GPR55-Mediated Effects in Colon Cancer Cell Lines

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Abstract

The cannabinoid-responsive G protein-coupled receptor GPR55 and its endogenous ligand L- α -lysophosphatidylinositol (LPI) have been reported to play a role in several cancers. A proliferation-enhancing effect of GPR55 has been described for several cancer cell lines and LPI has been found elevated in cancer patients. The aim of this study was to investigate whether GPR55 signaling had an effect on the proliferation of colon cancer cell lines. Using cell viability assays and Western blotting, we show that stable overexpression of the GPR55 receptor led to a growth advantage of SW480 cells per se. Proliferation of native colon cancer cell lines, however, was not affected by pharmacological manipulation of GPR55. Interestingly though, GPR55 signaling was responsive to treatment with both the GPR55 agonist LPI and the antagonist CID16020046 in the overexpressing cancer cell lines. This was evident through significantly increased or decreased levels of phosphorylated ERK1/2, respectively. Taken together, our findings suggest that GPR55 is constitu-

tively activated in overexpressing colon cancer cells affecting ERK1/2 phosphorylation and cell proliferation.

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Introduction

The G protein-coupled receptor 55 (GPR55) has been found to be responsive to a variety of natural and synthetic cannabinoids, including anandamide, virodhamine, and Δ^9 -tetrahydrocannabinol and has, therefore, been suggested to belong to the class of cannabinoid receptors [1, 2]. GPR55, however, has no significant sequence similarity with the classical cannabinoid receptors CB₁ or CB₂ [3] and is rather related to the purinergic receptor P2Y₅, as well as to the G protein-coupled receptors 23 and 92 (GPR23 and GPR92), which have been shown to be lysophosphatidic acid receptors [4–6]. GPR55 does not possess the typical “cannabinoid binding pocket” but rather exhibits a deep vertical binding pocket for long, thin inverted L- or T-shaped ligands [7] and, accordingly, its endogenous ligand was found to be a lysophospholipid, namely L- α -lysophosphatidylinositol (LPI) [8]. In particular, it is believed that LPI carrying an arachidonic

acid moiety is the most potent endogenous agonist of GPR55 [9].

GPR55 signaling has been implicated in a variety of cancers. In squamous cell carcinomas, breast and pancreatic cancer, and in glioblastomas, GPR55 expression levels are upregulated, positively correlating with aggressiveness [10, 11]. In vivo, GPR55 promotes carcinogenesis in mouse models of skin, pancreatic, and colorectal cancer [10, 12, 13]. Furthermore, GPR55 expression has been demonstrated in several cancer cell lines, such as breast, prostate, ovarian, glioblastoma, and colon cancer cells [11, 14, 15].

Since LPI has been found to be the endogenous agonist of GPR55 and since GPR55 is expressed in various cancers in an aggressiveness-related manner, a role for the LPI/GPR55 axis in cancer has been postulated [16]. Overexpression of GPR55 enhanced, whereas silencing reduced the proliferation of breast cancer, glioblastoma, and skin carcinoma cells, and the main signaling pathway through which GPR55 exerts its proliferative effects in cancer cells was identified to be ERK1/2 phosphorylation [10, 11].

We have recently found that GPR55 promotes colorectal carcinogenesis [13] and that serum LPI levels were increased in colorectal cancer patients [15]. We, therefore, set out to investigate whether LPI had direct effects on the proliferation of colon cancer cell lines and whether GPR55 overexpression would alter signal transduction in these cells.

Materials and Methods

Cell Culture

Colon cancer cell lines HCT116, SW480, SW620, HT29, and DLD-1 were obtained from Interlab Cell Line Collection (Genova, Italy), and CaCo-2 from ATCC (Manassas, VA, USA). HCT116 and HT29 were maintained in McCoy's 5A, SW480, SW620, DLD-1, and CaCo-2 in DMEM, each supplemented with 10% FBS (all Life Technologies, Vienna, Austria), 2 mM L-glutamine, and 1% penicillin/streptomycin (both PAA Laboratories, Pasching, Austria). All cell lines were maintained at 37°C, 5% CO₂ in a humidified atmosphere.

