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RESEARCH PAPER

Caloric restriction increases levels of taurine in the intestine and stimulates taurine uptake by conjugation to glutathione

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Abstract

Our previous study indicated increased levels of taurine-conjugated bile acids (BA) in the intestine content of mice submitted to caloric restriction (CR). In the current project, we found increased levels of free taurine and taurine conjugates, including glutathione (GSH)-taurine, in CR compared to *ad libitum* fed animals in the mucosa along the intestine but not in the liver. The levels of free GSH were decreased in the intestine of CR compared to *ad libitum* fed mice. However, the levels of oxidized GSH were not affected and were complemented by the lack of changes in the antioxidative parameters. Glutathione-S transferases (GST) enzymatic activity was increased as was the expression of GST genes along the gastrointestinal tract of CR mice. In the CR intestine, addition of GSH to taurine solution enhanced taurine uptake. Accordingly, the expression of taurine transporter (TauT) was increased in the ileum of CR animals and the levels of free and BA-conjugated taurine were lower in the feces of CR compared to *ad libitum* fed mice. Fittingly, BA- and GSH-conjugated taurine levels were increased in the plasma of CR mice, however, free taurine remained unaffected. We conclude that CR-triggered production and release of taurine-conjugated BA in the intestine results in increased levels of free taurine what stimulates GST to conjugate and enhance uptake of taurine from the intestine.

Keywords: Caloric restriction; Bile acids; Taurine; Glutathione; Intestine.

1. Introduction

Caloric restriction (CR) is one of the primary intervention tools applied for weight loss and health maintenance, showing remarkable health benefits. CR lowers the incidence of multiple diseases and diminishes the rate of age-specific mortality, resulting in extended lifespan [1-5]. The metabolic adaptation to CR and the origin of its beneficial effects stem from a synchronized response of a composite network of pathways which results in multiple whole-body outcomes including reduction of inflammation, resting metabolic rate, body temperature, fat metabolism, and enhanced insulin sensitivity [1,6-8]. A reduction of oxidative stress is claimed to be among the factors contributing to the beneficial outcomes of CR [9-11]. Reduced glutathione (GSH) is one of the most important non-enzymatic antioxidants which serves as a scavenger of free radicals, aids in the reduction of H_2O_2, and takes part in detoxification. Together with glutathione peroxidases (GPx) and glutathione S-transferases (GSTs), GSH forms the glutathione system, which is abundant in the gastrointestinal (GI) tract [12,13]. GPx catalyzes the reduction of reactive oxygen species, mainly hydrogen peroxide on the cost of oxidation of GSH to GSH disulfide (GSSG) [14]. Afterward, to complete the cycle and maintain oxidative balance, GSH reductase (GR) converts GSSG back to GSH [15]. GSTs function as a family of enzymes, which catalyze the conjugation of GSH to different electrophilic substrates, thereby producing water-soluble compounds that are further directed to excretion via urine and bile [16]. The expression of GSTs is directly regulated by nuclear factor erythroid-derived 2-like 2 (Nrf2) which is one of the primary transcription factor modulating responses to oxidative stress on the gene expression level. Nrf2 controls also the production of GSH by stimulating the expression of rate-limiting enzymes in GSH synthesis: GSH synthetase (GS) as well as a glutamate-cysteine ligase (GCL) composed of catalytic (GCLC) and a modifier unit (GCLM) [17-20]. Similarly, Nrf2 induces the expression of γ-glutamyl transpep-

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tidase (GGT), an enzyme present in cell membranes of many organs including the intestine, and taking part in GSH metabolism [20,21].

Bile is produced by the liver and mainly consists of water, bilirubin, and bile acids (BAs). Upon secretion to the small intestines, BAs enhance lipid uptake. Once formed by activation of the key enzyme of the classic synthesis pathway Cyp7a1, BAs are conjugated to glycine or taurine. Contrary to humans, in mice, only a minor fraction (ca. 5%) of BAs is conjugated to glycine and the majority (ca. 95%) to taurine [22]. Taurine is one of the most abundant free amino acids in the body [23]. It has anti-inflammatory [24-27], antioxidative [26,28-31], and osmoprotective [30,32] properties. In the intestine of an immunosuppressive mouse model, taurine increases the number of some immune cells and total cells in Payer's patches [33], it reduces the growth of harmful bacteria, increases the production of short-chain fatty acids [34], and attenuates induced colitis [35,36]. In human intestinal epithelial Caco-2 cells, taurine stimulates the expression of anti-inflammatory factors [37]. The impact of taurine is primarily controlled by its concentration, which relies on TauT transporter as well as biosynthetic enzymes cysteine dioxygenase (CDO) and cysteine sulfinate decarboxylase (CSAD) [38]. Therefore, taurine can be also synthesized and the process takes place mainly in the liver [39]; however, the main source of taurine is dietary protein [40].

