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RESEARCH PAPER

Butyrate, a typical product of gut microbiome, affects function of the AhR gene, being a possible agent of crosstalk between gut microbiome, and hepatic drug metabolism

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Abstract

Modulation of gut microbiome composition seems to be a promising therapeutic strategy for a wide range of pathologic states. However, these microbiota-targeted interventions may affect production of microbial metabolites, circulating factors in the gut-liver axis influencing hepatic drug metabolism with possible clinical relevance. Butyrate, a short-chain fatty acid produced through microbial fermentation of dietary fibers in the colon, has well established anti-inflammatory role in the intestine, while the effect of butyrate on the liver is unknown. In this study, we have evaluated the effect of butyrate on hepatic AhR activity and AhR-regulated gene expression. We have showed that AhR and its target genes were upregulated by butyrate in dose-dependent manner in HepG2-C3 as well as in primary human hepatocytes. The involvement of AhR has been proved using specific AhR antagonists and siRNA-mediated AhR silencing. Experiments with AhR reporter cells have shown that butyrate regulates the expression of AhR target genes by modulating the AhR activity. Our results suggest also epigenetic action by butyrate on AhR and its repressor (AHR) presumably through mechanisms based on HDAC inhibition in the liver. Our results demonstrate that butyrate may influence the drug-metabolizing ability of liver enzymes *e.g.*, through the interaction with AhR-dependent pathways.

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Keywords: Butyrate; Aryl hydrocarbon receptor; Cytochromes P450; Drug metabolism; gut-liver axis.

1. Introduction

The gut microbiome, an aggregate genome of trillions of microorganisms residing in the human gastrointestinal tract (GIT), is now known to play a critical role in human health and predisposition to diseases [1]. Although the composition of the gut microbiota is relatively stable, it could be affected by wide range of factors such as diet [2], probiotics [3], administration of drugs (especially antibiotics) [4], and birth mode [5]. Changes in the

structure and diversity of the microbiome are also associated with large array of pathologic states including inflammatory bowel diseases (IBDs) [6], metabolic diseases as obesity [7] and diabetes [8], atherosclerosis [9], and neuropsychiatric disorders [10]. Based on this extensive list, it is clear that the influence of gut microbiota far exceeds the local impact on the GIT and reaches distant organs as well.

Liver – the center of drug metabolism – may be affected by the changes in the gut microbiota composition and bacterial metabolites production. Indeed, recent studies have reported that the gut-derived products may affect not only intestinal barrier function and immune homeostasis in the gut, but also can alter the hepatic metabolism and stimulate inflammation in the liver [11]. Other studies demonstrate the connection between small metabolites produced by bacteria in the gut and progression of hepatic diseases linked to inflammation such as hepatic fibrosis, non-alcoholic steatohepatitis (NASH), and non-alcoholic fatty liver disease (NAFLD) [12]. Hepatic inflammation causes impaired drug

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metabolism and, hence, may indirectly influence the pharmacotherapy. It has been found that gut microbiota and its metabolites are involved in the biotransformation of drugs [13,14] and several recent studies have shown that the gut microbiota can affect the pharmacokinetics of orally taken drugs with implications for their oral bioavailability [15–18].

Short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, are organic acids produced by the gut microbiota mainly during the fermentation of nondigestible polysaccharides in the intestinal lumen. Loads of papers show the importance of SCFAs in the maintenance of health and the development of diseases [19,20]. Among SCFAs, butyrate has earned particular consideration for its significant role in cellular energy metabolism and intestinal homeostasis and for its anti-inflammatory and anti-carcinogenic effect [21]. The mechanisms of action of butyrate are various; many of these are related to its potent regulatory effects on gene expression, including genes encoding enzymes involved in drug metabolism.

Studies on germ-free (GF) mice, lacking the intestinal bacteria, have shown that microbiome-derived metabolites may play an important role in regulation of the expression of hepatic biotransformation enzymes - including cytochromes P450 (CYPs) [22–24]. CYPs are key enzymes involved in the initial drug metabolism in humans [25]. Expression of CYPs is regulated by specific nuclear receptors like aryl hydrocarbon receptor (AhR). AhR is a ligand-induced transcription factor that belongs to the basic helix-loop-helix Per-Arnt-Sim family and regulates number of genes such as *CYP1A1*, *CYP1A2*, *CYP1B1* [26].

Apart from dioxin (TCDD), the first identified AhR ligand, a wide range of ligands were identified including microbiota-derived metabolites such as phenazines and indole derivatives [27,28]. Besides its ability to tune xenobiotic metabolism, the ligand dependent activation of AhR plays an important role in adaptive immunity [29] and inflammation [30] in the GIT. While the effect of butyrate in the GIT has been studied intensively and butyrate has been shown to activate AhR and induce *CYP1A1* gene expression in human intestinal epithelial cells [31], the potential impact of butyrate on the processes in the liver is scarce.

In this study, we have investigated whether butyrate may interfere with hepatic drug metabolism by regulating the CYP enzymes expression through AhR signaling pathway.

2. Materials and methods

2.1. Chemicals

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxine) and sodium butyrate were from Sigma-Aldrich (Prague, Czech Republic). CH-223191 (2-Methyl-2H-pyrazole-3-carboxylic acid-(2-methyl-4-o-tolylazophenyl) -amide, an antagonist of TCDD-mediated AhR activation [32,33], was from Calbiochem (Merck, Darmstadt, Germany). Acetonitrile, methanol, and HPLC columns were from Merck (Darmstadt, Germany). Western blotting was done using a chemiluminescence kit from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic). All chemicals were of highest purity available.

2.2. Cell cultures

HepG2-C3 cells (ATCC) were cultured as recommended in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, Penicillin-Streptomycin (100 U mL⁻¹-100 µg mL⁻¹; Gibco), in a 5% CO₂ humidified atmosphere at 37°C. Cells were used at low passage number (<20) to ensure constitutive AhR expression.

Huh7 (human hepatocellular carcinoma) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in antibiotic-free Dulbecco's modified Eagle's medium (DMEM, 10569010, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, F2442, Sigma-Aldrich) at 37°C in a humidified incubator under 5% CO₂.

HepG2-Lucia AhR reporter cells (InvivoGen) were grown according to the manufacturer's instructions in Minimum Essential Medium Eagle - Alpha Modification

Table 1
Clinical characteristics of the liver donors.