Generation of GPR55-Overexpressing Cell Lines

To stably overexpress GPR55, HCT116 and SW480 cells were transfected with a pcDNA3.1 construct coding for GPR55 with an N-terminal hemagglutinin tag (3xHA-GPR55), as described [17]. The transfection was performed with Lipofectamine 2000 according to the manufacturer's protocol. Transfected cells were selected with 4 mg/mL G418 (Life Technologies) 48 h after transfection, grown to confluence, and subsequently sorted on a FACSaria (BD Biosciences, Franklin Lakes, NJ, USA) as described previously [13]. Sorted cells were maintained as HCT116-GPR55 (HCT55) and SW480-GPR55 (SW55) in their respective media, which were

supplemented with 0.5 mg/mL G418. All assays involving pharmacological manipulation of GPR55 were performed after serum starvation and in the absence of serum and antibiotics.

Cell Viability Assays

For comparison of native and GPR55-overexpressing HCT116 and SW480, cells were seeded at 7.5×10^4 cells/mL in 96-well plates. Mitochondrial activity was assessed after 24, 48, and 72 h using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA). For treatment with LPI (Sigma, Vienna, Austria) or the GPR55 antagonist CID16020046 (ChemDiv, San Diego, CA, USA), cells were seeded at 1.5×10^5 cells/mL. After letting them adhere overnight, cells were starved in McCoy's 5A without serum for 24 h. Substances were then added at the indicated concentrations and cell viability was measured after 24, 48, and 72 h.

RNA Extraction, Reverse Transcription to cDNA, and qRT-PCR

RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany), treated with DNA-free DNA Removal Kit (Life Technologies), and reverse transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA), all according to the manufacturer's instructions. Quantification of gene expression was done by real-time PCR (CFX Connect Real-Time System, Bio-Rad, Vienna, Austria) using SsoAdvanced Universal SYBR Green Supermix and validated primers for GPR55 (ID: qHsaCED0001676) and GAPDH (ID: qHsaCED0038674, all Bio-Rad). Relative gene expression was assessed according to the $\Delta\Delta C_q$ -method.

Western Blotting

Protein was extracted using RIPA buffer (Thermo Scientific, Rockford, IL, USA) supplemented with protease and phosphatase inhibitors (both Roche Applied Science, Vienna, Austria). Lysates were centrifuged at 15,000 g for 15 min at 4°C, and protein concentrations of the supernatants were determined with Pierce BCA Protein Assay Kit (Thermo Scientific). For Western blot analysis, protein lysates were supplemented with 6× Laemmli buffer containing β-mercaptoethanol and denatured at 95°C for 5 min. Proteins were separated on 4–12% Tris-Glycine Gels (Thermo Scientific) and blotted onto polyvinylidene difluoride membranes (Bio-Rad) using a wet transfer system (100 V, 75 min, 4°C). Membranes were blocked in Tris-buffered saline/Tween 20 buffer containing 5% non-fat dry milk for 1 h at room temperature. Primary antibodies were then applied overnight at 4°C: pERK1/2 (CST 9101, 1:1,000), ERK1/2 (CST 4695, 1:1,000), or α-tubulin (CST 2125, 1:1,000). Horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit, Jackson ImmunoResearch #111-036-045, 1:5,000) was then applied for 1 h at room temperature. Detection was performed on a ChemiDoc Touch Imaging System using Clarity Western ECL Blotting Substrate (both Bio-Rad). Immunoblot images were analyzed with Image Lab 5.2 software (Bio-Rad).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA). Comparison of two treatment groups was done using two-tailed Student's *t* test, and one-way ANOVA with Tukey's post hoc test was performed when comparing more than two groups. Data were assumed to be statistically significant if $p < 0.05$.

Fig. 1. **a** All examined CRC cell lines expressed GPR55, with SW480 cells showing the highest expression levels. Data are shown as means + SD as determined for three different passages. **b, c** Cell viability of neither HCT116 nor SW480 cells was altered after treatment with LPI or CID16020046 (0–15 μ M) for 72 h (**b**) or 48 h (**c**). Cell viability assays were performed in sextuplicate in the absence of serum and after serum starvation ($n \geq 5$).

