JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Modulation of Key Elements of the Wnt Pathway by Apple Polyphenols

MELANIE KERN, GUDRUN PAHLKE, YUFANYI NGIEWIH, AND DORIS MARKO*

Institute of Applied Biosciences, Section of Food Toxicology, University of Karlsruhe (TH), Fritz-Haber-Weg 2, 76131 Karlsruhe, Germany, and Division of Food Chemistry and Environmental Toxicology, Department of Chemistry, University of Kaiserslautern, Germany

Glycogen synthase kinase-3β (GSK3β) is one of the key elements of the Wnt pathway involved in the regulation of β-catenin homeostasis. The inhibition of GSK3β kinase activity might lead to the onset of β-catenin/TCF/LEF-mediated gene transcription, representing a potentially mitogenic stimulus. Apple polyphenols have been shown to mediate several biological effects that might be of interest with respect to chemoprevention. The objective of the study was to elucidate whether apple polyphenols also modulate key elements of the Wnt pathway, an effect that might limit the usefulness of these compounds for the prevention of carcinogenesis. A polyphenol-rich apple juice extract (AE02) was found to effectively inhibit the kinase activity of GSK3β, immunoprecipitated from HT29 cells. Treatment of HT29 cells with AE02 for 24 h resulted in a concentration-dependent decrease of the cellular GSK3β kinase activity. The inhibition of the kinase activity in HT29 cells was observed at polyphenol concentrations corresponding to the concentration of the constituents in the original apple juice. The apple characteristic dihydrochalcone glycoside phloridzin was found to be inactive up to 500 μM. The free aglycon phloretin as well as the flavonol quercetin effectively inhibited isolated GSK3β, but did not affect the respective kinase activity within HT29 cells. In accordance with the inhibition of GSK3β kinase activity by AE02, treatment of HT29 cells resulted in a significant decrease of phosphorylated β-catenin. However, the total intracellular β-catenin level was also found to be diminished, indicating that the interference of the apple constituents with GSK3β was not associated with a stabilization of β-catenin in HT29 cells. In line with these results, TCF/LEF-mediated gene transcription remained unaffected by treatment with AE02 as shown in a reporter gene approach. We can assume from the results that at consumer-relevant concentrations apple polyphenols do not mediate growth-stimulating effects in HT29 cells via the Wnt pathway.

KEYWORDS: Phloretin; phloridzin; quercetin; glycogen synthase kinase-3β; β-catenin; colon carcinoma; HT29

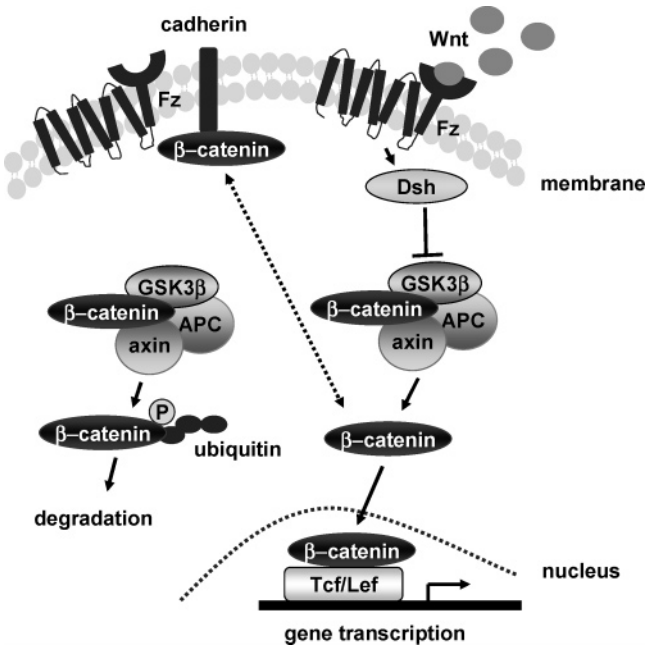
INTRODUCTION

Colorectal cancer is one of the leading causes of cancer mortality in Western countries. Lifestyle and diet are proposed to play an important role in colon carcinogenesis. Epidemiological studies have shown that colorectal cancer incidence could be significantly modulated by dietary intake of flavonoids with fruits and vegetables (*1*−*4*). In particular, compounds within the huge class of polyphenols have been discussed as contributing to the prevention of colon cancer (*5*−*7*). In colon carcinogenesis, the Wnt pathway (**Scheme 1**) appears to play an important role (*8*−*11*), with the serine/threonine kinase GSK3β being one of the key elements, regulating intracellular β-catenin levels. In the absence of pathway stimulation, β-catenin protein is destabilized by a cytoplasmatic complex consisting mainly of axin, adenomatous polyposis coli (APC) protein, and glycogen synthase kinase-3β (GSK3β). In a resting cell, β-catenin is marked for degradation by GSK3β-mediated phosphorylation, maintaining low cellular levels of β-catenin. Activation of the Wnt receptor complex, consisting of a seven-transmembrane receptor frizzled (Fz) and co-receptors, leads to the activation of disheveled (Dsh), finally suppressing GSK3β-mediated β-catenin phosphorylation. Nonphosphorylated β-catenin accumulates within the cell and translocates into the nucleus. As a consequence TCF/LEF-driven gene transcription is induced, representing a proliferative stimulus. However, the induction of β-catenin-mediated transcription is not necessarily limited to the stimulation of the Fz receptor, but can also be achieved by the inhibition of GSK3β kinase activity. Recently, a series of low molecular inhibitors of different structural classes have been reported to possess GSK3β inhibitory properties (*12*−*15*). The interference with GSK3β activity might imply an undesired proproliferative signal, limiting the use of respective compounds in the prevention of colon

* Corresponding author. Telephone: +49-(0)721-6082936. Fax: +49-(0)721-6087254. E-mail: doris.marko@lmc.uni-karlsruhe.de.

