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Glycocholic acid and butyrate synergistically increase vitamin D-induced calcium uptake in Caco-2 intestinal epithelial cell monolayers

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ABSTRACT

Background: Roux-en-Y gastric bypass (RYGB) substantially decreases intestinal calcium absorption and may eventually lead to bone resorption. This is likely a consequence of bile diversion from the alimentary limb, as the presence of bile seems necessary for vitamin D-mediated calcium uptake. We recently suggested that the mediating mechanism may be a down-regulation of the vitamin D co-activator heat-shock protein (Hsp)90β. Recent evidence suggests that vitamin D may have effects on both active and passive calcium absorption.

Aim: To identify mechanisms in vitro that may be responsible for the decreased calcium absorption after RYGB. We hypothesized that bile, alone or in concert with nutritional compounds, could be of importance.

Material & methods: Caco-2 cells were grown confluent on semi-permeable membranes in a double-chamber setup to mimic small intestinal mucosa. The effect of bile acids chenodeoxycholic, lithocholic, glycocholic and taurocholic acid, with and without the addition of the fatty-acid butyrate, were tested for their effects on Hsp90β expression and active and passive calcium-flux monitored using radioactive ^{45}Ca.

Results: We initially found that whole human bile, but only together with the fatty acid butyrate, potently induced Hsp90β expression. In line with this, a single bile acid, e.g. glycocholic acid (GCA), in combination with butyrate, increased Hsp90β expression (40 ± 13% vs. GCA, butyrate or vehicle alone; $p < 0{,}001$; $n = 14–25$). Further, this combination together with vitamin D increased the passive gradient-driven flux of calcium, compared to stimulation with vitamin D alone or in combination with either GCA or butyrate (880 ± 217% vs. vitamin D and GCA or butyrate, or vitamin D only; $p = 0{,}01–0.006$; $n = 5–11$). Surprisingly, this combination had no effect on active calcium transport in the absence of calcium gradient.

Conclusion: The combination of GCA and butyrate increased gradient-driven calcium uptake up to 9-fold in Caco-2 intestinal epithelial cells, but had no effect on active calcium absorption. This effect was mediated via the vitamin D receptor co-activator Hsp90β.

1. Introduction

One of the most common and effective surgical treatments of morbid obesity and its severe metabolic complications is Roux-en-Y gastric bypass (RYGB) (Schafer, 2017). The surgical re-routing of the small intestine however also leads to decreased uptake of micro-nutrients from the intestinal lumen, e. g. vitamin D-induced calcium absorption. Therefore, patients are advised to take lifelong vitamin D and calcium supplementation to counteract this, albeit the long-term efficacy of the prevention has still not been established. Several reports during the last years have shown negative effects of RYGB on bone mineralization, and now recently even indicate an increased risk of osteoporotic fractures, despite the supplementation therapy, which suggests that the supplementation is not effective in preventing the decreased calcium absorption and in the long-run osteoporosis (Yu et al., 2017; Rousseau et al., 2016; Axelsson et al., 2018; Ahlin et al., 2020). It was recently reported that RYGB leads to an up to almost 80% decreased fractional calcium absorption, despite adequate vitamin D levels (Schafer, 2017). It has also been shown that the risk of osteoporotic fractures is increased in particular after RYGB more than other bariatric surgery methods, and is thus not only a consequence of the weight decrease (Rousseau et al., 2016; Ahlin et al., 2020; Nakamura et al., 2014; Lu et al., 2015). Therefore, it is of importance to try finding mechanisms that can explain this effect, and make it possible to find treatments to reverse the decreased calcium absorption after RYGB.

Calcium homeostasis is regulated primarily by parathyroid hormone

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(PTH), and a reduction in the serum calcium concentration stimulates release of PTH from the parathyroid glands, which enhances calcium reabsorption in the kidney as well as calcium resorption from bone. PTH also stimulates the conversion of 25-hydroxyvitamin D (25(OH)D) to active form of vitamin D, $1,25(OH)_2D3$ (vitamin D3) which in turn enhances intestinal calcium absorption (Hewitt et al., 2018). Intestinal calcium is absorbed by two different mechanisms; 1) active vitamin D-mediated, energy-dependent saturable transcellular transport, and 2) passive, diffusional, non-saturable paracellular passage (Christakos et al., 2011). Active transcellular calcium transport occurs mainly in the proximal intestine and is thought to consist of three major steps: 1) entry of luminal calcium into the enterocyte cell through the apical calcium channel transient receptor potential vanilloid type 6 (Trpv6), the expression of which is increased by vitamin D via the vitamin D receptor (VDR): 2) binding to the calcium binding protein calbindin-D9k and diffusion across the cytosol: and 3) transport of calcium across the basolateral membrane and into the blood by the intestinal plasma membrane calcium ATPase (PMCA1b). Passive paracellular calcium transport takes place throughout the entire length of the intestine and predominates over the active transport in the distal regions (Christakos et al., 2011). Passive calcium transport has been assumed to mainly be related to the concentration of calcium in the intestinal lumen. Because passive paracellular transport is non-saturable, its relative contribution to the total absorbed calcium increases with increasing calcium intake. Under most circumstances, it accounts for the majority of all calcium absorption. With low calcium intake less of the passive transport occurs, but the efficiency of the active transcellular process is augmented by increased secretion of PTH and production of vitamin D3. While paracellular transport has traditionally been thought to be independent of vitamin D, recent research has revealed that vitamin D3 also promotes paracellular calcium diffusion by increasing junctional ion permeability *e.g.* via the permissive tight junction protein claudin-2 (Chirayath et al., 1998; Fujita et al., 2008; Diaz de Barboza et al., 2015; Zhang et al., 2015). Claudin-12 has also been described to influence passive calcium uptake (Fujita et al., 2008).