Liver identification	Sex	Age	Pathology
PHH 449	M	66	Cholangiocarcinoma
PHH 450	M	66	Cholangiocarcinoma
PHH 451	F	20	Adenoma
PHH 457	M	76	Hepatocellular carcinoma
PHH 462	M	73	Metastasis of colic adenocarcinoma
PHH 463	M	69	Organ donor

(α -MEM; Gibco USA), 10% (v/v) heat-inactivated fetal calf serum (Gibco), 1% non-essential amino acids (Gibco), Penicillin-Streptomycin (100 U mL⁻¹-100 µg mL⁻¹; Gibco), 100 µg mL⁻¹ Normocin (InvivoGen) and 100 µg mL⁻¹ Zeocin (InvivoGen). To measure AhR activation, 20 µL samples were added in triplicate to a 96-wells plate (Corning, USA). Thereafter, 180 µL cell suspension (~20,000 cells per well) were added to each well and the plate was incubated for 24 h at 37°C in a CO₂ incubator. After incubation, 20 µL stimulated cell supernatant were transferred into a white-walled clear bottom 96-wells plate (Corning), followed by addition of 50 µL QUANTI-Luc (InvivoGen). Luminescence was measured using a spectrophotometer Tecan Infinite M200 (Schoeller Instruments, Czech Republic). AhR activation was expressed as fold change of the untreated control.

2.3. Human liver samples and preparation of primary human hepatocytes (PHH)

Liver samples were obtained from resections performed in adult patients for medical reasons unrelated to our research program or from donors when the liver was considered unsuitable for organ transplantation. Their use has been approved by French National Ethics Committees and legal instance, and by the French Graft Institute "Agence de Biomedecine," respectively. Regarding livers from non-transplantable organ donor, the informed consent of the donor's family was obtained by the Service de la Coordination Hospitalière (CHU Montpellier). For resections, written or oral consent was obtained from the patients.

Liver samples were obtained from the Biologic Resource Center of Montpellier University Hospital (CRB-CHUM; <http://www.chumontpellier.fr>; Biobank ID: BB-0033-00031), and this study benefitted from the expertise of Dr. Benjamin Rivière (hepatogastroenterology sample collection) and Dr. Edouard Tuailon (CRB-CHUM manager). The clinical characteristics of the liver donors are given in Table 1. PHHs were prepared as described previously and cultured in collagen-coated dishes at a density of 1.7 × 10⁵ cells/cm² in ISOM medium [34, 35]. At day 3 post-isolation, cells were incubated with the different compounds or with equal amounts of vehicle (DMSO: final concentration 0.1%). All treatments were done in duplicates.

2.4. Small Interfering RNA transient transfection

Adherent PHHs were transfected with 20 nM non-targeting siRNA (scrambled, siSC) or siRNAs specific for AhR (Dharmacon, Lafayette, CO) at day 1 and day 3 after seeding using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, USA). HepG2-C3 cells were transfected with 20 nM non-siSC or siAhR using Lipofectamine RNAiMAX at day 1 before seeding and at day 2 post-seeding. At day 3 post-seeding, cells were pretreated for 2 h with butyrate and subsequently with TCDD. The total time of incubation with butyrate was 24 h. All treatments were done in duplicates.

2.5. RNA isolation and PCR

After extraction with Trizol reagent (Invitrogen), 500 ng of total RNA was reverse-transcribed using a random hexaprimer and the MMLV Reverse Transcriptase Kit (Invitrogen). Quantitative PCR was performed using the Roche SYBER Green reagent and a LightCycler 480 apparatus (Roche Diagnostic, Meylan, France) with the following program: one step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 10 s, annealing at 65°C for 15 s and elongation at 72°C for 15 s. The amplification specificity was evaluated by determining the product melting curve. Results are expressed as indicated in the figure legends. Primer sequences are given in Table 2. The relative mRNA expression was normalized to the expression of ribosomal protein large P0 (RPLP0), as recommended by Vandesompele et al. [36]. The Ct-values for RPLP0 were not affected by any of the treatments.

2.6. Protein preparation and western blotting

Total protein extracts were prepared using RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclear and cytoplasmic extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (ThermoFisher) according to the manufacture instructions. Buffers were supplemented with protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA) prior to use.

Table 2
Primer sequences.

Gene Name	Forward primer	Reverse primer
CYP1A1	TCCGGGACATCACAGACAGC	ACCTGGGGTTCATCACAA
CYP1A2	CATCCCCACAGCACAAACA	TCCCACTTGGCCAGACTTC
AhR	GTCGTCTAAGGTGTCTGCTGGA	CGCAAACAAGCCAAGTGGGTG
AhRR	TGACCTTGTCTTGACCC	CCATCCTCACTGTGCTTTC
p21	AGGTGGACCTGGAGACTCTCAG	TCCTCTGGAGAAGATCAGCCG
FGF21	CTGCAGCTGAAAGCCTTGAAGC	GTATCCGCTCTCAAGAAGCAGC
RPLP0	TCGACAATGGCAGCATCTAC	GCCTTGACCTTTTCAGCAAG

The protein concentration was determined by the bicinchoninic acid method, according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL, USA). Bovine serum albumin (Pierce Chemical Co.) was used as standard. Equal amounts of total proteins were separated on precast SDS-polyacrylamide gels (4–15%) (Bio-Rad, Marnes la Coquette, France), then transferred onto PVDF membranes (Bio-Rad). Membranes were incubated with mouse monoclonal anti-CYP1A1/2 (SC-53241, Santa Cruz), anti-GAPDH (SC-32233, Santa Cruz), anti-TATA Box binding protein (TBP, Neomarker), rabbit polyclonal anti-AhR (Enzo Life Sciences AG, Lausen, Switzerland) or goat polyclonal anti- β -actin (SC-1616, Santa Cruz) antibodies. Immunocomplexes were detected with horseradish peroxidase-conjugated mouse, rabbit or goat secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) followed by enhanced chemiluminescence reaction (Millipore, Molsheim, France). Chemiluminescence was monitored using a ChemiDoc-XRS+ apparatus (Bio-Rad Laboratories) and quantified using the Image Lab software (version 4.1).

2.7. Enzyme assay

Enzyme activity of CYP1A1/2 enzyme was measured in hepatic microsomes using phenacetin as a substrate according to the established method [37]. High-performance liquid chromatography analyses (performed in three replicates) of samples with formed metabolite paracetamol were performed using the Prominence system (Shimadzu, Kyoto, Japan) with UV detection.

2.8. Statistics

The normal distribution of data was tested using Shapiro-Wilk test. For statistical evaluation Student's paired two-tailed t-test and the one-way ANOVA with the Tukey's *post hoc* test was done using the Statistica 12 software (Dell, obtained from StatSoft, Prague, Czech Republic). The number of experiments and replicates are given in each figure legend. When evaluating the student's test, we proceeded as follows; in the first step, we verified the normal distribution of the tested data via Shapiro-Wilk test. When, we confirmed the normal distribution of the data, we continued by the student test. A similar procedure was chosen for the one-way ANOVA.

3. Results

3.1. Butyrate enhances the expression of AhR target genes in liver

It has been found earlier that intestinal microbiome may affect the expression of liver biotransformation enzymes [22–24]. Products of bacterial metabolism in the intestinal lumen may be responsible for this effect and an increasing number of microbiome-derived metabolites have been shown to drive the AhR activation in the GIT [27]. To assess whether the same effect will be proved in the liver, the human hepatocellular carcinoma cell line HepG2-C3 and the primary culture of human hepatocytes (PHHs), considered as the gold standard model of liver metabolism, were used. Experiments showed (by qRT-PCR) that in PHHs, AhR target genes CYP1A1, CYP1A2 and AhR repressor AHRR were upregulated upon 24 h incubation with butyrate in dose dependent manner (Fig. 1A). The mRNA expression of CYP1A1 and AHRR was significantly increased at all selected concentration of butyrate also in HepG2-C3 cells after 24 h incubation (Fig. 1B).