10.1021/jf0606611 CCC: $33.50
Published on Web 08/23/2006

Scheme 1. Simplified Scheme of the Wnt Pathway[a]



[a] APC, adenomatous polyposis coli protein; Dsh, disheveled; Fz, frizzled receptor; GSK3β, glycogen synthase kinase-3β; LEF, lymphoid enhancer factor; TCF, T-cell factor.

carcinogenesis. Polyphenol-rich apple extracts have been reported to possess bioactive properties, suppressing human colon cancer cell growth in vitro (*16*−*19*). In a rat colon carcinogenesis model a consumer-relevant apple juice has been shown to decrease DNA damage, hyperproliferation, and aberrant crypt foci development induced by dimethylhydrazine treatment (*20*). Thus, apple polyphenols might be of interest with respect to chemoprevention. However, little is known thus far about the impact of apple polyphenols on key elements of the Wnt pathway. In the present study, we investigated whether apple polyphenols modulate GSK3β activity in human colon carcinoma cells and whether subsequently β-catenin homeostasis and TCF/LEF-driven gene transcription are affected. For comparison, the apple characteristic dihydrochalcone phloridzin (PHD), the respective aglycon phloretin (PHL), and the flavonol quercetin (QUE) were included in the testing. The objective of the study was to elucidate whether the interference of apple polyphenols with the Wnt pathway might be taken into account as a potential limitation of the usefulness of these compounds in terms of chemoprevention.

MATERIALS AND METHODS

Chemicals. Polyphenol-rich apple juice extract was produced as reported previously (*17*, *21*) and provided by Prof. Dietrich from the State Research Institute Geisenheim, Germany. PHL and PHD were purchased from Roth (Karlsruhe, Germany), and QUE was obtained from Sigma Aldrich, Germany. SB-216763 was purchased from Tocris, UK. For all assays, compounds and extract were freshly prepared by dissolving in DMSO shortly before the experiment. The final concentration of DMSO in the different test systems did not exceed 1%.

Cell Culture. The human colon carcinoma cell line HT29 and human embryonic kidney cells HEK-293 were purchased from the German Collection of Microorganism and Cell Culture in Braunschweig, Germany. Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/L glucose, without sodium pyruvate, supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS) in humidified atmosphere at 37 °C and 5% CO_2. Cell culture medium and the supplements were obtained from Invitrogen Life Technologies, Germany.

Immunoprecipitation of GSK3β. A quantity of 5×10^6 cells was seeded per Petri dish (145 cm^2) and grown for 48 h. Before harvesting, the medium was removed and cells were washed with 5 mL of phosphate buffered saline (PBS). Harvesting and lysate preparation were performed at 4 °C. HT29 cells were scraped in 0.5 mL of lysis buffer (25 mM Tris-HCl, pH 7.4, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 0.27 M sucrose, 10 mM Na-β-glycerophosphate, 5 mM Na-pyrophosphate, 0.5% (v/v) Triton-X 100, 2 mM Na_3VO_4; 2% (v/v) protease inhibitor cocktail (Roche Diagnostics, Germany), 0.1% (v/v) β-mercaptoethanol, and 0.1% microcystin were added just before lysis). Thereafter, the lysate was homogenized thoroughly and subsequently centrifuged for 10 min (20000*g*, 4 °C). The colorimetric procedure of Bradford (*22*) was applied to determine the content of protein using bovine serum albumin as a standard.

A quantity of 2.5 μL of monoclonal GSK3β antibody (BD Transduction Laboratories, Lexington, KY) was incubated with 50 μL of activated Protein G-sepharose (Sigma Aldrich, Germany) for 60 min at 4 °C with intermittent mixing (every 5 min). A quantity of 600 μg of total protein from cell whole extract was added to the antibody G-sepharose complex. The reaction volume was supplemented to a final volume of 1 mL with PBS, and the reaction was performed overnight at 4 °C on a shaker.

GSK3β Kinase Assay. Kinase activity was determined in kinase buffer (50 mM Tris HCl, pH 7.4, 1 mM EGTA, 0.15 M NaCl, 0.03% Brij-30, 0.1% (v/v) β-mercaptoethanol) according to published methods (*23*, *24*). In a final volume of 150 μL, 15 μL of the substrate peptide (0.5 mg/mL, RRAAEELDSRAG*p*SPQL, Biosyntan GmbH, Germany), 30 μL of [γ-^{32}P]-ATP (0.2 μCi/30 μL, 75 mM $MgCl_2$, 500 μM ATP), 15 μL of protein kinase inhibitor (PKI tide, Alexis, Lausen, Switzerland) (10 μM), and 15 μL of the respective test compound were incubated with 15 μL of sample and kinase buffer (60 μL for sample from treated cells and 75 μL for sample from untreated cells) for 15 min at 37 °C. A quantity of 3×35 μL of kinase reaction mix was spotted onto P81 phosphocellulose paper and air-dried. Filters were washed three times with 0.75% phosphoric acid and once with aceton allowing to air-dry. Phosphorylated substrate was determined by liquid scintillation counting.