As reported previously [41], we measured increased levels of the taurine-conjugated BA taurocholic acid (TCA) and tauroursodeoxycholic acid (TUDCA) in the intestinal content of mice submitted to CR compared to *ad libitum* feeding. In the current study, we explored the consequences of a CR-triggered increase in the levels of intestinal taurine-conjugated BA. We hypothesized that CR-associated elevated levels of taurine-conjugated BAs are accompanied by increased levels of free taurine that stems from deconjugation in the intestine.

2. Materials and Methods

2.1. Animal care and experimental procedures

Male C57Bl/6 mice purchased from Janvier Labs (Le Genest-Saint-Isle, France) were kept under a 12-h light/12-h dark cycle in standard specific-pathogen-free (SPF) conditions. The animals were fed a V153x R/M-H auto diet from SSNIFF-Spezialdiäten GmbH (Soest, Germany) and housed with free water access. Mice aged 12 weeks were randomly divided into experimental groups of control *ad libitum* fed (ad lib) or CR mice. The study was confirmed on a second independent group of animals. During the dissection, some samples were lost resulting in seven to nine biological replicates in the first group and eight biological replicates in the second group. The animals were assigned ID not associated with the experimental group; therefore, throughout sample and data analysis group assignment was not known. The groups did not differ significantly in body weight when starting the experimental procedures. Animal food intake was measured for one week prior to the intervention to determine the amount of chow diet to be given under CR. The mice from the CR group underwent 14 days of CR that consisted of a ~ 25% reduction of daily food intake. The CR mice were fed one time per day with the defined amount of chow. This extent of CR is efficient in triggering CR-related phenotypes but prevents excessive body weight loss as presented previously [41]. The samples of the stomach and intestinal mucosa as well as the liver were collected during dissection, snap-frozen, and stored at -80°C until use.

During the desing and performance of the experiment the ARRIVE guidlies have been followed. All animal experimentation protocols were approved by the Bundesministerium für Wissenschaft, Forschung und Wirtschaft, Referat für Tierversuche und Gentechnik (BMBWF-66.006/0008-V/3b/2018). All experiments were carried out according to animal experimentation Animal Welfare Act guidelines.

2.2. Intestinal sacs assay

The freshly dissected small intestine was divided into five even parts. Part four, counting from the duodenum was flushed with PBS, and ends were loosely tied with a thread leaving a four cm-long sac. A blunted needle was introduced and the intestine was filled with 200 µl of taurine (25 mg/ml) or taurine (25 mg/ml) and GSH (61.5 mg/ml) solutions. The sacs were closed tightly and incubated in a 37°C water bath in 10 ml of prewarmed Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA). Three 200 µl medium samples were collected over 1.5 h for measurement of taurine transport.

2.3. Protein concentration and activity assays

The levels of GSH and GSSG, as well as the activity of GST, GR, GPx (all from BioVision, Milpitas, CA, USA), and Nrf2 (Abcam, Cambridge, UK), were assessed using commercial assay kits according to the manufacturer's indications.

2.4. Electron spin resonance

Frozen samples were cut into 15 µg pieces and 144 µl of oxygen-free KHB and 6 µl of oxygen-free 10 mM CMH solution were added. The samples were incubated for 60 min in a 37°C shaking incubator and quickly spun down. 100 µl of the solution from each sample was transferred to a fresh tube and snap-frozen in liquid nitrogen until measuring. Electron spin resonance (ESR) measurements were performed at 150 K in a capillary tube (100 µl), which was placed into a high sensitivity resonator (Bruker ER 4122SHQE), using an X-band Bruker Elexsys-II E500 EPR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) with a modulation frequency of 100 kHz and a microwave frequency of 9.4 GHz. Spectra were recorded every 20 s, averaging every 10 consecutive spectra. The sweep width was 450 G, the sweep time 20 s, the modulation amplitude 5 G, the center field 3400 G, the microwave power 20 mW, and the resolution was 1024 points. EPR spectra were simulated and the area under the curve was determined by double integration of the spectrum. A reference-free quantitation of the number of spins was performed, as has been described previously [42].