Moreover, gained data were confirmed at the level of enzyme activity (using phenacetin as substrate), when PHHs treated with butyrate exhibit significantly higher levels of functional CYP1A1/2

than untreated control cells (Fig. 1C). The enzyme activity was increased by almost 50% in cells treated with 0.5 mM butyrate for 24 h, which may modulate the response to drugs metabolized by this important biotransformation enzyme.

3.2. Butyrate increases the expression of AhR-target genes in an AhR-dependent manner

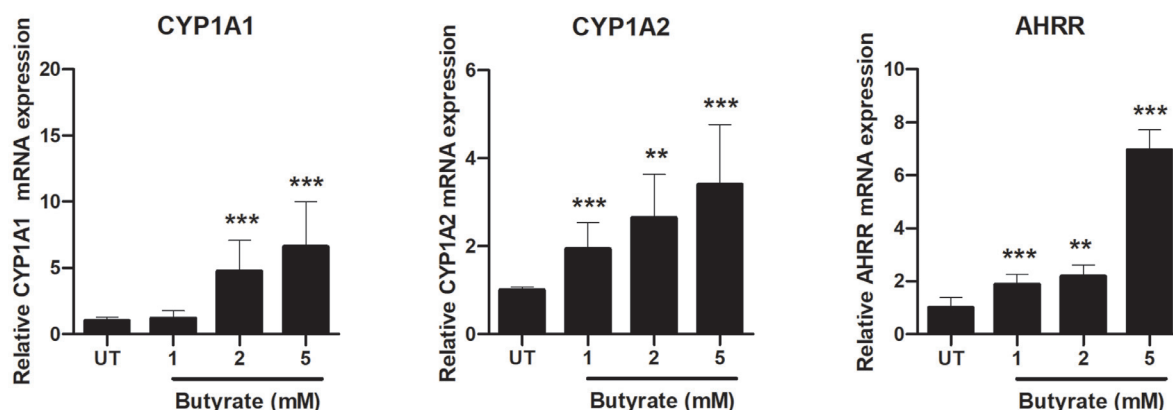
The AhR involvement in upregulation of selected target genes by butyrate was assayed using siRNA. As shown in Figure 2A, the basal mRNA expression of AhR was significantly reduced in silenced PHHs compared with PHHs transfected with non-targeting siRNA (siSC). The increase in CYP1A1 and CYP1A2 mRNA expression upon incubation with butyrate or TCDD was reduced in AhR-silenced PHHs compared with PHHs transfected with siSC (Fig. 2A). The obtained data suggest that butyrate induces the expression of CYP1A1/1A2 mRNA in an AhR-dependent manner. On the contrary, the induction of AHRR mRNA expression was not diminished in silenced PHHs suggesting different mechanism behind butyrate effect (Fig. 2A). Finally, AhR activation by butyrate was observed in HepG2-C3 cells where the basal mRNA expression of AhR was reduced by approximately 90% in silenced cells compared with control cells transfected with non-targeting siRNA (Fig. 2B). As a consequence, the protein expression of AhR was reduced by siRNA silencing in HepG2-C3 cells. This effect of AhR siRNA was accompanied by a significant reduction of the mRNA expression of CYP1A1 (induced by butyrate or TCDD) in HepG2-C3 cells (compared to control cells, Fig. 2B). Further, the observed increase in CYP1A1/1A2 mRNA expression by butyrate was, at least partially, reduced when cells were pre-incubated with AhR antagonist CH-223191 (a known inhibitor of TCDD-mediated AhR-dependent transcription [32]) for 90 min before application of butyrate or TCDD in PHHs (Fig. 2C). The same trend has been observed in HepG2-C3 cells, where pretreatment with CH-223191 significantly diminished the upregulation of CYP1A1 mRNA expression (Fig. 2D), suggesting that butyrate may work as a low AhR activator. Specific AhR antagonist and siRNA-mediated AhR silencing demonstrated that butyrate induces the expression of CYP1A1/1A2 in an AhR-dependent manner.

3.3. Butyrate induces AhR translocation and activation in cell lines

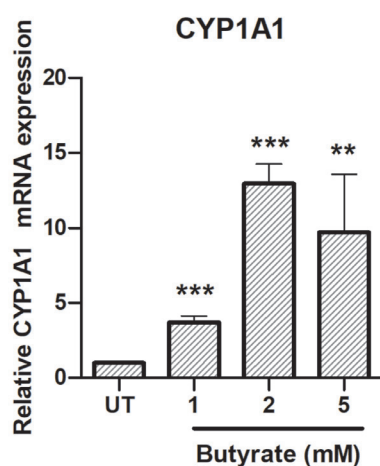
Next, the ability of butyrate to activate AhR was investigated using HepG2-Lucia AhR reporter cells. A significant increase of AhR activity by butyrate in dose dependent manner was observed, although the induction was lower than in the case of TCDD (Fig. 3A). The observed increase in luciferase activity was reduced when cells were pre-incubated with AhR antagonist CH-223191 for 90 min before stimulation with butyrate or TCDD (Fig. 3A). These results indicate that butyrate activates AhR in liver cells.

Subsequently, a nuclear translocation assay was used to further investigate a potential role of butyrate as a AhR agonist in the hu-

A PHH



B HepG2-C3



C PHH

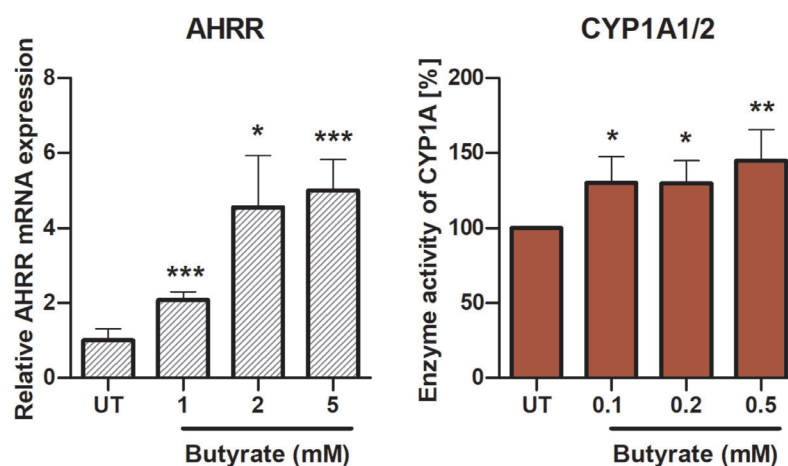


Fig. 1. Butyrate induces the mRNA expression of AhR responsive genes in HepG2-C3 cells and primary human hepatocytes (PHHs). (A) Expression of CYP1A1, CYP1A2 and AHRR mRNA in PHHs treated for 24 h with butyrate (1, 2, and 5 mM). The results are expressed as mean±S.D.; N=5 (five donors), in each experiment duplicate measurements were performed. (B) Expression of CYP1A1 and AHRR mRNA evaluated after 24 h treatment with butyrate (1, 3, and 5 mM) in HepG2-C3 cells. The results are expressed as mean±S.D.; N=3, in each experiment duplicate measurements were performed. (C) PHH treated with butyrate exhibit higher levels of functional CYP1A1/2 enzymes. The enzyme activity of CYP1A1/2 was measured in the PHHs treated with butyrate (0.1, 0.2, and 0.5 mM) for 24 h. Control (100%) represents systems in the absence of butyrate. Results are expressed as mean±S.D.; N=3 (three donors), in each experiment duplicate measurements, were performed. Values significantly increased (* P <.05, ** P <.01, *** P <.001) in comparison with the control are labeled.