Preparation of Cellular Extract. A quantity of 1.5×10^6 cells was seeded per Petri dish (57 cm^2) and allowed to grow for 48 h. Thereafter, the FCS content of the culture medium was reduced to 1% for 24 h. The cells were incubated for 24 h, serum-free, with test compounds dissolved in DMSO (final DMSO concentration of 1%). Cells were abraded at 4 °C in 0.2 mL of lysis buffer as described above. Thereafter, the lysate was homogenized thoroughly and centrifuged for 10 min (20000*g*, 4 °C). The supernatant was used for immunoprecipitation and the subsequent GSK3β kinase assay as well as for Western blot analysis.

Western Blot Analysis. A quantity of 20 μL of cellular extract (40 μg protein) was separated by SDS-PAGE (10% acrylamide gel), and proteins were transferred onto a nitrocellulose membrane. Blocking the membrane for nonspecific protein binding was carried out for 1 h in 5% (w/v) dry milk (Roth, Germany) in TBS/Tween-20 (0.1%). Western blot was performed using antibodies against human GSK3β (46 kDa, BD Transduction Laboratories) and human β-catenin (92 kDa, Cell Signaling Technology) as well as phosphorylated β-catenin (Ser33/Ser37/Thr41; 92 kDa. Cell Signaling Technology) following the manufacturer's protocol. Peroxidase-conjugated immunoglobulin G (IgG) against rabbit (Cell Signaling Technology) and mouse (Santa Cruz Biotechnology) was employed as a secondary antibody. α-Tubulin (54 kDa, Santa Cruz Biotechnology) was used as a loading control. The respective chemoluminescent signals (LumiGLO, Cell Signaling Technology) were analyzed using the Lumi-Imager with the Lumi-Analyst 3.0 software for quantification (Roche Diagnostics, Germany). Arbitrary light units were plotted as test over control (%), with the control representing cells treated with 1% DMSO (solvent control).

TCF/LEF Reporter Gene Assay. A quantity of 5.0×10^5 HEK-293 cells per Petri dish (57 cm^2) was seeded and allowed to grow for 72 h. Thereafter, cells were transiently transfected with 5 μg of the luciferase reporter constructs (TOPflash or FOPflash, Upstate Biotechnology) using FuGENE-6 transfection reagent (Roche Diagnostics, Germany). Twenty-four hours after transfection, cells were detached

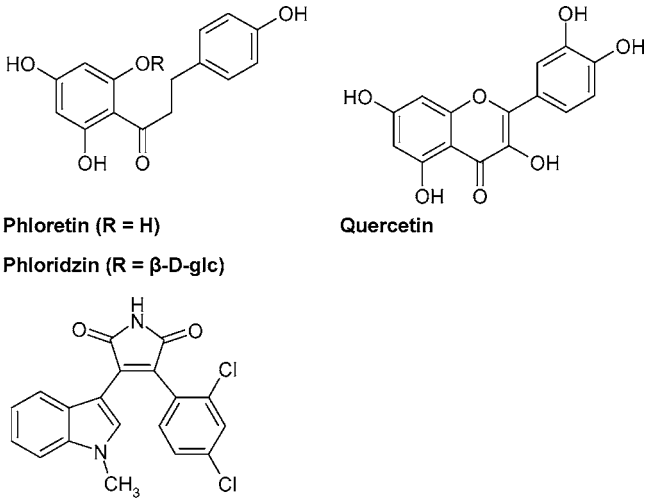


Figure 1. Chemical structures of the tested polyphenols and the selective GSK3 inhibitor SB216763.

and reseeded into a 12-well dish at a density of 200 000 cells per well and grown for 24 h. Cells were incubated at 37 °C for 24 h with test compounds or 1% DMSO and lysed in cell culture lysis buffer (500 μL per well, Promega, Germany). Luciferase activity was measured as arbitrary light units using a luminometer and was normalized for cell number variation with respect to protein concentration.

RESULTS

Inhibition of Immunoprecipitated GSK3β Activity. The effect of polyphenol-rich apple juice extract (AE02), PHL, PHD, and QUE (**Figure 1**) on immunoprecipitated GSK3β from HT29 cells was determined in a kinase assay as phosphorylation of the substrate peptide RRAAEELDS RAG*p*SPQL. Incubation of immunoprecipitated GSK3β with apple extract for 15 min led to a concentration-dependent inhibition of enzyme activity with an IC_{50} value of 329 $\pm$ 30 μg/mL (**Figure 2A**). The dihydrochalcone PHL inhibited the kinase activity of isolated GSK3β with an IC_{50} of 123 $\pm$ 27 μM (**Figure 2B**). In contrast, the respective glycoside PHD was inactive up to 500 μM (data not shown). From the flavonoids tested, the flavonol QUE exhibited the strongest inhibitory properties against isolated GSK3β (IC_{50} of 7.5 $\pm$ 1.8 μM) (**Figure 2C**).