2.5. Gene expression

RNA was isolated from intestinal scrapings using the RNeasy mini kit (Qiagen, Hilden, Germany). Samples were thawed in lysis buffer, disrupted using a syringe and needle, and processed following the manufacturer's recommendations. SuperScript II Reverse Transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA) was used for the reverse transcription step. Quantitative real-time PCR (qRT-PCR) reactions were carried out using the QuantStudio 6 Flex Real-Time PCR System with the SYBR Green PCR Master Mix (both from Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The primers used are listed in the supplementary data file. The Ct values for each tested gene were normalized to Ct values of Eef1a1. The presented results show $\Delta\Delta$Ct averaged for biological replicates for each experimental group.

2.6. GSH and taurine conjugates detection

The protocol was adapted from the method of Ito et al. [43] and Budinska et al. [44]. Frozen liver and intestinal mucosa samples were cut on dry ice to the size of 7–10 mg and homogenized. Liver samples were transferred into Precellys homogenizing tubes with 1.4 mm ceramic beads and according to the sample weight nine times the volume of ethanol absolute at -20°C was added. Liver samples were homogenized in the Precellys 24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) twice for 15 s at 5000 rpm, vortexed for 30 s, and incubated at -20°C. Intestinal samples were transferred into 1.5 ml Eppendorf tubes and were disrupted in five thawing and freezing cycles. Next, nine times the volume of ethanol absolute -20°C was added, samples were vortexed for 30 s. After the homogenization, liver and intestinal samples were handled alike. Samples were incubated at -20°C for 24 h and centrifuged for 10 min at 18000 g, the supernatants were transferred to new tubes, and, to remove the remaining debris, the centrifugation step was repeated. The supernatants were transferred into HPLC vials in a thermostatic autosampler kept at 4°C. 60 µl of intestinal sacs samples were diluted with 600 µl ethanol, vortexed, incubated for 20 min at -20°C, and centrifuged at 15000 g for 15 min at 4°C. The supernatant was dried in a SpeedVac concentrator for 45 min at 60°C, then dissolved in 70 µl ethanol. Samples (10 µl) were analyzed by LCMS in negative modus using an LCMS-8040 Liquid Chromatograph Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan) with an Atlantis T3 3 µm column (2.1 × 15 0mm, Waters, Milford, MA, USA). The column temperature was 40°C. The mobile phases consisted of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The gradient was maintained at an initial 5% B for 2.5 min, to 20% B at 8 min, and was set back to 5% B at 9 min with a hold for one minute. The area under the curve (AUC) corresponding to each molecule was quantified and used to compare samples.

2.7. Identification of conjugates

Standards of GSH and taurine (both from Sigma-Aldrich, St. Louis, MO, USA) were prepared in 70% ethanol. To induce the conjugation of taurine to GSH 150 mmol of each was weighted in the same 2 ml Eppendorf tube, 70% ethanol was added, vortexed shortly, and was incubated for 30 min at room temperature. Standards were separated with the column and HPLC gradient described above and

were fragmented with an LC-MS system (LCMS-80-40, Shimadzu, Korneuburg, Austria). The MS instrument was operated in multiple reaction mode (MRM) with the following settings: nebulizing gas flow 3L/min, drying gas flow 12L/min, desolvation line temperature 250°C, and heat block temperature 350°C. Argon was used as the collision-induced dissociation (CID) gas with a collision energy of 20eV. The fragmentation pattern was compared to METLIŃs database. To identify GSH and taurine conjugates, samples were screened for precursor ions producing similar fragmentation patterns to GSH and taurine. For validation that the identified compound contains taurine or GSH, samples were analyzed with the multiple reaction monitoring (MRM) setting. For example, the fragmentation pattern of metabolite m/z 249 shows taurine and the specific taurine fragment, according to the METLIN database. The exact mass of the selected precursor ions was measured using an LC-ESI-TOF-system consisting of an Ultimate 3000 (Thermo Fischer Scientific, Waltham, MA, US) and a micrOTOF-Q II (Bruker Daltonics, Bremen, Germany) with an Atlantis T3 3 μm column (2.1 × 150 mm, Waters, Milford, MA, USA). The column temperature was 40°C. The mobile phases consisted of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The gradient was maintained at an initial 5% B for 2.5 min, to 20% B at 8 min, and was set back to 5% B at 9 min with a hold for one minute. The exact mass and fragmentation pattern of the selected precursor ions were checked against the METLIN database. The compounds identified as GSH or taurine conjugates are listed in supplementary table 2.

