SDS sample buffer, boiled for 5 min at 95°C followed by SDS–PAGE and blotted on PVDF membrane (Millipore). Blots were blocked with either 1% low-fat milk powder [for phospho-β-catenin, β-catenin (both from Cell Signaling Technology), β-actin, phospho-PP2A (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PP2A (BD Transduction Laboratories), α-tubulin, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), glucose-6-phosphate dehydrogenase (G6PDH) and 0.5% low-fat milk powder [for cyclin D1 (Neomarkers, Fremont, CA, USA), c-myc, c-met (both from Santa Cruz Biotechnology)] in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) for 1 h at room temperature and were incubated overnight at 4°C with primary antibody in blocking buffer. Subsequently, blots were washed with TBST and incubated with, depending on primary antibody, a secondary HRP-linked antibody (goat-α-mouse, swine-α-rabbit, goat-α-rabbit, goat-α-rat, all from DAKO, Glostrup, Denmark) or 0.5% low-fat milk powder [for phospho-PP2A, PP2A (both from Santa Cruz Biotechnology™, Beverly, MA, USA)]. Immunoprecipitation for PP2A activity determination

DLD-1 and SW480 cells were treated with 1 mg/ml mesalazine during a time course: 0, 5, 15, 30, and 60 min. Afterwards, total cellular proteins were extracted in a phosphate-free buffer (10 mM Tris HCl, pH 7.6, 100 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, 2 mM EGTA, 1% Triton-X-100, 0.1 mM MnCl2, 0.1 mg/ml BSA, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM Pefablock) sonicated and spun down. A-specific binding was blocked by preincubation for 2 h with protein-G sepharose beads (Sigma). After centrifugation, supernatants were incubated overnight at 4°C with anti-PP2A catalytic site α antibodies (1: 2000). Subsequently, samples were incubated with protein-G sepharose beads for 2 h at 4°C. Hereafter, beads were washed in phosphate-free buffer before the addition of 200 μl of phosphate-free buffer. Immunoprecipitates were used for PP2A activity determination.

PP2A activity determination

Phosphatase activity was assessed by using the malachite green-based phosphatase assay kit (Upstate Biotechnology) that measures phosphate release. Assays were run according to the manufacturer’s instructions. Briefly, 25 μl per sample were incubated with 100 μM of phosphopeptide (amino acid sequence: KRTPTRR, obtained from the kit), in a total volume of 50 μl. Reaction was started by addition of the phosphopeptide and conducted for 5 min at room temperature. The reaction was stopped by the addition of 100 μl malachite green solution. After 15 min of color development, the release of phosphate was quantified by measuring the absorbance at 650 nm in a microtiterplate reader. The amount of released phosphate (pmol) was
Fig. 1. Mesalazine downregulates the Wnt/β-catenin pathway. (A) Left panel: SW480 cells were transiently co-transfected with either pTOPFLASH (contains optimal binding sites for TCF) or pFOPFLASH (in which the optimal binding sites for TCF are mutated) luciferase reporter constructs as well as a pCMV-Renilla luciferase construct to serve as an internal control for transfection efficiency and potential toxicity of treatments, followed by incubation with various concentrations of mesalazine (as indicated) for 48 h. Afterwards, cells were lysed and analyzed using the luciferase assay system (Roche). Incubations were performed in triplicate and experiments were repeated three times. The presented results are represented as light units, normalized to the internal control. Right panel: no effects of mesalazine were seen in SW480 cells transfected with other reporter assays; an ISRE-reporter (upper graph) and a TAL-reporter (lower graph) (for more information, see Materials and methods). (B) Western blot analysis of DLD-1 and SW480 cells after treatment with various concentrations of mesalazine (as indicated) for 72 h. A dose-dependent decrease of cyclin D1, c-myc and c-met expression is shown. β-Actin was used as a loading control. This blot is representative of three individual experiments. All blots were quantified and are shown in bar graphs (right panels) (*P-value < 0.05). The bar graph (left panel) shows the relative amounts of Wnt target expression corrected for β-actin loading controls. (C) DLD-1 and SW480 cells were incubated with 1 mg/ml mesalazine for several hours (as indicated). Nuclear fractions were isolated and western blot analysis was performed. A reduction in β-catenin expression in the nuclear fraction is shown. Expression of β-actin and TCF4 in the nuclear samples were assayed as a control to assess purity of the isolation (β-actin is not expressed in the nuclear fraction; TCF4 is expressed in the nuclear fraction) and as a control for equal loading. These blots are representative of two individual experiments, performed in duplex. All blots for β-catenin were quantified and are shown in the bar graph (right panel) (*P-value < 0.05).
then calculated from a standard curve (0–2000 pmol). Percentages of PP2A activity in mesalazine-treated cells are compared with basal PP2A activity measured in untreated cells.