man liver. In Huh7 cells, butyrate at the both selected concentrations induced a slight nuclear translocation of AhR after 8h and 24h with peak at 8h of incubation. Therefore, butyrate performed the similar effect on the AhR nuclear translocation as a typical AhR agonist, TCDD (Fig. 3B). Slight induction of AhR nuclear translocation by butyrate in dose dependent manner was observed in HepG2-C3 cells, which was reduced when cells were pre-incubated with AhR antagonist CH-223191 (Fig. 3C). Altogether, the obtained data provide the evidence that butyrate may work as a low AhR activator.

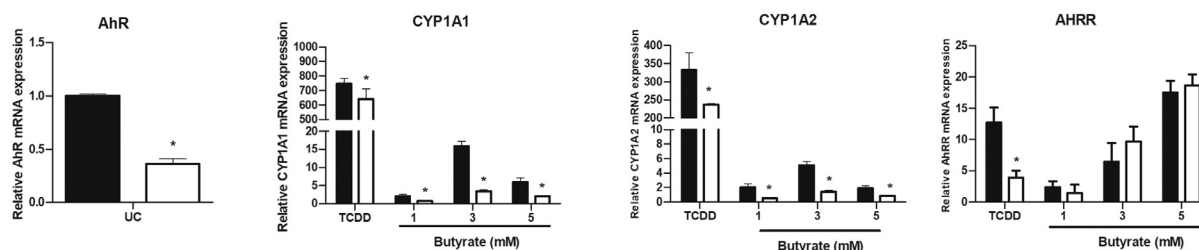
Butyrate enhances the AhR nuclear translocation. (B) Huh7 cells were incubated during 3 h, 8 h, and 24 h in presence of 1mM and 5 mM butyrate or 10nM TCDD. Nuclear extracts were blotted for AhR protein expression; its quantification is on the right. TBP was used as control for nuclear extract preparation. (C) Effect of CH-223191 on butyrate or TCDD induced AhR nuclear translocation in HepG2-C3 cells. HepG2-C3 cells were incubated or not with AhR ligand antagonist (CH-223191 3.33 μ M) prior stimulation with 1 mM and 5 mM butyrate and 10nM TCDD for 24 h. Nuclear ex-

tracts were blotted for AhR protein expression; its quantification is on the right. TBP was used as control for nuclear extract preparation. UT: Untreated.

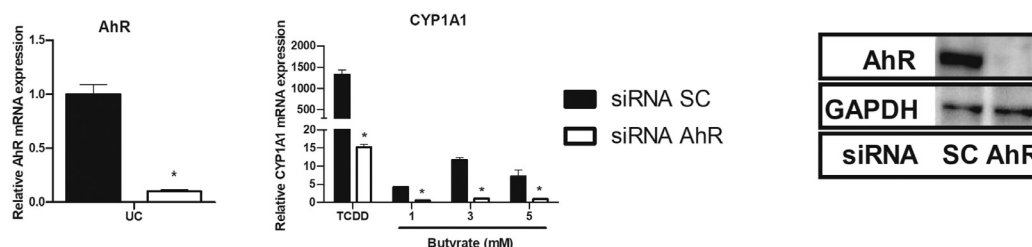
3.4. Butyrate potentiates TCDD-mediated AhR activity

The combined effect of the prototypical AhR activator TCDD and butyrate was also examined. Interestingly, in PHHs the strong induction of CYP1A1 mRNA expression by TCDD was amplified in dose dependent manner by butyrate (Fig. 4A). Experiments with HepG2-C3 cells have showed the same results; TCDD alone strongly increased CYP1A1 mRNA expression and its 24 h co-incubation with 1 mM, 3 mM, and 5 mM butyrate led to a further increase in dose dependent manner (Fig. 4B). Moreover, it was observed that a significant increase in AhR activation by TCDD was amplified in dose dependent manner by butyrate in HepG2-Lucia AhR reporter cells (Fig. 4C). These results were rather unexpected for an agonist and suggest a complex effect of butyrate on the AhR signaling pathway.