We recently described how bone mineral density (BMD) decreases independently of weight after RYGB (Elias et al., 2014). We proposed a mechanism for the bone loss by impaired intestinal calcium uptake caused by decreased activation of vitamin D-dependent calcium absorption mechanisms, possibly mediated by decreased expression of the VDR co-activator heat-shock protein (Hsp)90β (Angelo et al., 2008). The decreased Hsp90β expression and compromised calcium uptake following RYGB surgery might in part be caused by the diversion of bile from the upper small intestine, based on studies originally performed in the 1930-ies showing that bile deviation substantially decreases vitamin D-mediated calcium absorption (Greaves and Schmidt, 1933). However, it is possible that the deviation of other nutritional factors from the intestinal mucosa may also contribute to this. For instance, the mucosa of the proximal small intestine is not exposed to fatty acids because decreased fat digestion due to the absence of bile and pancreatic lipase in the alimentary limb. Therefore, we hypothesized that exposure of the mucosa to either bile, or a combination of bile and other components, *e.g.* fatty acids could improve calcium absorption after RYGB.

The specific aim of this project was therefore to 1) screen different bile acids, alone or in combination with butyrate for the induction of Hsp90β protein expression, and 2) to examine the effect of that combination of bile acid and butyrate on active and passive calcium transport in vitro using ^{45}Ca.

2. Materials and methods

2.1. Cell cultures

Caco-2 cells at passage 45 (Sigma-Aldrich, Stockholm, Sweden) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies Invitrogen AB, Lidingo, Sweden), with 10% Foetal Bovine Serum (FBS) (Life Technologies), 1% NonEssential Amino Acid (NEAA) (Life Technologies), 100 IU/mL Penicillin Streptomycin (Pen-Strep) (Life Technologies). The cells were incubated in 37 °C with 5% CO_2 and cultured in a cell culture flask (BD Falcon®, VWR International, Stockholm, Sweden) and the medium was changed every other day. The cells were cultured for approximately 1 week before they were detached (0.25% trypsin-EDTA, Life Technologies) and seeded in 12 well plates with semipermeable filter inserts (3 μm pore size, BD Biosciences, Le Pont de Claix, France), 50,000 cells/well. At confluence the culture medium was changed to FBS-free in both the upper and lower well compartment and to the lower compartment also a mixture of 10 μL/mL insulin, 0.55 μg/mL transferring and 6.7 ng/mL selenium (ITS, Life Technologies) was added to establish cell polarity and structural differentiation. The cell monolayers were let to cultivate for 14 days before experimentation. After 14 days, some cells were fixed in Karnowsky's fixative overnight as previously describe (Helander and Fändriks, 2014) for morphological analysis.

2.2. Transepithelial electrical resistance (TEER) in Caco-2 cell monolayers

The resistance was measured to keep track of cell development. Electrical resistance of the Caco-2 cell monolayer grown on permeable membrane filter was measured using a voltmeter (Millipore Corporation, Burlington, Massachusetts, USA). Resistance was measured from day 1 in culture (before confluence) to day 14 in culture. In addition, the effect of treating 14-day-old cultures with bile acids and FA (see below) as well as vitamin D3 (Sigma-Aldrich), on TEER was examined. A value above 400 $Ohm*cm^2$ indicating a tight monolayer culture, was set as a criterion for utilization in below experiments.

2.3. Screening of bile acid and fatty acid effects

All Caco-2 cell experiments started on day 14 after seeding. The cells were exposed for 48 h with the following: 1:75 dilution (~1 mM) of whole human bile (coming from one patient with unknown identity for the researchers, going through laparoscopic cholecystectomy for bile stone disease where the gallbladder was emptied of bile due to medical/surgical reasons and the bile was collected instead of being discarded) 10 or 100 μM chenodeoxycholic acid (CDCA) (Sigma-Aldrich) as primary bile acid, 10 or 100 μM lithocholic acid (LCA) (Sigma-Aldrich) as secondary bile acid, and 10 or 100 μM glycocholic acid (GCA) and taurocholic acid (TCA) (Sigma-Aldrich) as conjugated bile acid and 10 mM of the short-chain fatty acid butyrate (Sigma-Aldrich) in FBS-free medium in the upper compartments. In a separate series of experiments, the Caco-2 cells were treated with vitamin D3 (10 or 100 nM) and the Hsp90β inhibitor Geldanamycin (10–100 μM) for the last 24 h (InvivoGen, San Diego, CA, USA).

After incubation, the resistance was measured and the cells were saved for protein expression. The cells were scraped into lysis buffer in protein kinase blocking solution (1% Triton x-100, EDTA; ethylenediaminetetraacetic acid) containing protein kinase inhibitor buffer (10 mM potassium phosphate buffer pH 6.8, 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS: Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete (Roche Diagnostics AB, Stockholm, Sweden). The samples were sonicated on ice and the homogenate was centrifuged (10,000*g* for 10 min at 4 °C) and the supernatant was analyzed for protein content by the Bradford method. Samples were kept at −80 °C until further analyses of protein levels.

2.4. Western blot analysis

Samples were diluted in SDS buffer and heated at 70 °C for 10 min before loaded on a NuPage 10% Bis-Tris gel using MOPS buffer (Invitrogen AB, Lidingö, Sweden). One lane on each gel was loaded