2.8. Bile acid analysis

Sample extraction and measurement of bile acids were carried out using a modified method by Rohn et al. [45]. Shortly, feces samples were weighted in Precellys homogenizing tubes with 1.4 mm ceramic beads, and nine times the volume of methanol absolute at -20°C was added. Samples were homogenized in the Precellys 24 Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) twice for 15 s at 5000 rpm, vortexed for 30 s, and centrifuged for 10 min at 5000 g at 4°C. The supernatants were transferred to new 1.5 ml Eppendorf tubes, and, to remove the remaining debris, the centrifugation step was repeated this time at 12000 g. Supernatants were transferred into new Eppendorf tubes and after third centrifugation, supernatants were directly transferred into an HPLC vial. Samples (10 μl) were analyzed by LCMS in positive modus using an LCMS-8040 Liquid Chromatograph Mass Spectrometer (Shimadzu Corporation, Kyoto, Japan) with an Atlantis T3 3 μm column (2.1 × 150 mm, Waters, Milford, MA, USA). The column temperature was 30°C. The mobile phase A consisted of water and eluent B was acetonitrile/methanol (3/1, v/v), both containing 0.1% formic acid and a concentration of 20mM ammonium acetate. The gradient was maintained from an initial 30% B for 5 min, to 100% B at 25 min, which was kept constant for 20 min. Afterward, the composition was set back to the initial ratio of 30% B within 2 min, followed by 10 min of re-equilibration.

2.9. Statistics

The differences in the intestine mucosa GST expression between the experimental groups were used to calculate the sample size. Within the study, data sets with two groups (CR and *ad libitum*) of seven to nine biological replicates were compared using a two-sided student's t-test to verify statistical significance. Additionally, for the *ex vivo* assay, statistical significance was assessed using one-way ANOVA with Bonferroni post-hoc corrections for four groups of five to seven replicates using SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). A p-value lower than 0.05 was considered statistically significant. The presented data stems from two technical replicates.

3. Results

3.1. CR increases the levels of free taurine and its conjugates along the intestinal mucosa but not in the liver

As we previously published, CR is associated with increased levels of taurine-conjugated BAs in the intestine [41]. To further explore this phenomenon, we studied the impact of CR on intestinal taurine levels. In order to mirror previous experimental conditions, male C57Bl/6 mice were submitted to 14 days caloric restriction receiving 75% of the amount of standard chow that the animals would voluntarily consume. The levels of taurine were measured in the mucosa along the small intestine. We detected increased levels of free taurine in the duodenum of CR compared to *ad libitum* fed mice (Fig. 1A). To verify how taurine could be utilized in the intestine, compounds containing taurine were analyzed. Multiple conjugates of taurine were detected and, accordingly, their levels were increased in the mucosa of CR animals (conjugate with molecular mass 249 is presented) (Fig. 1B). Among the compounds detected, we identified a GSH-taurine conjugate. The conjugate showed an increased concentration in intestinal mucosa samples of CR compared to *ad libitum* fed mice. However, in the duodenum, this difference was not statistically significant (Fig. 1C). The levels of free taurine, taurine conjugates, and GSH-taurine were statistically significantly elevated in the jejunum (Fig. 1D-F), and the ileum of CR animals (Fig. 1G-I). The differences in the amounts of the metabolites were more pronounced in the distal compared to the proximal intestine and the concentration of taurine was the highest in the ileum. The level of one of the taurine metabolites, taurine chloramine (TauCl), a compound with strong anti-inflammatory properties, was also increased in the ileum (Fig. 1J). While glycine levels were not affected by the diet (Fig. 1K). We further verified if CR also affects the liver in a similar way, but there was no difference in free taurine (Fig. 2A), conjugated taurine (Fig. 2B), or GSH-taurine conjugates (Fig. 2C) levels between CR and *ad libitum* mice. Importantly, the expression of genes associated with BAs synthesis (Cyp7a1), transport (Ntcp), and cysteine metabolism (Cdo) was upregulated in CR liver while that of TauT was not affected by the restriction (Fig. 2D). Contrary to the liver, the mRNA expression of Cdo was not affected by CR in the mucosa of the ileum (Suppl Fig. 1A).