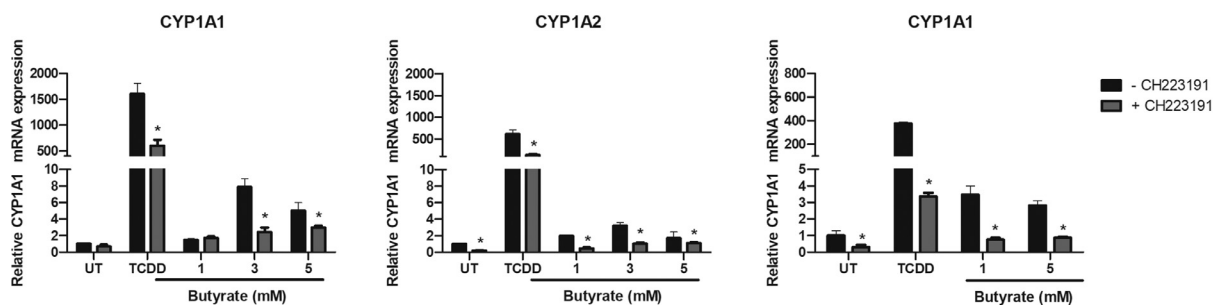
A PHH



B HepG2-C3



C PHH



D HepG2-C3

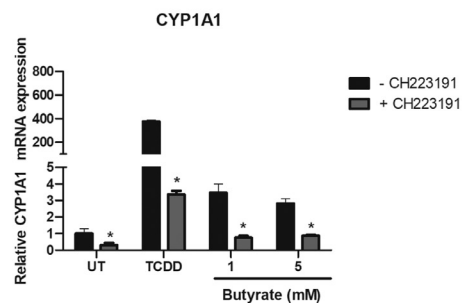


Fig. 2. Involvement of AhR in CYP1A1 and CYP1A2 mRNA up-regulation by butyrate. (A) RT-qPCR analysis of AhR, CYP1A1, CYP1A2 and AHRR mRNA expression in PHHs (Donor #462, measured in duplicates) after siRNA-mediated AhR down-regulation and incubation with 1-, 3- and 5-mM butyrate or 10nM TCDD for 24h. (B) AhR and CYP1A1 mRNA expression in HepG2-C3 cells transfected with control siRNA or AhR siRNA and incubated during 24 h with 1-, 3-, and 5-mM butyrate or 10nM TCDD. The results are expressed as mean \pm S.D.; N=3, in each experiment duplicate measurements were performed. To assess the efficiency of siRNA, AhR protein expression was also evaluated in HepG2-C3 cells transfected with control siRNA or AhR siRNA (right). (C) CYP1A1 and CYP1A2 mRNA expression in PHHs (Donor #463, measured in duplicates) was determined. PHHs were incubated or not with AhR ligand antagonist (CH-223191 3.33 μ M) prior stimulation with 1-, 3- or 5-mM butyrate or 10 nM TCDD for 24 h. (D) The same experiment was performed with HepG2-C3 cells and mRNA expression of CYP1A1 was evaluated. Data are expressed as mean \pm S.D., (N=3) and significantly different results compared to cells transfected with non-target siRNA are labeled (* $P \leq 0.05$).

3.5. Butyrate induces AhR gene and protein expression

As the effect of butyrate on the transcription of AhR-regulated genes was proven, and as butyrate potentiates TCDD-mediated AhR activity, a logical next step was to examine whether the expression of the AhR protein itself was influenced by butyrate in the PHHs and liver cell lines. The AhR mRNA expression was not affected by 1- and 2-mM butyrate but was significantly increased by 5 mM butyrate in all tested PHHs (N=5 donors) (Fig. 5A). The AhR mRNA expression was also significantly increased in HepG2-C3 cells after 24 h incubation with butyrate (Fig. 5B). In Huh7 cells, the AhR mRNA expression was increased by 5 mM butyrate in time-dependent manner. The strongest induction by 1 mM butyrate was reached after 6 h of treatment (Fig. 5C). The protein expression of AhR was enhanced by butyrate in Huh7 cells. After 8 h of incubation, the AhR protein expression increased and continued to rise in time and concentration-dependent manner (Fig. 5D).

Twenty-four h incubation with butyrate has also caused significant increase in AhR protein expression in HepG2-C3 cells (Fig. 5E).

3.6. Epigenetic action of butyrate on AhR

Question arises whether the induction of AhR mRNA and protein expression caused by butyrate depends on AhR activation. Conversely it may act through the inhibition of histone deacetylation that plays a role in the regulation of transcriptional activity of AhR promoter [38]. Butyrate effect on AhR expression was thus compared to valproic acid (VA), another short-chain fatty acids inhibitor of Class I and II HDAC [39]. Both molecules exhibit a similar effect on promoter activity of Renilla control plasmid (S3) [40].

The induction of AhR expression by 1 mM and 3 mM butyrate was significantly reduced by CH-223191 in PHHs (Fig. 6A). This is not the case for higher concentration (5 mM) of butyrate, or VA (Fig. 6A). By the same, the induction of AHRR mRNA expression by butyrate or VA was not reversed by CH-223191 (Fig. 6A).

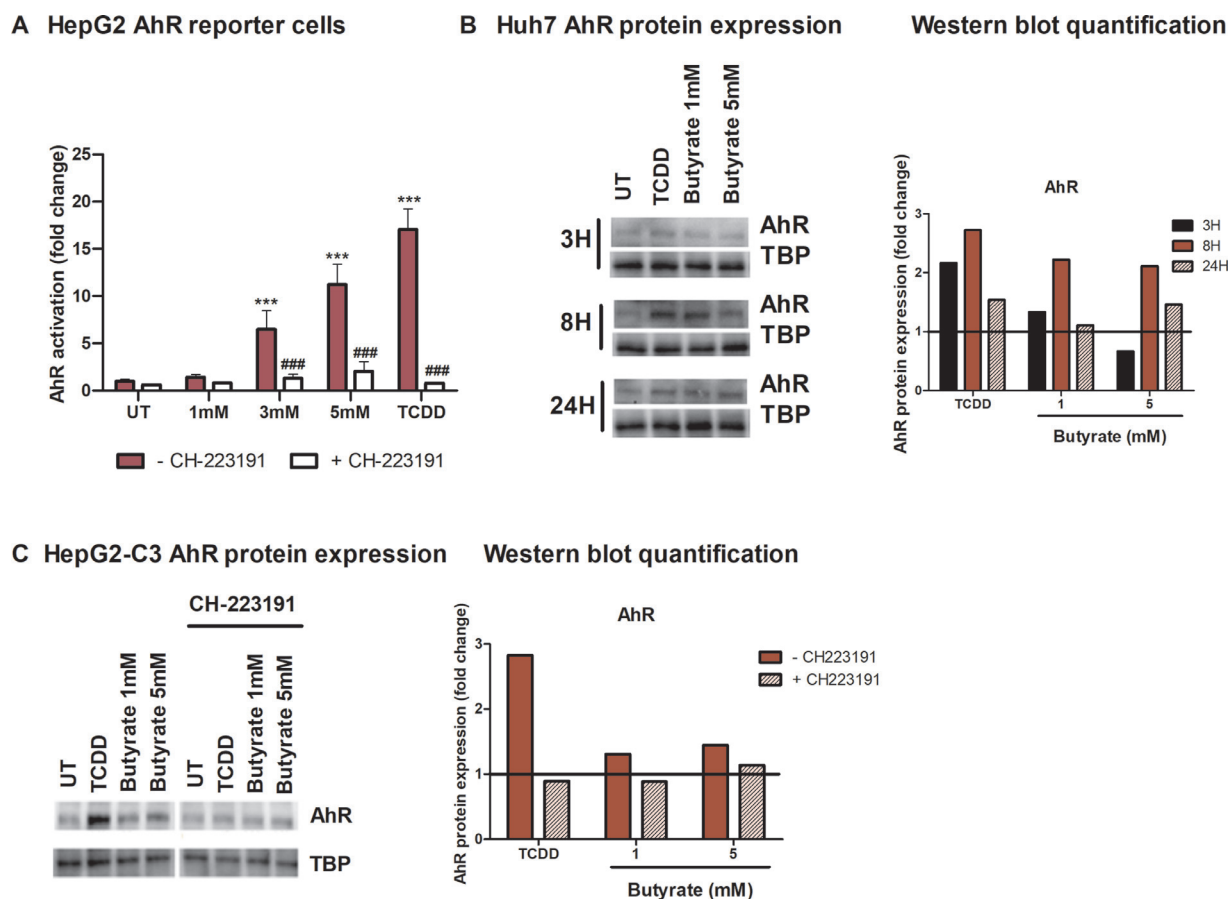


Fig. 3. Butyrate induces AhR translocation and activation in cell lines. (A) HepG2-Lucia AhR reporter cells was incubated with/without AhR ligand antagonist (CH-223191 3.33 μ M) prior stimulation with 1, 3, and 5 mM butyrate or 10 nM TCDD for 24 h. Data are expressed as means \pm S.D. (N=3), in each experiment triplicate measurements were performed. Values significantly increased in comparison with the un-treated (UT) controls are labeled (** P \leq .001), in comparison with the cells treated with TCDD only or in comparison with the cells treated with cells without antagonist pretreatment (### P \leq .001).