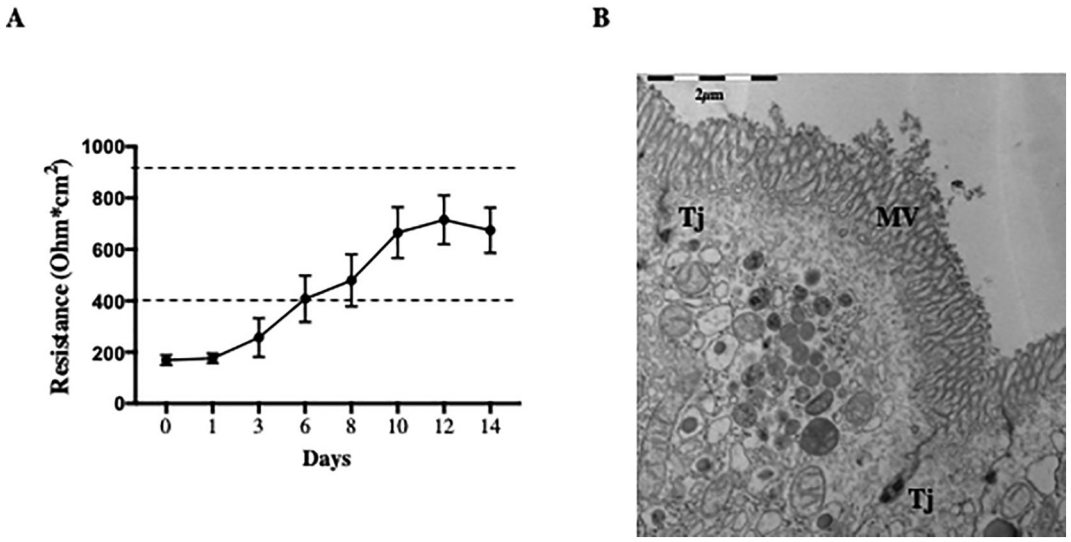

Fig. 1. Panel A shows the effect of cellular differentiation on transepithelial electrical resistance (TEER) in Caco-2 cells grown on semipermeable filter inserts. TEER was measured before seeding (day zero) and between day 1 (subconfluent, undifferentiated) and day 14 (confluent, fully differentiated). Values are means ± SD of seven 12 well-plates at each time. Representative electron microscopic image of Caco-2 cells monolayer with brush-border microvilli (MV) and tight junctions (Tj), shown in panel B.

with prestained molecular weight standards (SeeBlue, NOVEX, San Diego, CA, USA). After the electrophoresis, the proteins were transferred to a polyvinyldifluoride transfer membrane (Hybond, 0.45 μm, RPN303F, Amersham, Buckinghamshire, UK) using the iBlot dry blotting system (Invitrogen AB). The membranes were than washed with wash buffer (10 mM phosphate, 2.7 mM potassium chloride, 140 mM NaCl pH 7.45, 0.1% (v/v) Tween 20) and subsequently blocked in 0.2% (w/v) I-block reagent (Applied Biosystems, Bedford, MA) dissolved in wash buffer at room temperature before incubation with primary antibody against Hsp90β (ab80159, Abcam, Cambridge, UK), claudin-2 (51-6100, Invitrogen AB), claudin-12 (38-8200, Thermo Fisher Scientific, Rockford, USA), VDR (sc-1008, Santa Cruz Biotechnology, Dallas, USA) and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, IMG-5143A, Imgenex, San Diego, CA) overnight at 4 °C. After repeated washings with wash buffer, a HRP-conjugated secondary antibody (#7074, Cell Signaling Technology) was applied for 1 h at room temperature and visualization was carried out using the WesternBright Quantum reagents (K-12042, Advansta Corporation, Menlo Park, CA, USA). The signal intensities of specific bands were detected and analyzed using a Chemidox XRS cooled charge-couple device camera and the Quantity One software (BioRad Laboratories, Hercules, CA). GAPDH was used as a control for equal loading and for each tested sample the optical density (OD) of primary antibody divided by the corresponding GAPDH signal is presented in results. Each time the membrane was incubated with a new primary antibody, the previous antibody was first removed using stripping buffer (Re-Blot Plus Mild Solution (10×), Millipore, Temecula, CA).

2.5. Calcium flux experiments

Caco-2-cells were cultured on membranes as described above until culture day 14. In the first calcium transport experiment, cells were treated in both the apical and basolateral compartment with vehicle, or 1, 10, or 100 nM vitamin D3 for 24, 48, or 72 h, for validation of the protocol as well as to confirm previously results by Giuliano and Wood (1991) and Fleet and Wood (1994). In the next series of experiments, the cells were cultured for another 4 days in the presence of vehicle, or 0.05, 0.1 or 1 mM bile acid alone or in combination with 0.5, 1 or 5 mM of the short-chain fatty acid butyrate or the long-chain fatty acid oleic acid (Sigma-Aldrich) in the upper compartment. During the last 48 h the cells were also treated with 10 nM vitamin D3 in both compartments or the Hsp90β inhibitor Geldanamycin (10 μM) in the apical compartment in the last 24 h. In all experiments bile acid, fatty acid and vitamin D3 were diluted in DMEM. Control treatments comprised vehicle for vitamin D diluted to the same extent as the most concentrated vitamin D stock used in a particular experiment (0.1% or less final ethanol concentration).

The above described Caco-2 cell culture experiments were ended after 72 h. The cells were treated differently in the active and passive calcium transport experiments. In the active calcium transport experiments, 1 μCi/mL ^{45}Ca (Calcium-45, Perkin Elmer, Upplands Väsby, Sweden) was placed in the apical reservoir mixed with bile acid, butyrate, vitamin D3 and Geldanamycin, and the apical-to-basal flux was followed from the hot side (H) to the cold side (C). In the passive calcium transport experiments, the calcium flux assessments were performed by applying a lumen-to-basolateral concentration-gradients of Ca^{2+} as previously has been described by Jantarajit et al. (2007). One μCi/mL ^{45}Ca was placed in the apical reservoir mixed with 4, 40 or 80 mM Ca^{2+}, bile acid, fatty acid, vitamin D3 and Geldanamycin. The amount of ^{45}Ca radioactivity in 150 μL samples was measured in a beta counter (DSA-based liquid scintillation counters, Wallac 1409, Turku, Finland), after mixing the samples with 1 mL liquid scintillation cocktail (Ultima Gold XR, Perkin Elmer). Calcium transport was expressed as nanomoles calcium transported to the serosal compartment per hours per well during 90 min from 30 min to 2 h. Each treatment was performed in at least three wells in each experiment and all experiments were conducted at least three times.