**Transient transfection with siPP2A catalytic site α**

DLD-1 cells at 40% confluence were transiently transfected with PP2A catalytic subunit α SMARTpool® siRNA reagent (Upstate Biotechnology). Transfections were carried out using Effectene transfection kit (Qiagen) according to the manufacturer’s instructions. Briefly, cells were transfected with 5 µl of PP2A catalytic subunit α SMARTpool® siRNA reagent (final concentration: 100 nM), 8.8 µl enhancer and 30 µl Effectene transfection reagent for 24 h. Hereafter, the transfection medium was replaced by a culture medium for 24 h, followed by the replacement of the culture medium with one containing 0.5% FCS for another 16 h, and then treated with various concentrations of mesalazine (0.5, 1 and 2 mg/ml mesalazine) for 1 h. Afterwards, cells were washed in PBS and scraped into 200 µl cell lysis buffer (described in western blot analysis), sonicated and spun down. Subsequently, lysates were mixed with 3x SDS sample buffer. Transfection efficiency was assessed by expression of PP2A catalytic site α by western blot analyses (on average 52% decreased expression).

**Transient transfection with PP2A constructs**

DLD-1 cells at 40% confluence were transiently transfected with an N-terminal triple (HA)-tagged PP2A catalytic subunit C construct (30). One construct in which tyrosine 307 of the catalytic subunit is mutated to a phenylalanine ([HA]3-CY307F) was used to eliminate the site of phosphorylation and one wild-type catalytic subunit ([HA]3-CWT) constructed as control (30). The constructs were kindly provided by Dr D.L.Brautigan from the University of Virginia, Charlottesville, Virginia, USA. Transfections were carried out using Effectene transfection kit (Qiagen) according to the manufacturer’s instructions. Briefly, cells were transfected with 0.4 µg construct ([HA]3-CWT or [HA]3-CY307F, 1.6 µl enhancer and 10 µl Effectene transfection reagent for 24 h. Hereafter, the transfection medium was replaced by a culture medium for 24 h, followed by the replacement of the culture medium with one containing 0.5% FCS for another 16 h, and then treated with 1 mg/ml mesalazine for 1 h. Afterwards, cells were washed in PBS and scraped into 200 µl cell lysis buffer (described in western blot analysis), sonicated and spun down. Subsequently, lysates were mixed with 3x SDS sample buffer. Transfection efficiency was assessed by expressing the site of phosphorylation α by western blot analyses (on average 52% decreased expression).

**Results**

**Mesalazine inhibits the Wnt/β-catenin pathway**

The Wnt/β-catenin pathway controls the transcription of genes through the binding of a complex of β-catenin and Tcf/ Lef to specific promoter elements. The activity of this final step in the Wnt/β-catenin pathway can be measured using a luciferase reporter construct (28). SW-480 cells were transiently transfected with the pTOPFLASH (Tcf optimal binding site) or pFOPFLASH (containing a ‘far from optimal’ Tcf binding site) construct together with a pCMV-Renilla luciferase construct to act as an internal control for transfection efficiency and potential toxicity of treatments. Figure 1A, left panel, shows that mesalazine downregulates the constitutively active β-catenin/Tcf signaling found in SW-480 cells in a dose-dependent manner. The specificity of the Tcf reporters was confirmed using pTOPFLASH construct (TOPFLASH construct in which the optimal Tcf binding sites are mutated; data not shown). Furthermore, no effects of mesalazine were seen on other reporter assays (an ISRE-reporter and TAL-reporter), which demonstrated that mesalazine specifically targeted the Wnt/β-catenin pathway (Figure 1A, right panel).