3.2. CR impacts GSH and GST but does not influence REDOX response in the jejunum mucosa

Since one of the identified taurine conjugates contained GSH, we verified the levels of reduced (GSH) and oxidized (GSSG) glutathione in the jejunum mucosa. The levels of GSH were significantly decreased in the CR compared to ad libitum fed mice (Fig. 3A), while GSSG concentration was not affected by CR (Fig. 3B). To validate whether the rate of de novo synthesis or utilization of GSH is modified during CR, expression of genes connected with GSH synthesis (Nrf2, Ggt, Gclc, and Gs) and antioxidant activity (Grx1, Grx2, GPx1, and GPx2), as well as activity of several of the factors, were measured in the mucosa of the jejunum. The mRNA level of Nrf2 was downregulated in the CR mice (Fig. 3C), although its transcriptional activity was not changed (Fig. 1D). Also, the expression of Ggt was downregulated (Fig. 3C). The expression of other genes connected with GSH synthesis and its anti-oxidative role was not affected (Fig. 3C). The activity of GR which is responsible for the conversion of GSSG to GSH was increased in the CR animals (Fig. 3E), whereas the activity of GPx was not significantly affected and showed high variability (Suppl Fig. 1B). To verify the functional outcome of the anti-oxidative role of GSH in the CR intestine, the total antioxidant capacity was assessed (Fig. 3F) and the levels of reactive oxygen species were measured by means of ESR (Fig. 3G) in the intestinal mucosa. Both of the parameters were not affected. Additionally, the expression on manganese superoxide dismutase (MnSOD), catalase (Cat), thioredoxin 1, and 2 (Trx1 and Trx2) were decreased in the intestinal mucosa of CR mice (Suppl fig. 1C). Therefore, we decided to focus on the conjugating function of GSH and found increased activity of GST in the intestine of CR compared to ad libitum fed mice (Fig. 3H). We also measured gene expression in six distinct parts of the GI tract (stomach, duodenum, jejunum, ileum, proximal colon, and distal colon) (Fig. 3I). Fittingly with the enzymatic activity results, the expression of Gst genes was consistently increased in mucosa of the different parts of the GI tract. However, the range of upregulation varied between different forms of Gst. Additionally, Gsta4 showed the opposite trend of decreased expression in CR compared to ad libitum mice in jejunum; however, not statistically significant.