2.6. Data analysis and statistics

Kruskal Wallis test and Mann-Whitney test for un-paired variables were used for analyses of differences in protein expression as well as calcium-flux measurements. All analyses were performed using Prism 6 for Mac OS X (GraphPad Inc., LaJolla, CA, USA). A p-value below 0.05 was considered significant.

3. Results

3.1. Effect of cellular differentiation on TEER

Cultures of Caco-2 cells were grown on semi-permeable membrane filters for up to 19 days (including the experiments). TEER was

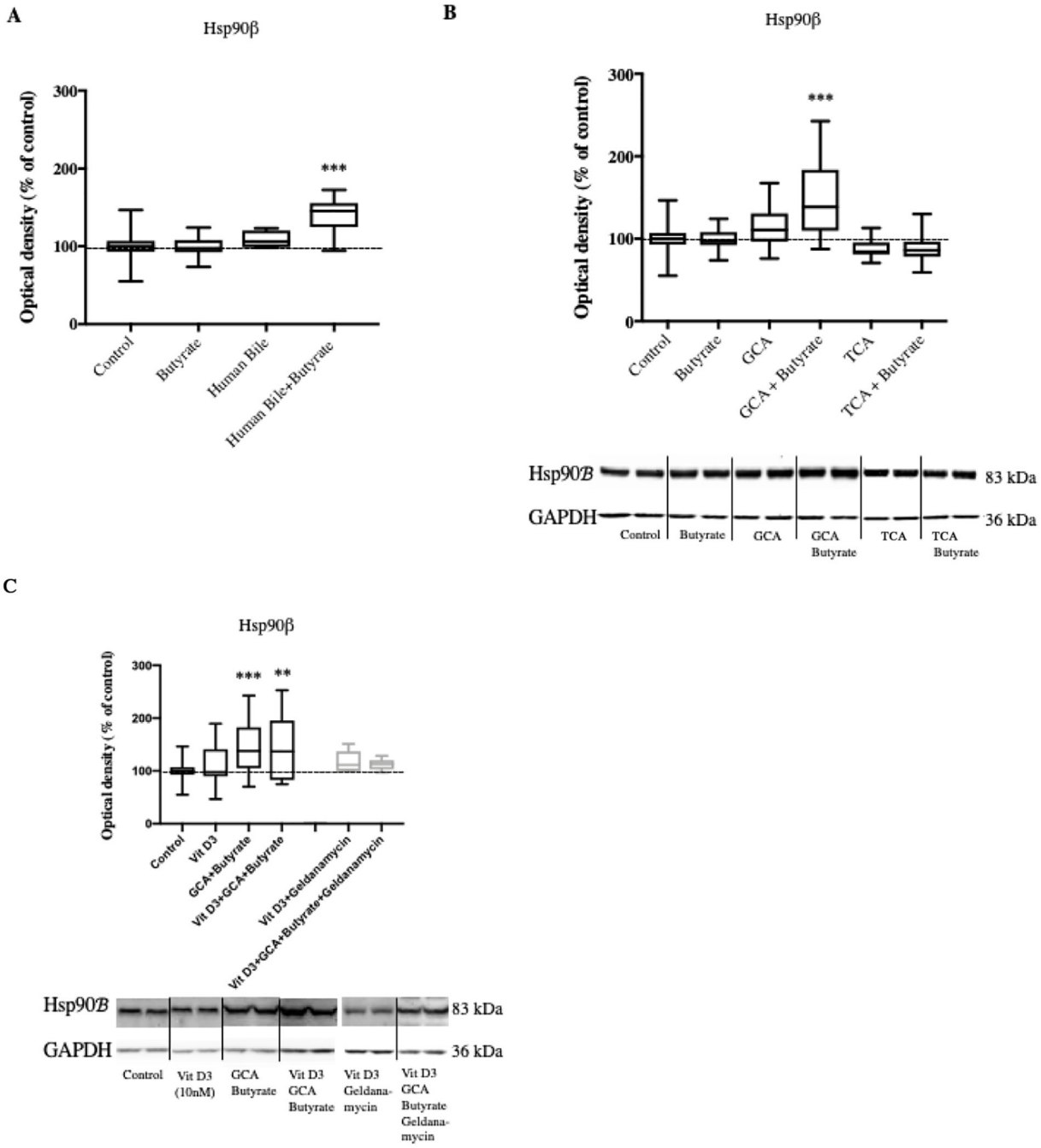

Fig. 2. Protein expression of the vitamin D receptor co-activator Heat-shock protein (Hsp) 90β. Panel A shows fourteen days cultured Caco-2 cells treated with whole human bile and the short-chain fatty acid butyrate (10 mM) alone or in combination. Panel B shows the effect of 10 mM glycocholic acid (GCA) or taurocholic acid (TCA) alone or in combination with butyrate (10 mM). Panel C shows the effect of vitamin D3 (Vit D3) alone or in combination with GCA and butyrate as well as the effect of addition of the Hsp90β-inhibitor Geldanamycin (10 μM). Values in panels A to C are min to max and each treatment was performed in three wells and the experiments were conducted three times. **($p < 0.01$); ***($p < 0.001$) denotes significant differences between control group and treated groups (Mann-Whitney).

measured on various days between days 1 (sub-confluent, undifferentiated) and 14 days (confluent, fully differentiated) (Fig. 1A). The resistance increased until day 10–12 where it usually culminated at around 700 Ohm ∗ cm^2. At this stage the cell line exhibited structural and functional patterns characteristic of mature small intestinal enterocytes, such as brush-border microvilli and the presence of tight junctions (Fleet and Wood, 1999). Wells, where resistance was below 400 at day 10–12 or increased above 900 Ohm ∗ cm^2, were omitted from further experimentation in order to get as homogeneous conditions for the experiments as possible. The histological appearance of the monolayer Caco-2 cultures is at this stage very similar to human intestinal epithelium (Fig. 1B).