Results

Since we had recently found that GPR55-deficient mice had a significantly lower tumor burden in various models of colorectal cancer [13], we investigated whether these effects were mediated by the epithelial cells. Therefore, we analyzed six commercially available colon cancer cell lines for their GPR55 expression. Indeed, all cell lines expressed GPR55 mRNA, with SW480 cells expressing ~50-fold more than the other tested cell lines (Fig. 1a). Thus, we decided to use the SW480 cells and another cell line with low GPR55 expression levels, i.e., HCT116 cells, for further experiments. Cell viability assays with the endogenous GPR55 agonist LPI or the GPR55 antagonist CID16020046, however, showed that proliferation of neither HCT116 nor SW480 cells was affected by pharmacological manipulation of GPR55 (Fig. 1b, c). GPR55-related signaling pathways were also examined. Surprisingly, treatment with LPI (concentrations from 0.1 to 10 μ M for 1–30 min) or CID16020046 (0.1–10 μ M for 10–30 min) did not alter expression levels of phosphorylated p38, AKT, STAT3, or ERK1/2 in these cells. Ca^{2+} release from intracellular stores after LPI treatment was not observed either (data not shown).

Therefore, we next created colon cancer cell lines that stably overexpressed GPR55 (Fig. 2a, b). As a result, SW55 cells showed significantly increased proliferation compared to SW480 cells (Fig. 2c). HCT55 cells showed the same trend but did not reach statistical

significance (Fig. 2d). Concomitantly, SW55 cells had increased levels of ERK1/2 phosphorylation compared to SW480 cells when grown in complete medium. HCT55 cells, again, showed the same trend, but expression levels were not statistically different from HCT116 cells (Fig. 2e, f). Interestingly, these differences were abolished upon serum starvation, hinting at constitutive activation of GPR55 in overexpressing cells in the presence of serum.

GPR55-overexpressing cells were then treated with LPI, and ERK1/2 phosphorylation was examined via Western blotting. Indeed, pERK1/2 expression was increased by ~70% in HCT55 cells and by ~260% in SW55 cells after treatment with 1 μ M LPI for 10 min (Fig. 3a). In accordance with this observation, ERK1/2 phosphorylation was also altered in the GPR55-overexpressing cells after treatment with the GPR55 antagonist CID16020046. In HCT55 cells, pERK1/2 expression was reduced by ~35% and in SW55 cells by ~50% after treatment with 10 μ M for 30 min (Fig. 3b). Importantly, these effects were not observed in native HCT116 and SW480 cells.

Since GPR55 signaling was apparently functional in overexpressing cells, we next investigated whether it also had an effect on the proliferation of these cells. As shown in Figure 4a, b, however, treatment with LPI did not alter cell viability in neither HCT55 nor SW55. Treatment with CID16020046 also did not have an effect on the growth of GPR55-overexpressing cells (Fig. 4c, d).

Fig. 2. a, b Real-time qPCR was performed to show GPR55 mRNA levels in overexpressing colon cancer cell lines SW480 (**a**) and HCT116 (**b**). Data are shown as mean + SD ($n = 3$). **c, d** Cell viability was increased in GPR55-overexpressing cells compared to native SW480 and HCT116 cells when cells were grown in complete medium. Data are shown as mean + SD from at least five independent experiments performed in sextuplicate. **e, f** Concomitantly, ERK1/2 phosphorylation was increased in GPR55-overexpressing cells. A representative Western blot is shown in **e**. **f** Data from three independent experiments are shown as mean + SD. * $p < 0.05$; ** $p < 0.01$.

Fig. 3. a HCT55 and SW55 cells were treated with 1 μM LPI or vehicle control for 10 min. A representative blot of three independent experiments is shown. Bars are mean + SD. **b** HCT116, HCT55, SW480, and SW55 cells were treated with 10 μM CID16020046 for 10 or 30 min or with vehicle control. In GPR55-overexpressing cells, a decrease in pERK1/2 levels was observed after CID16020046 (CID) treatment, whereas this effect was absent in native HCT116 and SW480 cells. A representative blot of three independent experiments is shown. Experiments were performed after serum starvation and in the absence of serum. ** $p < 0.01$; *** $p < 0.001$.