Inhibition of Cellular GSK3β Activity. To address the question whether the inhibitory effects of apple polyphenols on isolated GSK3β are of relevance within the cell, HT29 cells were incubated with the test compounds for 24 h prior to the immunoprecipitation of GSK3β and the respective kinase assay. The apple juice extract AE02 inhibited intracellular GSK3β kinase activity in a concentration-dependent manner with a maximum of about 50% at 500 μg/mL (**Figure 2A**). In contrast, PHL showed no significant effect on intracellular GSK3β activity in HT29 cells up to 500 μM (**Figure 2B**). Similar results were observed with PHD (data not shown). Also, QUE failed to suppress GSK3β kinase activity within HT29 cells (**Figure 2C**). At higher QUE concentrations (100 μM) even a slight but significant increase in the cellular GSK3β kinase activity was observed.

Effect on GSK3β Protein Content. Considering the effect of apple polyphenols on the activity of GSK3β within HT29 cells, we investigated whether the modulation of enzyme activity is associated with changes in the amount of GSK3β protein. In contrast to the observed decrease in GSK3β kinase activity, treatment of HT29 cells with AE02 for 24 h resulted in an increase of GSK3β protein content as detected by Western blot

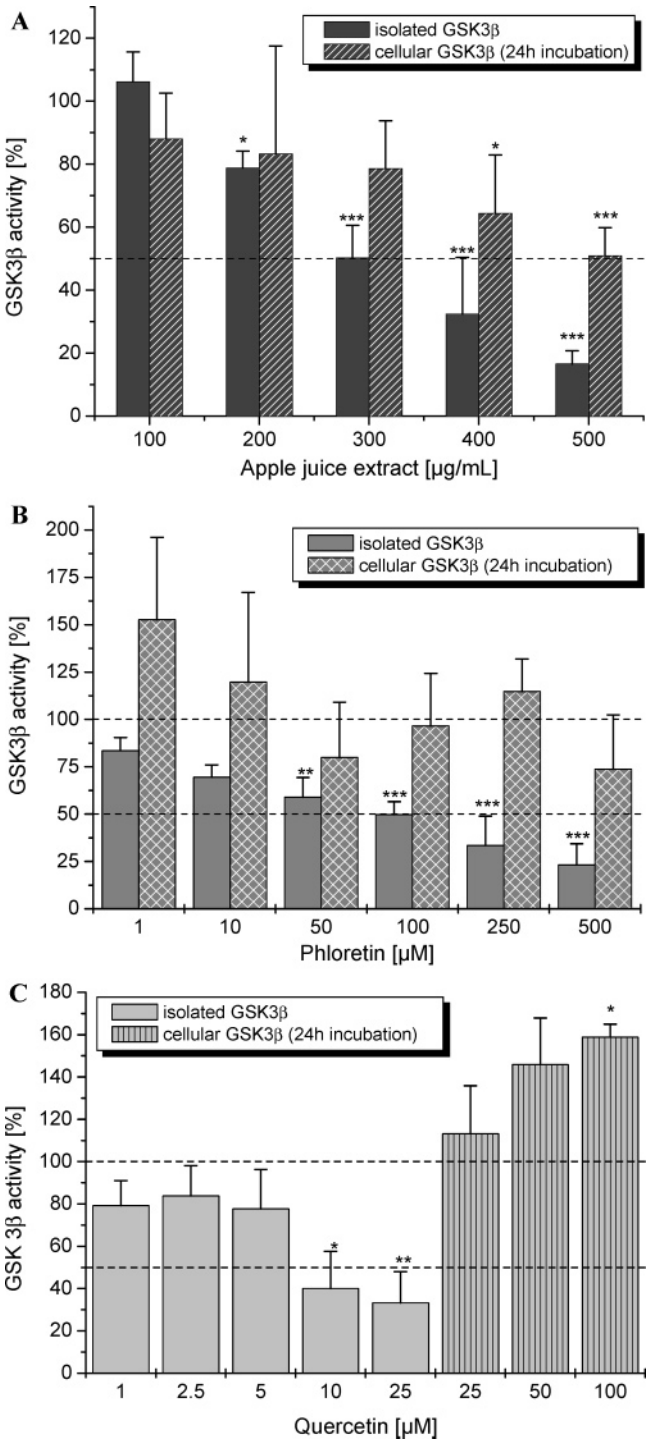


Figure 2. Inhibition of GSK3β kinase activity in HT29 cells was determined as phosphorylation of the substrate peptide RRAAEELDSRAGpSPQL by (A) apple polyphenols (AE02), (B) phloretin (PHL) and (C) quercetin (QUE). The test compounds were either directly added to the immunoprecipitated enzyme or were used for treatment of HT29 cells for 24 h prior to the immunoprecipitation of GSK3β and the subsequent kinase assay. The data presented are the mean $\pm$ SD of at least three independent experiments (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$).

analysis (**Figure 3A**,**B**). This increase in the amount of GSK3β protein was exclusively observed after treatment with AE02, but was not detected after incubation with PHL (**Figure 3C**,**D**) or QUE (data not shown).