3.2. Effect of bile and fatty acids on Hsp90β protein expression

Surprisingly, we found that whole human bile in combination with a short fatty acid, butyrate, increased Hsp90β expression in human intestinal epithelial Caco-2 cells, whereas either factor separately had no effect (Fig. 2A). In the next experiment, Caco-2 cells were stimulated with the single conjugated bile acids, GCA (10 or 100 μM) or TCA (10 or 100 μM), or the primary bile acid CDCA (10 or 100 μM) or the secondary bile acid LCA (10 or 100 μM) either alone or in combination

with butyrate. GCA in combination with butyrate increased the Hsp90β protein expression significantly, at a comparable level to whole bile ($p < 0.001$), while TCA had no such effect. A western blot panel presenting the effects of GCA, TCA and butyrate is shown in Fig. 2B. Neither the primary bile acid CDCA nor secondary bile acid LCA had any effect on Hsp90β expression (data not shown). Further it was tested whether vitamin D with or without GCA and butyrate affected the expression of Hsp90β (Fig. 2C). Therefore, Caco-2 cells were incubated with GCA and butyrate for 4 days with the addition of vitamin D3 for the last 48 h. Vitamin D did not affect the expression of Hsp90β either by itself or in combinations. Geldanamycin, a Hsp90β-inhibitor, tended to reduce Hsp90β expression but not significantly ($p > 0.05$) (Fig. 2C).

3.3. Effect of bile and fatty acids on Claudin-2 and Claudin-12 protein expression

Experiments were then performed to determine whether the passive calcium transport mechanism via tight-junctions would be regulated in a vitamin D- and Hsp90β-dependent manner. Caco-2 cells were incubated with GCA and butyrate for 4 days with the addition of vitamin D3 and Geldanamycin for the last 48 and 24 h respective. The expression of the permissive tight-junction proteins claudin-2 and claudin-12 was measured. The expression of claudin-2 and claudin-12 was not affected by GCA or butyrate. However, treatment of the Caco-2 cells with vitamin D3 significantly increased the claudin-2 and claudin-12 protein expression ($p < 0.05$). Further, Geldanamycin, significantly reduced claudin-2 expression in comparison with vitamin D3 alone or in combination with GCA and butyrate ($p < 0.05$, Fig. 3A). However, the expression of claudin-12 was not affected by Geldanamycin (Fig. 3B).

3.4. Effect of bile and fatty acids on VDR protein expression

Furthermore, we studied whether VDR would be regulated in a vitamin D- and Hsp90β-dependent manner. The expression of VDR was not affected by vitamin D3, GCA or butyrate. However, treatment of the Caco-2 cells with Geldanamycin significantly reduced VDR expression ($p < 0.05$, Fig. 3C).

3.5. Calcium flux validation experiments

In the first validation experiment, the effect of vitamin D3 on active calcium transport in Caco-2 cells was determined in 14-days old monolayer cultures with equal concentrations of calcium on the luminal and basolateral sides, with the addition of 1, 10 or 100 nM vitamin D3 for the last 4 days. With increasing concentrations of vitamin D3, there was an apparent dose-dependent increase in active calcium transport. Calcium transport was measured at 24, 48 and 72 h of exposure. A significant increase in calcium transport in the presence of 10 nM vitamin D3, but not vehicle, was seen after 48 h treatment ($+13.1 \pm 1.3$ nmol/cm^2 × h, $p < 0.05$, Fig. 3A). Longer periods of vitamin D3-treatment did not seem to result in further augmentation of the rate of calcium transport (not shown) but are in accordance with the previous studies by Giuliano and Wood (1991) and Fleet and Wood (1994). We therefore decided to proceed with measurements of calcium flux at 48 h using concentrations of 10 nM vitamin D3.

3.6. Active calcium transport

The effect of pretreatment of Caco-2 cells with GCA and butyrate for 4 days, in combination with 10 nM vitamin D3 during the last 48 h was determined. The active calcium transport was evaluated for different doses of GCA (0.05, 0.1 or 1.0 mM) and butyrate (0.5, 1 or 5 mM). The maximal apical-to-basal flux concentrations were estimated to occur at 0.1 mM GCA and 0.5 mM butyrate. Butyrate itself increased the TEER at concentrations higher than 1 mM ($p < 0.05$), whereas GCA, vitamin D3 as well as Geldanamycin at different concentrations did not affect the TEER on the Caco-2 cell monolayers (see Fig. 4C). Fig. 4A summarizes the effect of vitamin D3 separately and in combination with butyrate, or with GCA, or with these two in combination. Active calcium flux was significantly increased similarly in all groups compared to the vehicle treated controls ($+7.1$ nmol/cm^2 × h), but the magnitude was marginal. The rate was also similar between the different treatments ($+13.1$–18.2 nmol/cm^2 × h, p = not significant). Geldanamycin did not affect calcium flux in relation to vitamin D3 alone (Fig. 4A).