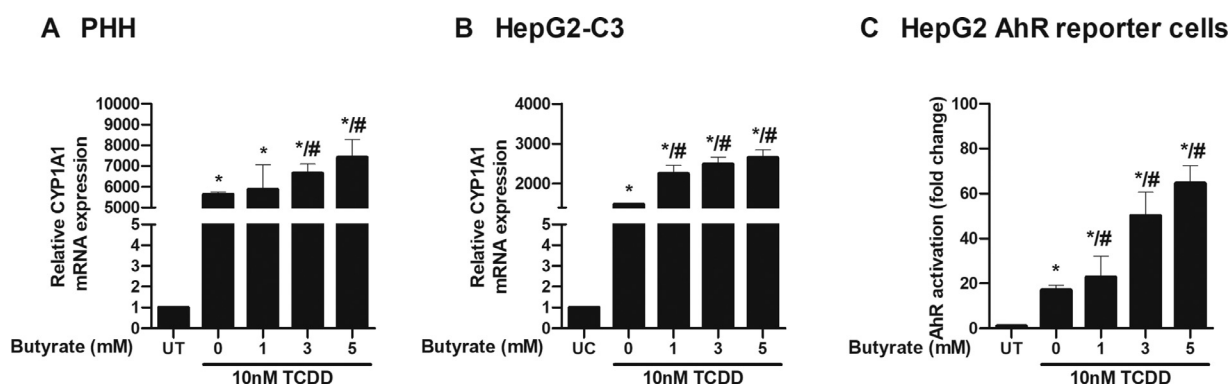


Fig. 4. Butyrate potentiates the effect of TCDD on the CYP1A1 mRNA expression in (A) PHH (B) HepG2-C3. Expression of CYP1A1 mRNA in PHHs treated with 10nM TCDD alone or in combination with butyrate (1, 3, and 5 mM). The results are expressed as mean \pm S.D.; N=3, in each experiment duplicate measurements were performed. (C) HepG2-Lucia AhR reporter cells were incubated with butyrate (1, 3, and 5 mM) and/or TCDD (10 nM) for 24 h and AhR activation was measured by luciferase activity in the supernatant. The results are expressed as mean \pm S.D.; N=3, in each experiment triplicate measurements were performed. Values significantly increased in comparison with the un-treated controls are labeled (* P \leq .05), in comparison with the cells treated with TCDD only or in comparison with the cells treated with cells without antagonist pretreatment (# P \leq .05).

Moreover, butyrate-mediated induction of AhR mRNA was not affected by the down-regulation of AhR expression thanks to siRNA (Fig. 2A). Fibroblast growth factor 21 (FGF21), which transcription was reported to be induced by butyrate through inhibition of HDAC3 in the liver [41] has shown a similar pattern observed for AhR and AhR mRNA.

In HepG2-C3 the AhR mRNA induction by 5 mM butyrate was not affected by CH-223191 pretreatment. Here again, the same pattern was observed for gene p21 (WAF1/CIP1), one of the most common genes induced by HDAC inhibitors [42]. Taken together, the results indicate the complex effect of butyrate on the AhR responsive genes, involving AhR activation and suggesting epigenetic regulation of AhR expression based on HDAC inhibition.