β-Catenin Homeostasis. We further addressed the question whether the inhibition of GSK3β kinase activity by apple polyphenols in HT29 cells affects the intracellular β-catenin

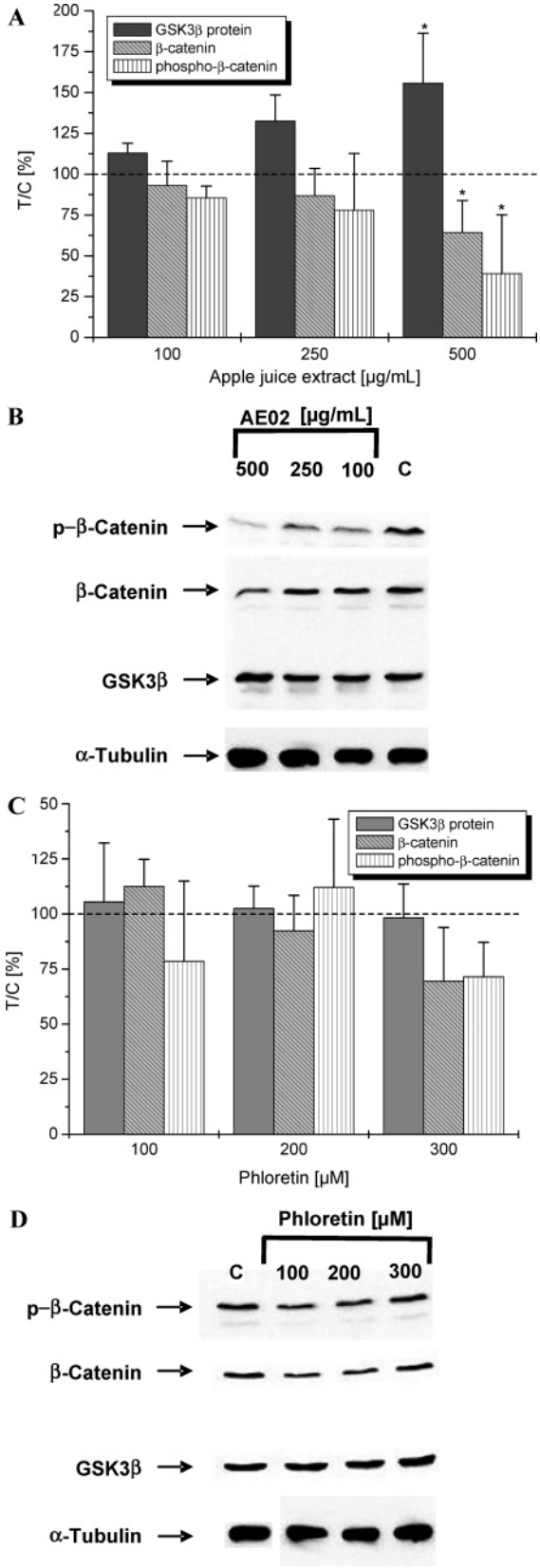


Figure 3. Western blot analysis of GSK3β protein, total β-catenin, and phosphorylated β-catenin in HT29 cells after 24 h incubation with (A + B) apple polyphenols (AE02) or (C + D) phloretin (PHL). The data are plotted as test over control (%) with the control being cells treated with 1% DMSO (solvent control). The data presented in (A, C) are the mean ± SD of at least three independent experiments with similar outcome (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$), with (B) and (D) showing respective representative Western blots.

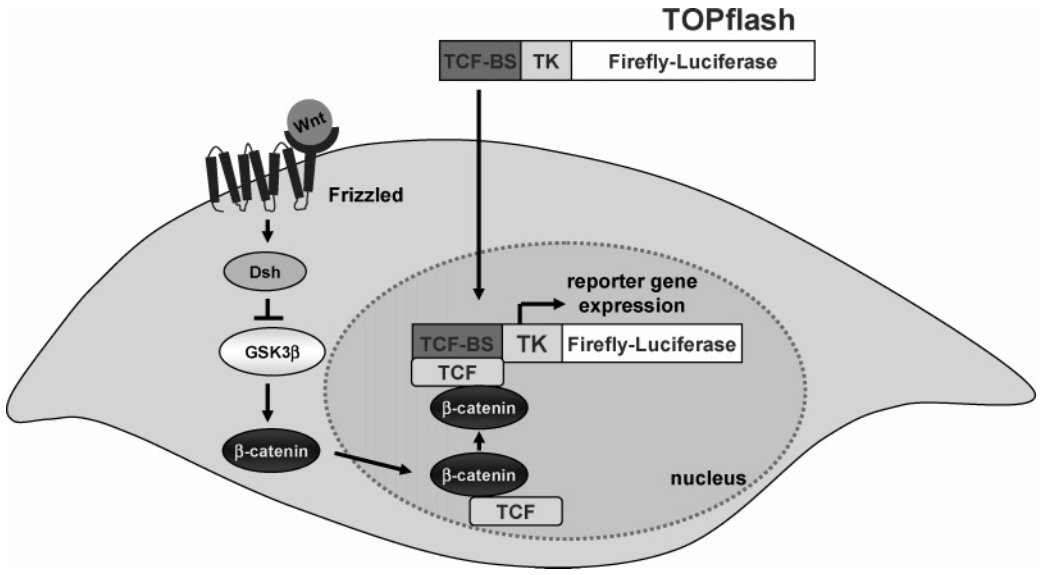
level. In resting cells GSK3β is constitutively active, enabling the phosphorylation of β-catenin and its proteasomal degradation. Inhibition of GSK3β kinase activity is expected to enhance the intracellular level of nonphosphorylated β-catenin. Western blot analysis was performed to investigate the impact of the test compounds on total β-catenin and phosphorylated β-catenin levels in HT29 cells. Incubation of HT29 cells with 10 μM of the selective GSK3 inhibitor SB216763 (**Figure 1**) for 24 h induced a strong decrease of phosphorylated β-catenin accompanied by a slight but significant increase in total β-catenin (data not shown). In contrast, the incubation with AE02 for 24 h resulted in a significant decrease of both total β-catenin and phospho-β-catenin in HT29 cells at a concentration of 500 μg/mL (**Figure 3A**,**B**). PHL did not significantly affect the intracellular levels of β-catenin and phosphorylated β-catenin (**Figure 3C**,**D**).