3.7. Passive calcium transport

Passive calcium transport was evaluated using increasing lumen to basolateral concentration-gradients of Ca^{2+} from 4, 40 to 80 mM in Caco-2 cells pretreated with bile acid, fatty acid, vitamin D3 and Geldanamycin. The apical to basolateral calcium flux at 4 mM CaCl2 was low (data not shown), but increased significantly at 40 and 80 mM compared to controls (Fig. 4B). The maximal transport velocity occurred after administration of 0.1 mM GCA and 0.5 mM butyrate in the presence of 40 mM luminal Ca^{2+}, and represented an up to almost 9-fold increase compared to vitamin D3 treated controls ($+1160 \pm 286$ vs. 130 ± 12 nmol/cm^2 × h, $p < 0.01$). At an 80 mM Ca^{2+}-gradient, the increase in calcium flux was 6.2-fold ($+820 \pm 57$ nmol/cm^2 × h, $p < 0.05$) at 0.1 mM GCA and 0.5 mM butyrate. When each factor was given separately, an increase in calcium flow relative to vitamin D3 treated controls was observed ($p < 0.05$) but with no significant difference between butyrate and GCA. Neither the second conjugated bile acid tested, TCA at 0.1 mM, nor the primary bile acid CDCA at 0.1 mM, had any effect on the calcium transport, separately or in combination with 0.5 mM butyrate. In contrast, a slight increase in calcium transport was observed when the Caco-2 cells were pretreated with 0.1 mM GCA in combination with 0.5 mM of the long-chain fatty acid, oleic acid, compared to vitamin D3 treated controls ($p < 0.05$). The maximum transport rate after administration of vitamin D3, GCA and butyrate in the presence of 40 mM luminal $Ca^{2+}$was significantly reduced in presence of the Hsp90β-inhibitor Geldanamycin ($p < 0.05$, Fig. 4B). Except for butyrate in high concentration (1-10 mM), none of the bile acids tested, nor vitamin D3 or Geldanamycin significantly affected the TEER in the Caco-2 cell monolayers (Fig. 4C). In contrast, during the 40 and 80 mM Ca^{2+}-gradient experiments, the resistance was reduced by 20 and 40% at the end of study respectively. However, the resistance was still high, which means that the cell layer was still intact. No change in resistance was found after administration of vitamin D3, GCA or butyrate (Fig. 4D).

4. Discussion

Traditionally, it has been assumed that active intestinal calcium absorption takes place only in the proximal small intestine and that passive absorption mainly takes place in the more distal small intestine. Recent evidence, however, suggests that passive paracellular calcium transport across the intestinal mucosal barrier, is regulated and may be coupled to active transcellular movement of calcium (Schafer, 2017; Christakos, 2012). Tight junctions are the major components regulating paracellular transport and claudins are clearly essential tight junction proteins for the paracellular permeability (Yu et al., 2009). Recently, Fujita et al. (2008) showed that vitamin D3 induced the expression of claudin-2 and claudin-12 in an intestinal epithelial cell line in vitro (Lee et al., 2015). Obviously, the exclusion of the duodenum from the passage of nutrients abolishes calcium uptake there. Further, it is possible that the diversion of bile acids and fatty acids (due to the absence of bile and pancreatic lipase) from the alimentary limb, may be part of causing the decreased calcium absorption. Therefore, we hypothesized that exposure of intestinal mucosal cells to either bile, or combinations of bile and fatty acids could improve calcium absorption after RYGB.