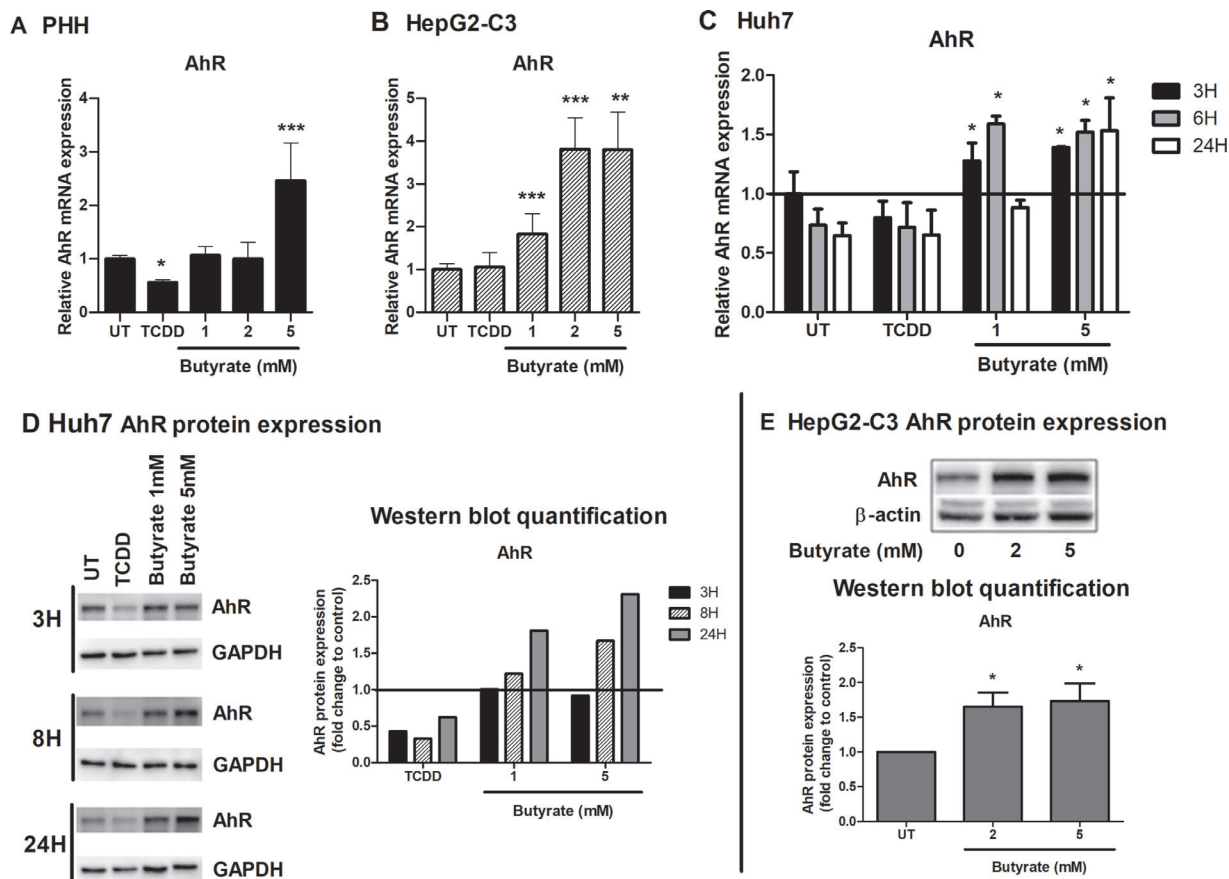


Fig. 5. Enhancement of AhR mRNA and protein expression by butyrate in hepatic cell lines or PHHs. (A) RT-qPCR analysis of AhR mRNA expression in PHHs and (B) HepG2-C3 (N=3) after incubation for 24 h with 1-, 2- and 5-mM butyrate or 10 nM TCDD. (C) Huh7 cells were incubated with 1 mM and 5 mM butyrate or 10 nM TCDD for 3, 6, and 24 h and AhR mRNA expression was determined. Each experiments were performed in duplicates and values significantly increased ($*P<.05$, $**P<.01$, $***P<.001$) in comparison with the control are labeled. (D) Huh7 cells were incubated during 3, 8, and 24 h with 1 and 5 mM butyrate or 10nM TCDD, and AhR cytosolic protein expression was determined. AhR protein expression was normalized to the expression of GAPDH; its quantification is on the right. UT : Untreated. (E) HepG2-C3 cells were incubated with 2- and 5-mM butyrate for 24 h. AhR protein expression was assessed and normalized to the expression of β -actin; its quantification is below the western blot. Results are expressed as mean \pm S.D, N=3. Values significantly increased in comparison with the control are labeled ($*P<.05$).

4. Discussion

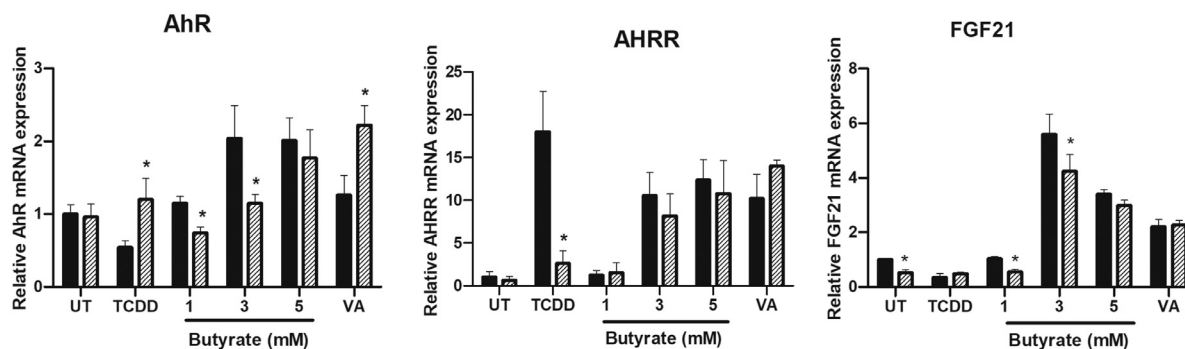
Gastrointestinal tract, with 100 trillion microbes, is a rich resource of microbial metabolites which may be responsible for indirect effect of gut microbiome on the whole-body metabolism [43]. Production of these microbiome-derived components may be affected by wide range of factors, among which, diet is a major agent driving the composition and metabolic activity of the gut microbiota. Recent studies have reported that the gut-derived products may affect intestinal barrier function and activate hepatic receptors altering the hepatic metabolism and stimulate inflammation [11]. As modulation of gut microbiome has become a promising therapeutic strategy more work needs to be done to understand how microbiome-generated molecules may interfere with the hepatic host's metabolism. These studies have also important clinical implications because the gut microbiota may contribute to several liver diseases such as hepatic fibrosis [44] and non-alcoholic fatty liver disease (NAFLD) [12].

Butyrate is a short-chain fatty acid produced in the colon through bacterial fermentation mainly of plant-derived polysaccharides [45]. Butyrate has well established anti-inflammatory role in the intestine [46], while the direct effect of butyrate on the liver is unknown. It is important to note that most of the butyrate is consumed by colonocytes [47]; however, at least part of bacteria-produced butyrate can enter the liver through the portal vein and

directly impact hepatic metabolism. The tendency to increase butyrate production through dietary supplementation to alleviate intestinal inflammation may simultaneously increase the portion of butyrate reaching the liver. Moreover, the fact that the intestinal barrier becomes more permeable during intestinal inflammatory diseases [48] highlights the relevance of the effect of butyrate on the liver.

Recently, studies on germ-free (GF) mice, devoid of intestinal bacteria, have shown that microbiome-derived metabolites may play an important role in regulation of the expression of hepatic biotransformation enzymes - including cytochromes P450 (CYPs) [23,24,49,50]. Selwyn et al. have shown that expression of hepatic CYPs is also affected by application of probiotic to GF mice [51]. It has been also published that the mRNA expression of *Cyp1a2* was significantly increased in the liver of GF mice and, interestingly, the colonization of these GF mice with probiotic bacterial strains restored the mRNA expression back to the conventional level [22]. These results highlight the importance of bacterial metabolites in the regulation of expression of *Cyp1a1/1a2* genes involving the AhR pathway; however, the exact molecular mechanisms remain mostly unknown. In view of the above, it is crucial to understand the molecular mechanism of the butyrate effect on AhR signaling pathway in the liver. So far, few molecules produced by commensal bacteria, such as indoles and other tryptophan derivatives, have been identified as AhR ligands [52,53].

A PHH mRNA expression



B HepG2-C3 mRNA expression

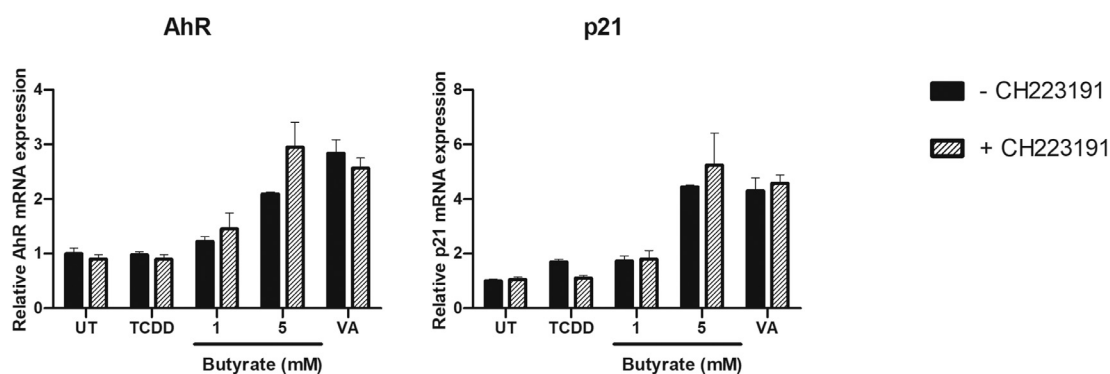


Fig. 6. Effect of butyrate on AhR. (A) RT-qPCR analysis of AhR, AHRR and FGF21 mRNA expression in PHHs (Donor #463, measured in duplicates) after siRNA-mediated AhR down-regulation. (B) HepG2-C3 cells incubated or not with AhR ligand antagonist (CH-223191 3.33 μ M) prior stimulation with 1, 3 and 5 mM butyrate, 5 mM valproic acid (VA) and 10 nM TCDD for 24h. mRNA expression of AhR and p21 was determined. Data are expressed means \pm S.D. (N=4) measured in duplicates and significantly different results compared to cells without antagonist pretreatment are labeled (* $P \leq 0.05$).