To confirm that mesalazine inhibits the Wnt/β-catenin pathway activity, protein levels of various Wnt/β-catenin targets were assessed by western blot analysis. DLD-1 and SW480 cells were incubated with various concentrations of mesalazine, and after 72 h the expression of cyclin D1, c-met and c-myc, all proteins known to be influenced via the Wnt/ β-catenin pathway, was assessed (31,32). As shown in Figure 1B, mesalazine induces a decrease in the expression of cyclin D1, c-met and c-myc in both cell lines. Further support for the notion that mesalazine decreases the activity of Wnt/β-catenin pathway was obtained from experiments in which nuclear fractions from DLD-1 and SW480 mesalazine-treated cells were investigated for the effect of mesalazine on the nuclear accumulation of β-catenin by western blot analysis. Mesalazine treatment reduced nuclear β-catenin levels (Figure 1C), which is in agreement with the effects observed with mesalazine on Wnt/β-catenin pathway activity. Inclusion in Figure 1C is the expression of β-actin and TCF4 in the nuclear samples. These were assessed as a control to assess purity of the isolation (β-actin is not expressed in the nuclear fraction; TCF4 is expressed in the nuclear fraction) and as a control for equal loading. Together, these experiments show that mesalazine efficiently reduces the activity of the Wnt/β-catenin pathway in colorectal cancer cells.

**Mesalazine leads to increased threonine 41/serine 45 phosphorylation of β-catenin in APC mutant cells**

To identify the interaction point of mesalazine with the Wnt/ β-catenin pathway, we examined the effect of mesalazine on the phosphorylation status of β-catenin. Phosphorylated β-catenin is targeted for ubiquitination and subsequent destruction and thus this phosphorylation is a controlling event in Wnt/β-catenin pathway activity (16,33). Mesalazine caused time- and dose-related (Figure 2A and B) increase in Thr41/Ser45 phosphorylation of β-catenin in DLD-1 and SW480, suggesting that increased phosphorylation of β-catenin underlies the effects of mesalazine on Wnt/ β-catenin pathway activity.

**Mesalazine has no effect on Wnt/β-catenin pathway in β-catenin mutant cells**

So far we have shown an inhibitory effect of mesalazine on the Wnt/β-catenin pathway in APC mutant cell lines with wild-type β-catenin. APC mutations are seen in ~85% of all colorectal cancers (16). If the increased Thr41/Ser45 phosphorylation of β-catenin underlies the effect of mesalazine on Wnt/β-catenin pathway activity, colon cancer cells in which the activity of the Wnt/β-catenin pathway is not regulated by this phosphorylation—that is, β-catenin mutant cells—should not respond to mesalazine with reduced activity of the Wnt/β-catenin pathway. Hence, we assessed the effect of mesalazine on the Wnt/β-catenin pathway in two β-catenin mutant cell lines: HCT116 and SW48 (19,34–36). HCT116 and SW48 cells were treated with 1 or 2 mg/ml mesalazine for 48 h (Figure 3A) and 72 h (Figure 3B) and the expression of the Wnt/β-catenin signal transduction target genes c-myc, c-met and cyclin D was assessed by western blot analysis and quantified. Levels of these various Wnt pathway targets is not influenced by mesalazine in either cell line, supporting the hypothesis that increased Thr41/Ser45
Fig. 2. Mesalazine incubation increases the phosphorylation of β-catenin in dose- and time-dependent manner in APC mutant cells. (A) Western blot analysis of DLD-1 and SW480 (both APC mutant cell lines) cells incubated with 1 mg/ml mesalazine during a time course (as indicated). A time-dependent increase of phosphorylation of β-catenin is shown. Blots were re-incubated with total β-catenin antibody as a loading control. These blots are representative of at least three individual experiments. All blots for phosphorylated β-catenin were quantified and are shown in the bar graph (right panel) (*P-value < 0.05, **P-value < 0.01, ***P-value < 0.001). (B) Western blot analysis of DLD-1 and SW480 cells treated with various concentrations of mesalazine (as indicated) for 60 min. A dose-dependent increase of phosphorylation of β-catenin is shown. Blots were re-incubated with total β-catenin antibody as a loading control. These blots are representative of three individual experiments. All blots for phosphorylated β-catenin were quantified and are shown in the bar graph (right panel) (*P-value < 0.05, **P-value < 0.01, ***P-value < 0.001).