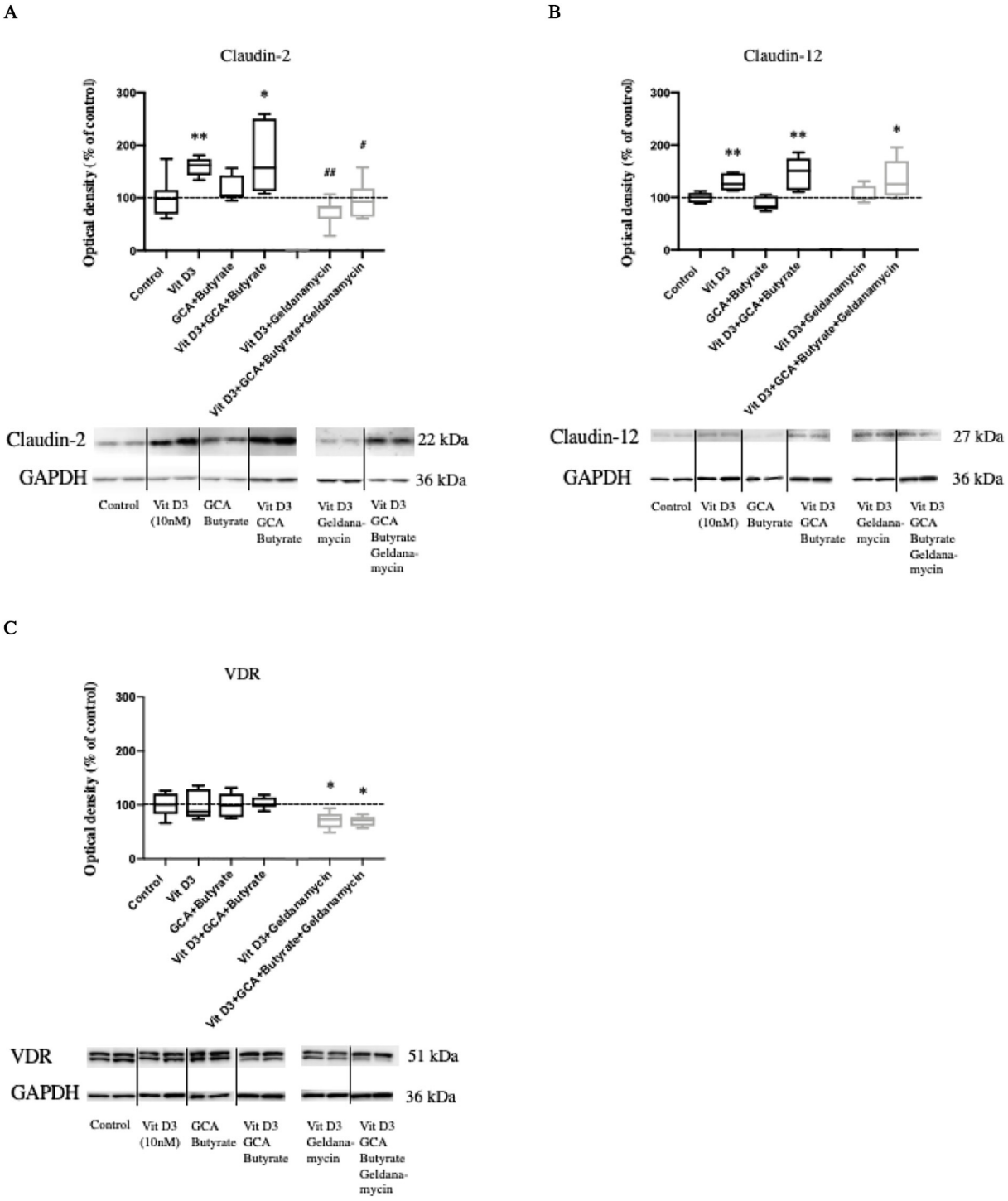

Fig. 3. Protein expression of the ion permissive tight-junction proteins claudin-2 and -12 and the vitamin D receptor (VDR). Panel A shows the effect of vitamin D3 (Vit D3) alone or in combination with 10 mM glycocholic acid (GCA), butyrate (10 mM), and the Hsp90β-inhibitor Geldanamycin on claudin-2 protein expression. Panel B shows the effect on claudin-12 protein expression. Panel C shows the effect on VDR. Values in panels A to C are min to max and each treatment was performed in three wells and the experiments were conducted three times. *($p < 0.05$); **($p < 0.01$) denotes significant differences between control group and treated groups. $^{\#}(p < 0.05)$; $^{\#\#}(p < 0.01)$ denotes significant differences between Vit D3, or the combination Vit D3, GCA, and butyrate with or without Geldanamycin (Mann-Whitney).

By global proteomics analyses of human jejunal mucosa from patients before and after RYGB, we have previously found that expression of the VDR co-activator Hsp90β was decreased (Elias et al., 2014). Further, we also found that the VDR-regulated calcium transporter protein TRPV6 was decreased, and that this was correlated with the down-regulation of Hsp90β (Elias et al., 2014). This indicated that Hsp90β is involved in the regulation of calcium transport by TRPV6 in a VDR-dependent fashion. In order to try finding ways to stimulate Hsp90β expression in Caco-2 cells, we exposed them to whole human bile, but found that this had no effect, unless also adding a fatty acid, butyrate, to the cell cultures. Butyrate by itself also had no effect. This indicated that bile and fatty acids act to increase Hsp90β in intestinal