Previously, two studies were published showing that butyrate enhanced the gene expression of AhR-responsive genes in human colon cancer cells [54] and in human intestinal epithelial cells [31]. In this work, the effect of butyrate on the hepatic gene expression of AhR and AhR-responsive genes was described. We have found that butyrate increases the mRNA level of *CYP1A1/1A2* and *AHRR*, the expression of which is controlled by AhR, in primary culture of human hepatocytes (PHHs) (Fig. 1A) and in the HepG2-C3 cells (Fig. 1B). The involvement of AhR in the *CYP1A1/1A2* mRNA up-regulation was confirmed by using HepG2-C3 cells and PHHs in which AhR was silenced by siRNA (Fig. 2A and B). The up-regulation of *CYP1A1/1A2* by butyrate was diminished in the both PHHs and HepG2-C3 cells, where pretreatment with specific AhR antagonist CH-223191 was performed (Fig. 2C and D), suggesting that butyrate may work as a low AhR activator. These experiments have demonstrated that butyrate induces the expression of *CYP1A1/1A2* in AhR-dependent manner in liver cells. Contrary to *CYP1A1/1A2*, the induction of mRNA expression of *AHRR* by butyrate was not diminished in silenced PHHs (Fig. 2A) indicating a different mechanism of butyrate action here.

Next, a hypothesis was tested that butyrate regulates the expression of AhR target genes by modulating the AhR transcriptional activity. With this aim, the ability of butyrate to activate AhR in HepG2-Lucia AhR reporter cells was investigated. A significant induction of AhR activity by butyrate in dose dependent manner was

observed, although the induction was lower than in the case of TCDD (Fig. 3A). The fact, that butyrate could not elicit the maximum possible response obtained with the full agonist TCDD in cell reporter assay support the role of butyrate as an AhR partial agonist. This ranks butyrate into the group of weak AhR activators together with other compounds in which a similar effect has been previously confirmed [55,56]. Moreover, the marked differences in the extent of *CYP1A1/1A2* induction observed in PHHs after incubation with TCDD or butyrate supported this finding (Fig. 2). The observed increase in luciferase activity was reduced when cells were pre-incubated with AhR antagonist CH-223191 for 30 min before stimulation with butyrate or TCDD (Fig. 3A). These results indicate that butyrate activates AhR in liver cells.

It has been recently published that the potential role of butyrate as a ligand of AhR in human intestinal epithelial cell line is supported by its ability to stimulate AhR protein translocation into the nucleus [31]. Here, it was found that butyrate induces a slight nuclear translocation of AhR also in hepatic cell line models (Fig. 3B and C), which was reduced in HepG2-C3 cells when pre-incubated with AhR antagonist CH-223191 (Fig. 3C). Altogether, the obtained data provide the evidence that butyrate promotes the AhR translocation to the nucleus and works as a partial agonist in the liver cells.

The mRNA and protein expression of AhR itself was slightly, but significantly, increased in PHHs and HepG2-C3 cells after 24

h of incubation with butyrate in dose dependent manner (Fig. 5A and B). This effect is probably associated with the ability of butyrate to inhibit HDAC [38] and thereby modulate (increase) constitutive activity of AhR gene promoter. This epigenetic effect of butyrate may be at least partly responsible for synergic effect of butyrate/TCDD treatment in *CYP1A1* mRNA expression observed in PHHs and HepG2-C3 cells (Fig. 4A and B) as well as in HepG2-Lucia AhR reporter cells (Fig. 4C) due to enhancement of total amount of AhR receptor. The same synergic effect of butyrate and TCDD was earlier reported in human colon cancer cells [54] and mouse hepatoma cells Hepa1c1c7 transfected with plasmid containing dioxin responsive elements [38]. Similar regulation of gene expression by HDAC has been described for p21 [57] and FGF21 [58]. Therefore, butyrate or valproic acid treatment may induce their transcription as well. Indeed, both genes mentioned have showed dose dependent enhancement of expression (Fig. 6A and B). This impact of butyrate was not reversed by CH-223191, refuting the role of butyrate-mediated AhR activation in this event. Moreover, it has been previously published that sodium butyrate stimulates expression of FGF21 in the liver by inhibition of HDAC 3 [41]. The results of modulation of AHRR expression are comparable to those of p21 and FGF21, this indicates an analogous mechanism of action based on HDAC inhibition.

Another interesting finding, supporting our hypothesis, is impact of butyrate and valproic acid on promoter activity of Renilla control plasmid (S3) suggesting the importance of butyrate related HDAC inhibition in our cell models. This statement is confirmed by the fact that the effect of valproic acid observed in our experiments has already been published by Phiel et al. [40].

CYPs are one of the key drivers of oral drug bioavailability, thus, from clinical point of view, it is significant that PHHs treated with butyrate exhibit higher levels of functional CYP1A1/2 enzymes (Fig. 1C). CYP1A1 and CYP1A2 can metabolize a broad range of foreign compounds and drugs [25], therefore butyrate may modulate the response to many drugs metabolized by these important biotransformation enzymes. Apart from drug metabolism, the CYP1As enzymes are responsible for unwanted effects such as activation of polycyclic aromatic hydrocarbons and other carcinogens and toxicants. Thus, their elevated activity in the liver may be associated with detrimental effects of gut-derived products on the liver function and metabolism.

5. Conclusion

Based on the experiments with cell models described here, one can reasonably expect that butyrate may influence the drug metabolizing ability of liver enzymes e.g., through the interaction with AhR dependent pathways. Presented results highlight the importance of gut microbiome-derived metabolites in processes involved in hepatic drug metabolism with possible clinical relevance. Due to increasing popularity of therapeutic interventions targeting gut microbiome, the better understanding of underlying molecular mechanism and clinically relevant microbiome-drug interaction is needed.

Author's contributions

Lenka Jourova: Conceptualization, Investigation, Writing - original draft, Data curation, Visualization, Eva Anzenbacherova: Supervision, Conceptualization, Funding acquisition, Zdenek Dostal: Investigation, Visualization, Writing - review & editing, Pavel Anzenbacher: Funding acquisition, Writing - review & editing, Philippe Briolotti: Investigation, Emilie Rigal: Investigation, Formal analysis, Martine Daujat-Chavanieu: Supervision, Writing - review & editing,

Visualization, Sabine Gerbal-Chaloin: Methodology, Investigation, Data curation, Visualization.

Author statement

All authors discussed the results and contributed to the final manuscript. The authors declare that they have no conflict of interest.

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Declaration of competing interests

All authors declare no commercial or financial conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2022.109042.

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