Fig. 3. Mesalazine does not affect the Wnt/β-catenin pathway in β-catenin mutant cells. (A) Western blot analysis of HCT116 and SW48 cells (both β-catenin mutant cell lines) were incubated with 0, 1 and 2 mg/ml mesalazine for 48 h. No differences in expression of cyclin D1, c-myc and c-met expression are seen. β-actin was used as a loading control. This blot is representative of at least two individual experiments. The bar graph (right panel) shows the relative amounts of Wnt target expression corrected for β-actin loading controls. (B) Quantified western blots of HCT116 cells that were incubated with 0, 1 and 2 mg/ml mesalazine for 72 h, showing the relative amounts of Wnt targets expression corrected for β-actin loading controls, of two individual experiments performed in duplex.
phosphorylation of β-catenin underlies the antagonizing effect of mesalazine on the Wnt/β-catenin pathway.

**PP2A inhibition by okadaic acid abrogates the effect of mesalazine on β-catenin phosphorylation**

The net phosphorylation status of a protein is dependent on kinase and phosphatase activity. It is known that calyculin A and okadaic acid, specific phospho-serine/phospho-threonine phosphatase inhibitors, induce hyperphosphorylation of β-catenin on serine–threonine residues (37). Furthermore, recent evidence has emerged that PP2A is a possible regulator of the Wnt/β-catenin pathway in colon cancer cells (25,26).

To investigate whether PP2A is an essential mediator of mesalazine effects on the Wnt/β-catenin pathway, we employed okadaic acid, a well-known inhibitor of PP2A. We treated DLD-1 and SW480 cells with various concentrations of mesalazine in the presence of okadaic acid, after a 30 min preincubation with okadaic acid (Figure 4A). In agreement with a role for PP2A in controlling β-catenin phosphorylation, we observed that treatment with okadaic acid increased the phosphorylation of β-catenin (Figure 4A). On preincubation of both SW480 cells and DLD-1 cells with 50 or 100 nM of okadaic acid [concentration commonly used in cell-based assays (38,39)], which efficiently inhibits PP2A without affecting protein phosphatase 1 (PP1) (40–42), the effect of mesalazine on β-catenin phosphorylation was no longer observed (Figure 4A). The same results were seen in DLD-1 and SW480 cells treated with 1.0 mg/ml mesalazine treated in a 60 min time course (an example using DLD-1 cells is shown in Figure 4B). This suggests that mesalazine leads to increased phosphorylation of β-catenin by inhibiting PP2A.

**Mesalazine decreases PP2A activity**

Having shown that when the PP2A inhibitor okadaic acid is used, there is no effect of mesalazine on β-catenin phosphorylation, together with new evidence that PP2A is a possible regulator of the Wnt/β-catenin pathway in colon cancer cells (22,23), we were prompted to investigate a possible effect of mesalazine on PP2A activity. To this end we evaluated the phosphorylation status of the inhibitory tyrosine 307 in PP2A by western blot analysis. As evident from Figure 5, mesalazine caused a time- and dose-dependent increase of phosphorylation of PP2A at this amino acid residue. These results suggest that mesalazine negatively influences PP2A enzymatic activity.

To establish whether increased tyrosine 307 phosphorylation actually corresponds with reduced PP2A activity we performed a malachite green assay. In this assay PP2A activity is measured by the release of Pi from the synthetic substrate KIpTIRR. Since this assay cannot fully differentiate between PP1 and PP2A activity, PP2A was first purified by immunoprecipitation and these samples were used for the malachite green assay. As evident from Figure 5C, mesalazine treatment (1 mg/ml, 60 min) reduced PP2A enzymatic activity in DLD-1 cells, which was consistent with the effects of mesalazine on β-catenin phosphorylation (P < 0.01). Similar data were observed in SW480 cells (data not shown).

To establish that the inhibitory effect of mesalazine on PP2A activity actually represents a PP2A-specific effect we compared the effects of this compound on PP2A phosphatase activity with the effect of mesalazine on total cellular phosphatase activity and with the effect of mesalazine on PP1-dependent dephosphorylation. Mesalazine neither influenced total cellular phosphatase activity nor did it affect PP1 enzymatic activity, whereas PP2A activity in the same samples was substantially reduced (data not shown). Furthermore, when we assayed the effect of mesalazine on the release of Pi from the synthetic substrate KIpTIRR, we could not detect any effect, which shows that the observed release of Pi from the synthetic substrate is not the effect of the treatment itself (data not shown). These results suggest that mesalazine negatively influences PP2A enzymatic activity.