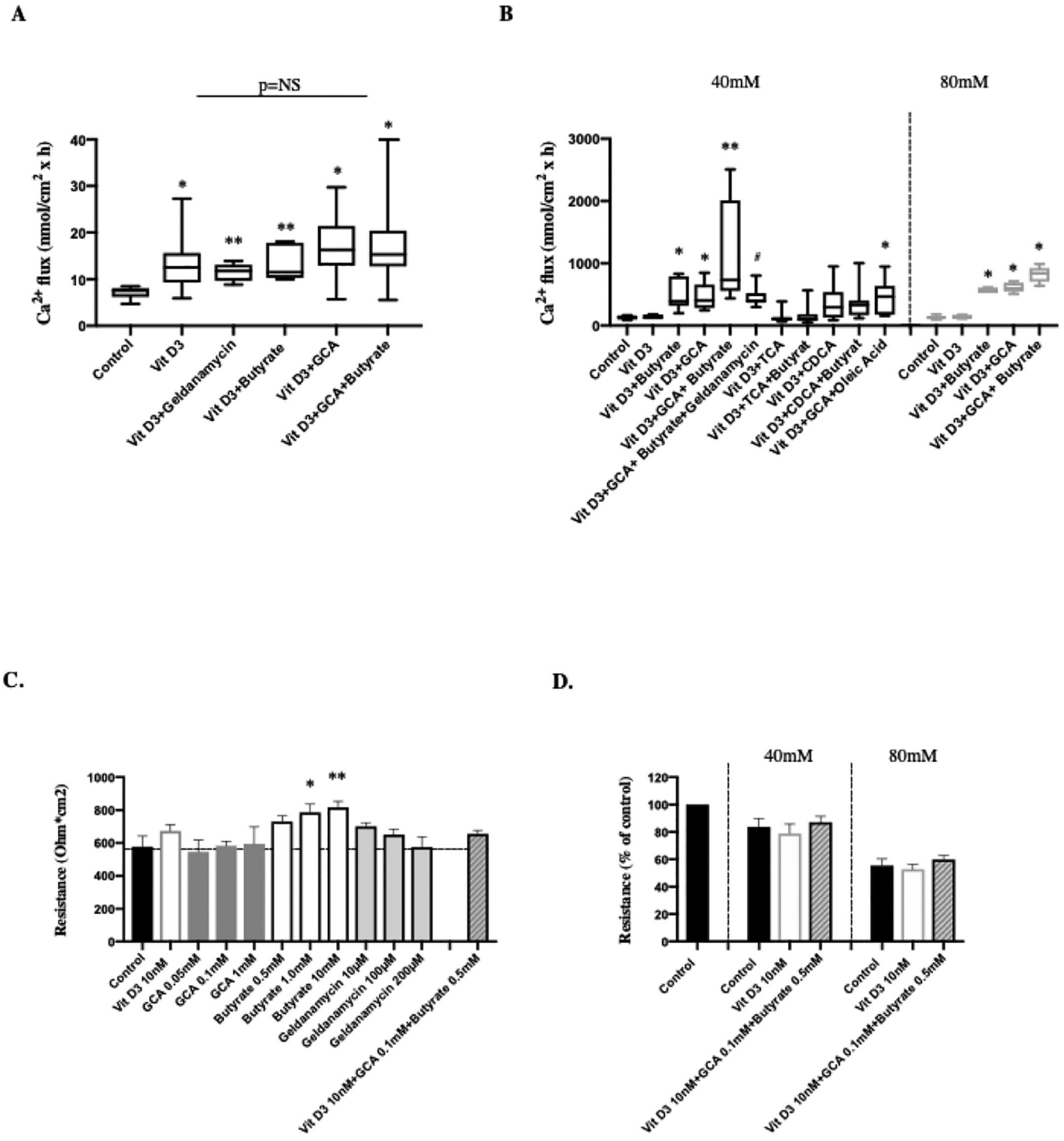

Fig. 4. Fourteen-days old Caco-2 cells grown on semipermeable membrane filters, then grown another 4 days in presence of a bile acid or fatty acid, or these two in combination. 10 nM 1,25 (OH)2D3 (Vit D3) was added for the last 48 h and Geldanamycin for the last 24 h. Panel A shows calcium flux for 0.1 mM glycocholic acid (GCA) and 0.5 mM butyrate tested with no Ca^{2+} gradient (1.8 mM concentration on both apical and basolateral sides). Panel B shows calcium flux for GCA, 0.1 mM taurocholic acid (TCA), chenodeoxycholic acid (CDCA), butyrate and 0.5 mM oleic acid tested in the presence of a Ca2+ gradient (40 mM or 80 mM on the apical side and 1.8 mM on the basolateral side). Values are min to max and each treatment was done in three wells and the experiments were conducted three times. Note the large scale difference between panels A and B. Panel C shows the resistance after the different treatments and Panel D shows the resistance after Ca2+ gradient. *($p < 0.05$); **($p < 0.01$) denotes significant differences between control group and treated groups. #($p < 0.05$) denotes significant differences between Vit D3, GCA and butyrate with or without Geldanamycin (Mann-Whitney).

epithelial cells in a synergistic fashion. This led us to screen single bile acids representing different groups of bile acids for a similar synergistic effect together with butyrate. Primary, secondary and conjugated bile acids were tested with or without the short fatty acid butyrate to screen for their effects on Hsp90β expression. Only GCA, a conjugated bile acid, in combination with butyrate significantly increased the Hsp90β expression. Neither, the other conjugated bile salt TCA, nor the primary CDCA or secondary LCA had any effect on Hsp90β expression.

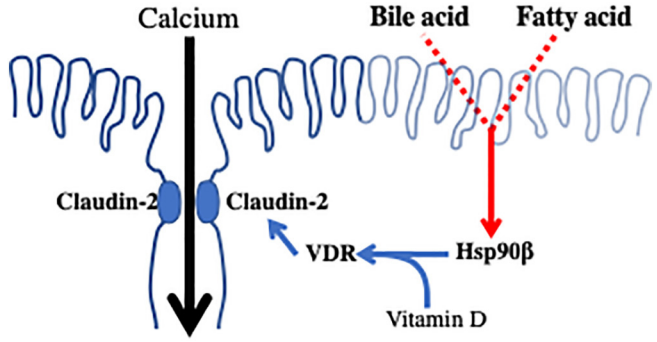

Fig. 5. A schematic presentation of the mechanism of effect of bile acid and fatty acid that synergistically act to increase Hsp90β expression. Hsp90β acts as a co-activator to the vitamin D receptor (VDR) to increase expression of the ion-permissive tight-junction protein claudin-2, to increase passive transcellular calcium flux.

In this study, we were able to confirm that the expression of claudin-2 and claudin-12 was vitamin D3-dependent and that Geldanamycin, a Hsp90β inhibitor, greatly reduced the expression of claudin-2, but not claudin-12. Therefore, this may represent a possible link between Hsp90β and passive calcium absorption. A number of bile acids together with short and long-chain fatty acids were screened to find combinations effective to increase trans-mucosal calcium transport. The study clearly shows that passive calcium transport was of much higher magnitude than active calcium transport in our in vitro model in the Caco-2 cell monolayers. Surprisingly, the combination of GCA and the short-chain fatty acid, butyrate, caused a synergistic increase of passive gradient-driven, but not active, calcium absorption. This is an interesting observation as the alimentary limb after RYGB completely lacks both bile acid and fatty acids which therefore may be a plausible cause of the reduced calcium uptake following RYGB surgery. A schematic presentation of the mechanism, i.e. the regulation of the paracellular calcium transport via VDR and Hsp90ß-mediated mechanism, is shown in Fig. 5. It should however be made clear that active and passive calcium transport occurs simultaneously (even at equimolar 1.8 mM calcium), albeit to varying degrees, but is described separately throughout the present work to elucidate their different mechanisms of action.

Patients undergoing RYGB are prescribed oral calcium and vitamin D supplementations. However, if the intestinal mucosa is rendered resistant to vitamin D-induced uptake of calcium, oral supplementation could be insufficient. In line with this, as mentioned above, recent data indicate that calcium uptake is significantly decreased after RYGB, even with sufficient vitamin D and calcium supplementations, and may lead to the compensatory increase of PTH levels that has widely been observed in RYGB patients (Schafer, 2017; Niu et al., 2019; Blom-Høgestøl et al., 2019). We recently described how bone mineral density (BMD) decreased independently of the weight-loss after RYGB but not after the restrictive bariatric method vertical banded gastroplasty, which does not involve re-routing of the alimentary tract (Elias et al., 2014). The main limitation of the current study is obviously that it was performed purely in a cell culture model and future in vivo studies will be necessary to establish to what extent the mechanisms described are of importance in a whole animal model.

5. Conclusions

The present study shows that in the presence of vitamin D, the conjugated bile acid GCA, in concert with the short fatty acid butyrate synergistically stimulated increased Hsp90β expression in Caco-2 cells. The same combination also increased passive calcium uptake up to 9-fold in these cells. We suggest that the mechanism is a vitamin D-mediated Hsp90β-dependent regulation of claudin-2. Future studies will be necessary to study whether the addition of a bile acid and a fatty acid to peroral vitamin D and calcium preparations also increase calcium uptake in vivo and whether this could improve calcium absorption after RYGB.

CRediT authorship contribution statement

Anna Casselbrant: Validation, Investigation, Writing - original draft.**Lars Fändriks:**Methodology, Data curation.**Ville Wallenius:**Conceptualization, Visualization, Writing - review & editing.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

VW and LF are co-founders and shareholders of Epicyt Pharma AB, that is investigating pharmacological possibilities to improve small intestinal calcium absorption.

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