Fig. 4. a, b Cell viability of GPR55-overexpressing cells was not altered by the treatment with LPI under serum-deprived conditions. Data shown are the means $\pm$ SD of sextuplicates measured after 24 and 48 h. **c, d** SW55 cells were treated with CID16020046 as indicated, but that did not diminish their growth advantage over SW480 cells. The experiment was performed in complete medium, and cell viability was assessed after 24 and 48 h. *** $p < 0.001$ untreated SW55 compared to SW480.

Discussion

The endogenous GPR55 ligand LPI has been established to promote tumorigenesis by activating signaling cascades related to cell proliferation, migration, and survival [16]. Recent work of our group had already revealed that GPR55 enhanced the migration and adhesion of colon cancer cells [15], but its role in cell proliferation had not yet been investigated. Therefore, we performed cell viability and signal transduction assays with two colon cancer cell lines.

The main findings of this study were that (i) pharmacologic manipulation of GPR55 did not alter ERK1/2 phosphorylation, i.e., the best described signaling pathway downstream of GPR55, and did not affect cell proliferation in native colon cancer cells, (ii) stable overexpression of GPR55 resulted in increased pERK1/2 levels and enhanced cell growth per se, and (iii) GPR55 signaling was responsive to pharmacological manipulation in GPR55-overexpressing cells, i.e., LPI further enhanced and CID16020046 abolished ERK1/2 phosphorylation.

A possible explanation for our finding that pharmacological activation of GPR55 did not result in downstream signaling in native colon cancer cells could lie in the pur-

ported cross-regulation of CB₁ and GPR55. In HEK293 cells that co-expressed CB₁ and GPR55, GPR55 signaling was found to be inhibited [18]. HCT116 and SW480 cells express both CB₁ and GPR55 receptors, and we hypothesized that GPR55 signaling may be inhibited in the presence of CB₁. Therefore, we stably overexpressed GPR55 in these cells. Indeed, we now observed a growth advantage of GPR55-overexpressing cells as compared to their native counterparts. Concomitantly, both HCT55 and SW55 had increased basal expression levels of phosphorylated ERK1/2. Treatment with LPI (1 μM) further enhanced ERK1/2 phosphorylation in both overexpressing cell lines. Interestingly, however, cell viability was still not enhanced by LPI in these cell lines, although the activation of signaling cascades was apparent. Treatment with CID16020046 decreased basal expression levels of phosphorylated ERK1/2 in HCT55 and SW55 cells but not in native HCT116 and SW480 cells. These data suggest that GPR55 can only be manipulated pharmacologically when it is expressed several fold higher than CB₁. Whether CB₁ actually inhibits GPR55 signaling in native colon cancer cells, however, still needs to be established.

Additionally, it is important to note that the enhanced cell proliferation in overexpressing cells was only ob-

served when cells were grown in complete medium, i.e., in the presence of serum. When cells were serum-starved, differences in growth rate were abolished. This suggests that factors present in the serum were responsible for the observed growth advantage. Since FBS is known to contain various endocannabinoids [19], we deemed it important to avoid any interference from other compounds that could act on GPR55 and, therefore, performed all pharmacological experiments after serum starvation and in the absence of serum. As detailed above, this setting did not allow us to uncover proliferation-related signaling mechanisms of LPI elicited on GPR55 in native cells. A possible explanation therefore could be that most signaling pathways downstream of GPR55 have been elucidated through functional assays using transfected cells. While some findings have been validated in untransfected cells, this is not the case for all studies [20]. It is thus conceivable that GPR55 signaling mainly affects migration and adhesion in native cells, as described previously [15], but enhances proliferation when overexpressed. This would corroborate the fact that GPR55 has been found upregulated in several cancers, and expression levels correlated with aggressiveness [11]. Before GPR55 can be considered as a target in cancer treatment, however, more studies are warranted to elucidate its enigmatic pharmacology.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

R.S. and C.H. designed and supervised the study. C.H., D.F., and M.K. performed cell culture experiments and biochemical analysis and analyzed the data. All authors participated in writing of the manuscript.

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