**The effect of mesalazine on the phosphorylation of Tyr307 of the catalytic subunit of PP2A is essential for its effect on β-catenin phosphorylation**

To further investigate whether the increased phosphorylation of β-catenin is directly linked to the increased phosphorylation and thus inhibition of PP2A, we used siRNA directed against the transcript of the catalytic α subunit of PP2A. As shown in Figure 6A, this procedure efficiently downregulated expression of the catalytic α subunit of PP2A in DLD-1 cells (on average 52% decreased expression). Furthermore, this procedure increased the phosphorylation of β-catenin, demonstrating that PP2A is an in vivo regulator of the β-catenin phosphorylation status. Importantly, under these conditions mesalazine lost the capacity to react to mesalazine treatment with further increased phosphorylation of β-catenin. Hence PP2A is essential for mesalazine effects on the Wnt/β-catenin pathway. Further support for this concept was obtained from experiments in which DLD-1 cells were transiently transfected with two N-terminal triple (HA)-tagged PP2A catalytic subunit C constructs (30). A wild-type catalytic subunit [(HA)₃-CWT] construct served as a control and a second construct in which tyrosine 307 of the catalytic subunit is mutated to a phenylalanine [(HA)₃-C307F] was used to modulate PP2A activity. Phosphorylation at tyrosine 307 inactivates PP2A. The antibody used in phosphorylated PP2A western blot analysis also recognizes phosphorylation at tyrosine 307 (shown in Figure 5). After transient transfection of DLD-1 cells with the (HA)-tagged PP2A constructs, cells were treated with 1 mg/ml mesalazine for 60 min. As expected, we observed an increase in the phosphorylation of β-catenin after 1 h incubation with 1 mg/ml mesalazine (Figure 6B) in the non-transfected cells. The same effect is seen in cells that were transiently transfected with the wild-type construct, whereas in the cells that were transiently transfected with the mutant construct, mesalazine had no effect on the phosphorylation of β-catenin (Figure 6B). To test the effect of PP2A inhibition in an additional assay, we performed a TOPFLASH assay in DLD-1 cells. The left panel of Figure 6C shows that mesalazine downregulates the constitutively active β-catenin/TCF signaling to the same extent as PP2A inhibition by okadaic acid. Furthermore, no effect of the combination of mesalazine and PP2A inhibition was seen, suggesting that the effect of mesalazine is via its effect on inhibition of the activity of PP2A. In the right panel we also show the TOPFLASH assay of β-catenin/TCF transcriptional activity in DLD-1 cells co-transfected with the same constructs used in Figure 6B. Mesalazine, okadaic acid and the combination of the two reduced the β-catenin/TCF transcriptional activity to the same extent in samples transfected with the wild-type PP2A construct. However,
the mutant PP2A construct alone reduced β-catenin/TCF transcriptional activity down to the same levels as seen with mesalazine treatment when using the wild-type construct. Treatment with mesalazine or okadaic acid was unable to further repress transcriptional activity, suggesting that the mutant construct, mesalazine and okadaic acid, which are all individually able to reduce β-catenin/TCF transcriptional activity, may act through the same mechanism.

Fig. 4. PP2A inhibition by okadaic acid abrogates the effect of mesalazine on β-catenin phosphorylation. Western blot analysis of DLD-1 and SW480 cells preincubated for 30 min with 100 nM okadaic acid or with vehicle control and subsequently incubated with various concentrations of mesalazine for 60 min or with 1 mg/ml mesalazine during a 60 min time course (as indicated) alone or in the presence of okadaic acid. A dose-dependent (A) and time-dependent (B) increase of phosphorylation of β-catenin is shown in cells treated with mesalazine, which is abrogated by the incubation with the PP2A inhibitor okadaic acid. Blots were reprobed with total β-catenin antibody as a loading control. These blots are representative of three individual experiments. All blots for phosphorylated β-catenin were quantified and are shown in bar graphs (*P-value < 0.05, **P-value < 0.01, ***P-value < 0.001).
We conclude that treatment of colon cancer cell lines with mesalazine promotes β-catenin phosphorylation by inactivation of PP2A. This is achieved by promoting the phosphorylation of Tyr307 on the catalytic subunit of PP2A, thereby inactivating it.