Impact on TCF/LEF-Mediated Gene Transcription. The AE02-mediated decrease in intracellular β-catenin and phosphorylated β-catenin led to the expectation that subsequently no activation of β-catenin/TCF/LEF-mediated gene transcription is induced. The modulation of β-catenin/TCF/LEF-mediated gene transcription was determined using a reporter gene approach (**Scheme 2**). Human embryonic kidney cells, Hek293, known to express an intact APC complex, were transiently transfected with the reporter gene construct TOPflash containing two sets of three copies of the TCF-binding site (wild type) upstream of the thymidine kinase (TK) minimal promoter and the open reading frame of the luciferase gene. Activation of the Wnt pathway is expected to result in cytosolic β-catenin accumulation. After translocation of β-catenin into the nucleus, its binding to respective transcription factors such as TCF/LEF leads to the onset of luciferase expression. FOPflash, containing mutated TCF binding sites, was used as a control for leakage of luciferase expression. Luciferase activity, measured as arbitrary light units, was normalized with respect to protein concentration. Treatment of transiently with TOPflash transfected Hek293 cells with the GSK3 inhibitor SB216763 induced an increase in luciferase activity of about 24-fold compared to cells incubated with the solvent control (1% DMSO) (**Figure 4**). In contrast, the apple juice extract AE02 did not affect luciferase expression up to 500 μg/mL (**Figure 4**).

DISCUSSION

In the present study, apple polyphenols were identified as potential inhibitors of GSK3β kinase activity in HT29 human colon carcinoma cells. The inhibition of enzyme activity occurs at polyphenol concentrations corresponding to the concentration of these compounds in the original apple juice, which represented a consumer-relevant apple juice blend (*17*). The even higher inhibitory effect of apple polyphenols on isolated GSK3β (**Figure 2A**) let us conclude that the enzyme is directly targeted by the respective apple constituent(s). Thus, within HT29 cells the suppression of GSK3β activity is most likely not a result of the interference of apple constituents with upstream signals, albeit an additional contribution by upstream effectors cannot totally be excluded. Interestingly, the concentration-dependent inhibition of enzyme activity is accompanied by increased GSK3β enzyme expression (**Figure 3A**,**B**), suggesting a counter steering response of the cells in the attempt to compensate the suppression of GSK3β activity. The effective inhibition of GSK3β activity by the complex apple juice extract AE02 raises the question which apple constituent(s) is(are) responsible. The apple characteristic dihydrochalcone PHD was found to be inactive against GSK3β up to 500 μM. With regard to the

Scheme 2. Reporter Gene Assay for the Detection of TCF/LEF-Mediated Gene Transcription Using Firefly Luciferase as a Reporter[a]



[a] APC, adenomatous polyposis coli; Dsh, disheveled; Fz, frizzled receptor; GSK3β, glycogen synthase kinase-3β; LEF, lymphoid enhancer factor; TCF, T-cell factor; TCF-BS, TCF binding site (wild type); TK, thymidine kinase minimal promoter; TOPflash, TCF reporter plasmid (Upstate, Lake Placid, NY).

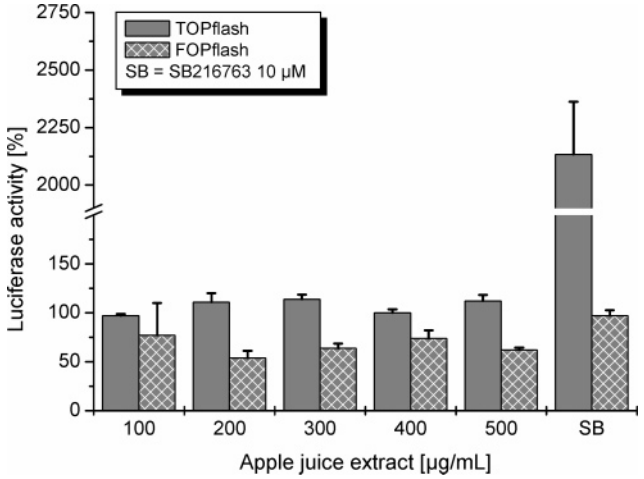


Figure 4. Modulation of luciferase expression as a measure for the stabilization of β-catenin by apple polyphenols (AE02). Hek293 human embryonic kidney cells were transiently transfected with the reporter gene constructs TOPflash and FOPflash (negative control vector containing mutated TCF binding sites). Cells were reseeded 24 h post-transfection prior to the incubation with AE02 for 24 h. Luciferase activity was determined 72 h after transfection and normalized with respect to the protein concentration. Data are presented as percent of the solvent control. The data presented are the mean ± SD of at least three independent experiments. The significances indicated are calculated compared to the control using Student's *t*-test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$).