**Discussion**

Apart from its anti-inflammatory characteristics, mesalazine treatment in ulcerative colitis is associated with a reduced incidence of colorectal cancer. Mesalazine therapy could...
therefore potentially be used as a chemopreventive agent for colorectal cancer, especially because unlike other NSAIDs mesalazine displays few side effects during long-time treatment. Rational use of mesalazine for chemoprevention, however, has been hampered by lack of insight into its molecular mode of action in colon cancer prevention. In the present study we provide evidence that mesalazine acts through PP2A inhibition to increase \( \beta \)-catenin phosphorylation, thereby reducing Wnt/\( \beta \)-catenin pathway activity.

Importantly, patients with sporadic polyps of the large bowel treated with mesalazine show a significant induction of apoptosis and a decrease in proliferation in colorectal mucosa (10), and in patients with colorectal cancer treated with mesalazine an induction of apoptosis and a decrease in proliferation is seen in tumor cells (9). Furthermore, a randomized double-blind multicenter trial conducted by Schmiegel et al. (43) showed a reduced recurrence rate of sporadic colorectal adenomas in high-risk patients treated with mesalazine. While in rodent models of colorectal cancer the effects of mesalazine are somewhat conflicting (44), most investigators find a chemopreventive effect of mesalazine analogs in colorectal cancer both in chemically and genetically induced colorectal cancer (8,45).

Fig. 6. Effect of mesalazine on \( \beta \)-catenin is mediated via the inactivation of PP2A by phosphorylation on Tyr307 of the catalytic subunit of PP2A. (A) DLD-1 cells were transiently transfected with PP2A catalytic subunit \( \beta \) SMARTpool\textsuperscript{\textregistered} siRNA reagent for 24 h. Hereafter, cells were incubated with various concentrations of mesalazine (as indicated). Western blot analysis shows a dose-dependent increase in \( \beta \)-catenin phosphorylation in the untransfected cells, which is not seen in cells transfected with siPP2A catalytic subunit \( \alpha \). Expression of PP2A catalytic subunit \( \alpha \) is used as control for the siPP2A catalytic subunit \( \alpha \) transfection. \( \beta \)-actin was used as a loading control. The bar graph (right panel) shows the relative amounts of phosphorylated \( \beta \)-catenin corrected for \( \beta \)-actin. (B) DLD-1 cells were transiently transfected with either wild-type or mutant PP2A constructs for 24 h. Hereafter, cells were incubated with 1 mg/ml mesalazine for 60 min. Western blot analysis shows an induction of phosphorylation of \( \beta \)-catenin after treatment with mesalazine in the non-transfected cells and in the cells transiently transfected with a PP2A construct containing a wild-type catalytic subunit. No effect of mesalazine is seen in the cells transiently transfected with a mutant construct of PP2A. \( \beta \)-actin was used as a loading control. These blots are representative of three individual experiments. The bar graph (right panel) shows the relative amounts of phosphorylated \( \beta \)-catenin corrected for \( \beta \)-actin. (C) DLD-1 cells were transiently either co-transfected with both pTOPFLASH and pCMV-\textit{Renilla} (left panel) or co-transfected with the pTOPFLASH, pCMV-\textit{Renilla} and with either wild-type or mutant PP2A construct (right panel) overnight. The transfection was followed by a preincubation with 100 nM okadaic acid or vehicle control for 30 min, and hereafter, the cells were treated with 1 mg/ml mesalazine, 100 nM okadaic acid or a combination of both (as indicated), for 1 h. Afterwards, cells were lysed and analyzed using the luciferase assay system (Roche). Incubations were performed in triplicate and experiments were repeated three times. The presented results are represented as light units, normalized to the internal control.
Most previous mechanistic studies of mesalazine have investigated its anti-inflammatory effect in models of inflammation (46–49). However, two articles have been published investigating the inflammation-independent properties of mesalazine: Gasche et al. (50) show that mesalazine improves replication fidelity, an effect that may be active in reducing mutations, which was independent of mismatch repair proficiency. Furthermore, Reinacher-Schick et al. (51) show that mesalazine induced mitotic arrest and apoptosis in HT-29 colon cancer cells. The concentrations of mesalazine used in these publications vary widely, ranging from 0 to 40 mM, and the treatment period was anywhere between 1 h and 7 days. The treatment periods used in our experiments are comparable with those used in the paper of Reinacher-Schick et al., and the concentrations we have used (1–2 mg/ml; or 7–13 mM) are the same as those found intraluminally and intramucosally in IBD patients receiving mesalazine maintenance treatment (7–14 mM), which is known to reduce colon cancer incidence (52,53). Frieti et al. (54) have shown that rectal application of mesalazine increases mesalazine concentrations to >40 mM, substantially in excess of those necessary to inhibit Wnt/β-catenin pathway activity in our experiments.