concentration of PHD in AE02 (32 μM) (*17*), PHD can be excluded to contribute to the GSK3β inhibitory properties of the extract. However, the free aglycon PHL, exhibiting GSK3β inhibitory properties, has not been detected thus far in the original apple juice extract (*17*, *19*, *21*). In contrast to the glycoside, PHL was identified as a potent inhibitor of isolated GSK3β (**Figure 2B**). But like PHD, PHL did not affect GSK3β kinase activity within HT29 cells (**Figure 2B**), suggesting that even if the free aglycon might be formed under in vivo conditions (e.g., in the gastrointestinal tract), PHL is not expected to affect substantially the GSK3β kinase activity in respective tissues.

The flavonol QUE has been identified as a potent inhibitor of isolated GSK3β kinase activity (**Figure 2C**). However, in the case of QUE the discrepancy between the effect on isolated GSK3β and the intracellular GSK3β activity was found to be even more pronounced than that for PHL (**Figure 2B**,**C**). Thus, despite substantial inhibitory properties against isolated GSK3β, QUE can be excluded as an effective modulator of GSK3β activity within HT29 cells. QUE itself appears not to be present as the free aglycon in the apple extract AE02 (*17*, *21*). Several QUE glycosides have been quantified thus far in AE02, with a total concentration of about 9 μM. In different test systems, these QUE glycosides were found to be inactive or at least less active than the respective aglycon (*17*, *19*, *25*). Therefore, considering that the free aglycon QUE did not inhibit the intracellular GSK3β activity, it is most unlikely that the QUE glycosides contribute to the effective inhibition of GSK3β activity by AE02 in HT29 cells. In summary, the polyphenol-rich apple extract AE02 contains constituents not identified thus far, directly targeting GSK3β, thereby effectively suppressing GSK3β kinase activity within HT29 cells.

The suppression of GSK3β kinase activity is expected to result in a decrease of phosphorylated β-catenin together with an increase of total β-catenin. Indeed, at a concentration of AE02 mediating a substantial inhibition of GSK3β kinase activity (500 μg/mL), a significant decrease of phosphorylated β-catenin was observed (**Figure 3A**,**B**). However, unexpectedly, the total β-catenin protein level was also found to be diminished (**Figure 3A**,**B**). Obviously, the inhibition of GSK3β kinase activity by AE02 is not associated with the stabilization of β-catenin in HT29. The effective suppression of the expected increase of the β-catenin protein level might arise from the interference of apple constituents with other upstream signals or by crosstalk with other signaling pathways. We previously reported that apple polyphenols potently inhibit the tyrosine kinase activity of the epidermal growth factor receptor, suppressing the activity of subsequent signaling elements (*17*). In SW620 colon carcinoma cells a procyanidin-rich apple extract was reported to induce apoptosis (*18*). The shutdown of signaling cascades crucial for the regulation of cell growth and/or the induction of apoptosis might at least contribute to the compensation of GSK3β inhibitory effects.

In accordance with the results on the β-catenin homeostasis, in the reporter gene assay, TCF/LEF-mediated gene transcription remained unaffected by treatment with AE02 up to 500 μg/mL (**Figure 4**). In contrast, the selective GSK-3 inhibitor SB216763, used as a positive control in our studies, significantly enhanced TCF/LEF-mediated luciferase expression (**Figure 4**), indicating a substantial activation of the Wnt pathway.

In summary, the results predict that apple polyphenols at consumer-relevant concentrations do not mediate growth stimulatory effects in HT29 human colon tumor cells via the Wnt pathway.

ACKNOWLEDGMENT

We gratefully acknowledge H. Dietrich and F. Will for supporting us generously with apple juice extract (AE02).

LITERATURE CITED

(1) Knekt, P.; Kumpulainen, J.; Jarvinen, R.; Rissanen, H.; Heliovaara, M.; Reunanen, A.; Hakulinen, T.; Aromaa, A. Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr*. **2002**, *76*, 560−568.

(2) Knekt, P.; Jarvinen, R.; Seppanen, R.; Hellovaara, M.; Teppo, L.; Pukkala, E.; Aromaa, A. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am. J. Epidemiol*. **1997**, *146*, 223−230.

(3) Le Marchand, L.; Murphy, S. P.; Hankin, J. H.; Wilkens, L. R.; Kolonel, L. N. Intake of flavonoids and lung cancer. *J. Natl. Cancer Inst.* **2000**, *92*, 154−160.

(4) Terry, P.; Suzuki, R.; Hu, F. B.; Wolk, A. A prospective study of major dietary patterns and the risk of breast cancer. *Cancer Epidemiol., Biomarkers Prev.* **2001**, *10*, 1281−1285.

(5) Depeint, F.; Gee, J. M.; Williamson, G.; Johnson, I. T. Evidence for consistent patterns between flavonoid structures and cellular activities. *Proc. Nutr. Soc.* **2002**, *61*, 97−103.

(6) Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **2000**, *52*, 673−751.

(7) Bruce, R. W.; Giacca, A.; Medline, A. Possible mechanisms relating diet and risk of colon cancer. *Cancer Epidemiol., Biomarkers Prev.* **2000**, *9*, 1271−1279.

(8) Bienz, M.; Clevers, H. Linking colorectal cancer to Wnt signaling. *Cell* **2000**, *103*, 311−320.

(9) Taipale, J.; Beachy, P. A. The Hedgehog and Wnt signaling pathways in cancer. *Nature* **2001**, *411*, 349−354.

(10) Polakis, P. Wnt signaling in cancer. *Genes Dev.* **2000**, *14*, 1837−1851.

(11) Radtke, F.; Clevers, H. Self-renewal and cancer of the gut: Two sides of a coin. *Science* **2005**, *307*, 1904−1909.

(12) Leclerc, S.; Garnier, M.; Hoessel, R.; Marko, D.; Bibb, J. A.; Snyder, G. L.; Greengard, P.; Biernat, J.; Wu, Y. Z.; Mandelkow, E. M.; Eisenbrand, G.; Meijer, L. Indirubins inhibit glycogen synthase kinase-3 beta and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *J. Biol. Chem*. **2001**, *276*, 251−260.

(13) Meijer, L.; Flajolet, M.; Greengard, P. Pharmacological inhibitors of glycogen synthase kinase 3. *Trends Pharmacol. Sci.* **2004**, *25*, 471−480.

(14) Meijer, L.; Thunnissen, A. M.; White, A. W.; Garnier, M.; Nikolic, M.; Tsai, L. H.; Walter, J.; Cleverley, K. E.; Salinas, P. C.; Wu, Y. Z.; Biernat, J.; Mandelkow, E. M.; Kim, S. H.; Pettit, G. R. Inhibition of cyclin-dependent kinases, GSK-3beta and CK1 by hymenialdisine, a marine sponge constituent. *Chem. Biol.* **2000**, *7*, 51−63.

(15) Smith, D. G.; Buffet, M.; Fenwick, A. E.; Haigh, D.; Ife, R. J.; Saunders, M.; Slingsby, B. P.; Stacey, R.; Ward, R. W. 3-Anilino-4-arylmaleimides: potent and selective inhibitors of glycogen synthase kinase-3 (GSK-3). *Bioorg. Med. Chem. Lett.* **2001**, *11*, 635−639.

(16) Liu, R. H.; Sun, J. Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *J. Agric. Food Chem.* **2003**, *51*, 1718−1723.

(17) Kern, M.; Tjaden, Z.; Ngiewih, Y.; Puppel, N.; Will, F.; Dietrich, H.; Pahlke, G.; Marko, D. Inhibitors of the epidermal growth factor receptor in apple juice extract. *Mol. Nutr. Food Res.* **2005**, *49*, 317−328.

(18) Gosse, F.; Guyot, S.; Roussi, S.; Lobstein, A.; Fischer, B.; Seiler, N.; Raul, F. Chemopreventive properties of apple procyanidins on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis* **2005**, *26*, 1291−1295.

(19) Veeriah, S.; Kautenburger, T.; Habermann, N.; Sauer, J.; Dietrich, H.; Will, F.; Pool-Zobel, B. L. Apple flavonoids inhibit growth of HT29 human colon cancer cells and modulate expression of genes involved in the biotransformation of xenobiotics. *Mol. Carcinog*. **2006**, *45*, 164−174.

(20) Barth, S. W.; Fahndrich, C.; Bub, A.; Dietrich, H.; Watzl, B.; Will, F.; Briviba, K.; Rechkemmer, G. Cloudy apple juice decreases DNA damage, hyperproliferation and aberrant crypt foci development in the distal colon of DMH-initiated rats. *Carcinogenesis* **2005**, *26*, 1414−1421.

(21) Schaefer, S.; Baum, M.; Eisenbrand, G.; Dietrich, H.; Will, F.; Janzowski, C. Polyphenolic apple juice extracts and their major constituents reduce oxidative damage in human colon cell lines. *Mol. Nutr. Food Res.* **2006**, *50*, 24−33.

(22) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(23) Welsh, G. I.; Patel, J. C.; Proud, C. G. Peptide substrates suitable for assaying glycogen synthase kinase-3 in crude cell extracts. *Anal. Biochem*. **1997**, *244*, 16−21.

(24) Cross, D. Assays for glycogen synthase kinase-3 (GSK-3). *Methods Mol. Biol*. **2001**, *124*, 147−159.

(25) Shen, S. C.; Chen, Y. C.; Hsu, F. L.; Lee, W. R. Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. *J. Cell Biochem.* **2003**, *89*, 1044−1055.

Received for review March 8, 2006. Revised manuscript received June 7, 2006. Accepted June 12, 2006. The study was performed as part of the Nutrition Net (www.nutrition-net.org) funded by Grant 01EA0101 of the German Ministry of Research and Education.

JF0606611