Interestingly, a discrepancy is noted in the kinetics of β-catenin phosphorylation and degradation, especially in the nucleus, lower β-catenin levels substantially lagging the increase in phosphorylation; this observation is even more evident for the SW480 cells than the DLD-1 cells. An explanation may be found in our observation that there is an increase in phosphorylated β-catenin in the nucleus upon mesalazine treatment (data not shown). It has been shown, however, that with our TCF-responsive reporter (pTOP-FLASH), only the unphosphorylated form of β-catenin has transactivating potential (55). This is supported by work from Sadot et al. (56), who show that phosphorylated β-catenin forms a transcriptionally inactive complex with the LEF-1, although it accumulates in the nucleus. However, differences between the two cell lines observed could be due to the slower induction of phosphorylation of β-catenin in SW480 (see Figures 2 and 4A).

Furthermore, after 60 min a reduced expression of β-catenin in SW480 whole cell lysates was observed. β-catenin exists in two pools (a cytoplasmic signaling pool and E-cadherin-membrane bound pool); therefore, the ratio between these two pools determines whether a decrease in the cytoplasmic pool is visible or not when assessing total levels of β-catenin. This is apparently not a constant factor between various cell lines, because in SW480 cells a visual reduction of total β-catenin levels is seen even without separating the structural and signaling pools of β-catenin.

It should further be noted when interpreting our results that western blot analysis to assess the phosphorylation of PP2A is a fairly insensitive assay, resulting in an increase of phosphorylation of β-catenin (assessed by western blot analysis) at time-points when no effect is seen in the phosphorylation of PP2A. Furthermore, levels of phosphorylated PP2A appear to be not very high after 60 min of mesalazine treatment, but a huge effect can be shown in the (sensitive) enzymatic activity assay at this same time-point. It is therefore impossible to compare both signals in terms of absolute levels, especially as western blot data are essentially non-linear and thus more qualitative than quantitative in nature. However, these observations show the cardinal importance of PP2A in the regulation of β-catenin phosphorylation.

A further important finding of this study is the involvement of PP2A in the regulation of Wnt/β-catenin pathway activity in colon cancer cells. Association between PP2A and the Wnt/β-catenin pathway in X. laevis embryos has been previously demonstrated. The catalytic subunit of PP2A (the subunit investigated in this paper) potentiated secondary axis formation, and when the Wnt/β-catenin pathway is activated by inducing stabilized β-catenin, the activity of the Wnt/β-catenin pathways is subsequently subject to regulation by this catalytic subunit and by inhibitors of PP2A (26). It has also been shown that the expression of the B56 PP2A subunit reduces the abundance of β-catenin and inhibits transcription of β-catenin target genes in mammalian cells and X. laevis embryo explants (25) and that the B56γ subunit of PP2A is required for Wnt/β-catenin signaling during embryonic development in X. laevis embryos (57). Finally, overexpression of the B56γ subunit of PP2A in rats resulted in severe alterations in fetal lung branching morphogenesis that correlated with suppression of β-catenin levels (58). Thus, the current body of existing literature is consistent with a role for PP2A in the control of the Wnt/β-catenin pathway as suggested by the present study, although the evidence presented to date has been mainly on a genetic level via the introduction of PP2A subunits.

In the present manuscript we show that PP2A enzymatic activity influences the phosphorylation status of β-catenin and β-catenin/TCF transcriptional activity and that mesalazine leads to reduced PP2A activity, which may explain its effects on Wnt/β-catenin pathway activity. As this observation demonstrates that mesalazine can directly influence the Wnt/β-catenin pathway downstream of the APC mutation (which is generally accepted to be both the first and the most common mutation in colorectal carcinogenesis), this activity may well explain the chemopreventive effects of mesalazine. Hence our data support more widespread use of mesalazine in the chemoprevention of colorectal cancer.

Conflict of Interest Statement: None declared.

References


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