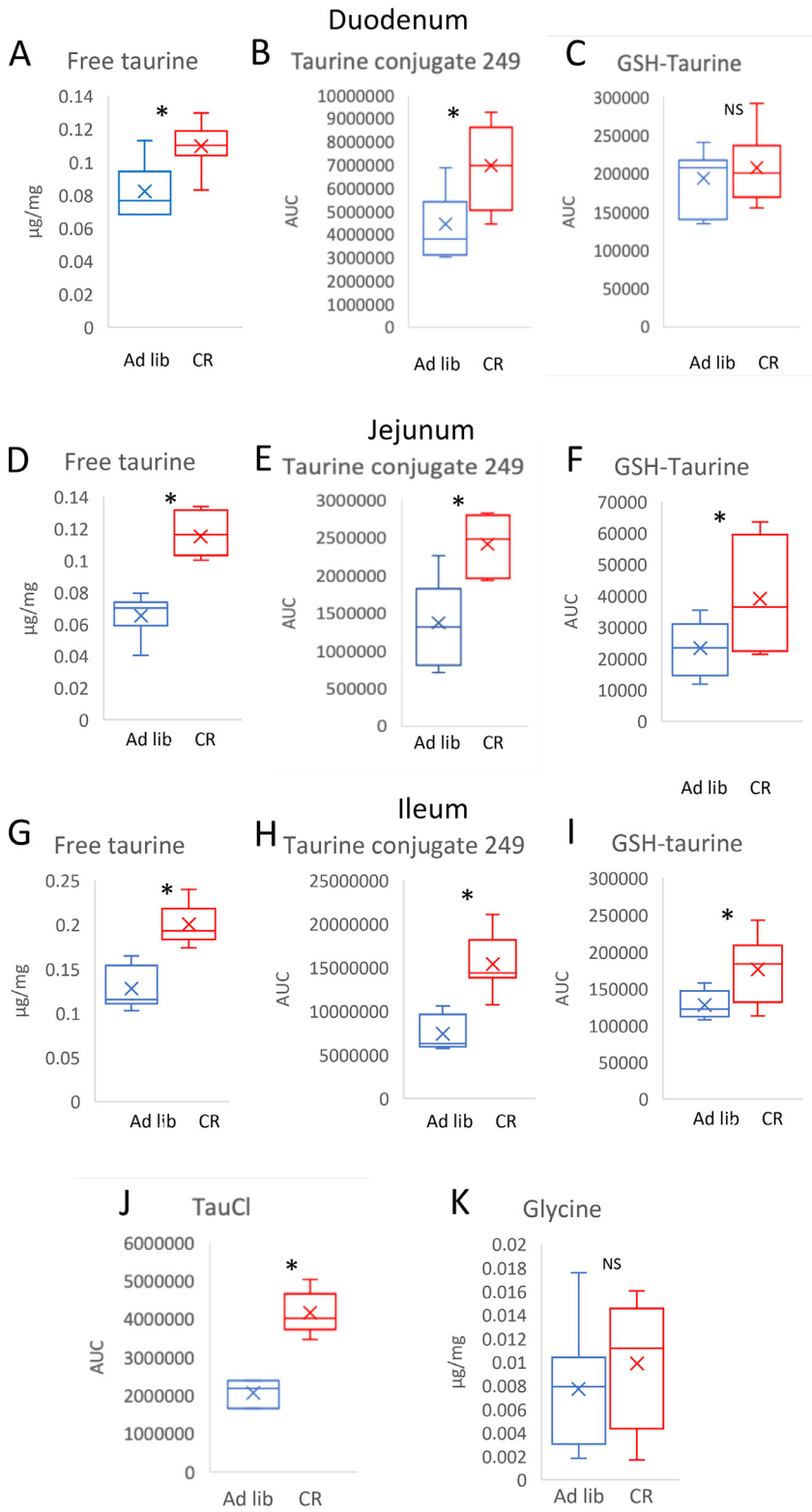

Fig. 1. Caloric restriction (CR) increases the levels of free taurine and its conjugates along the intestinal mucosa. The levels of free taurine (A, D, G), taurine conjugate with m/z 249 (B, E, H), and GSH-taurine conjugate (C, F, I) were measured in the mucosa of the duodenum (A-C), jejunum (D-F), and ileum (G-I). Symilarly, the levels of taurine chloramine (TauCl; J) and glycine (K) were assessed in the mucosa of ileum. Statistical significance was assessed using a two-tailed Student's t-test; $^{*}P<.05$; $n=6$-8. Error bars stand for ±SEM.

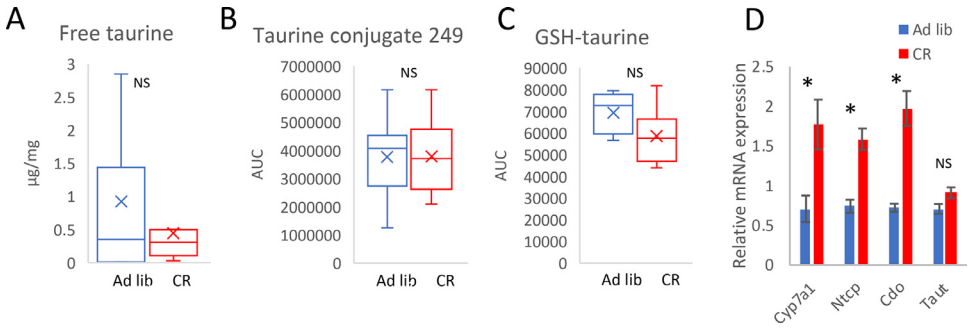

Fig. 2. CR does not affect the levels of taurine and its conjugates in the liver but regulates BA production and cysteine-related gene expression. The levels of free taurine (A), taurine conjugate with m/z 249 (B), and GSH-taurine conjugate (C) were measured in the liver. Gene expression was assessed in the liver of CR and *ad libitum* fed mice (D). Cyp7a1: cholesterol 7 α-hydroxylase; Ntcp: Na^+/taurocholate cotransporting polypeptide; Cdo: cysteine dioxygenase; TauT: taurine transporter. Two-tailed Student's t-test was applied to assess statistical differences between the groups; $^*P<.05$. Bars indicate the mean of eight to nine biological replicates ±SEM.

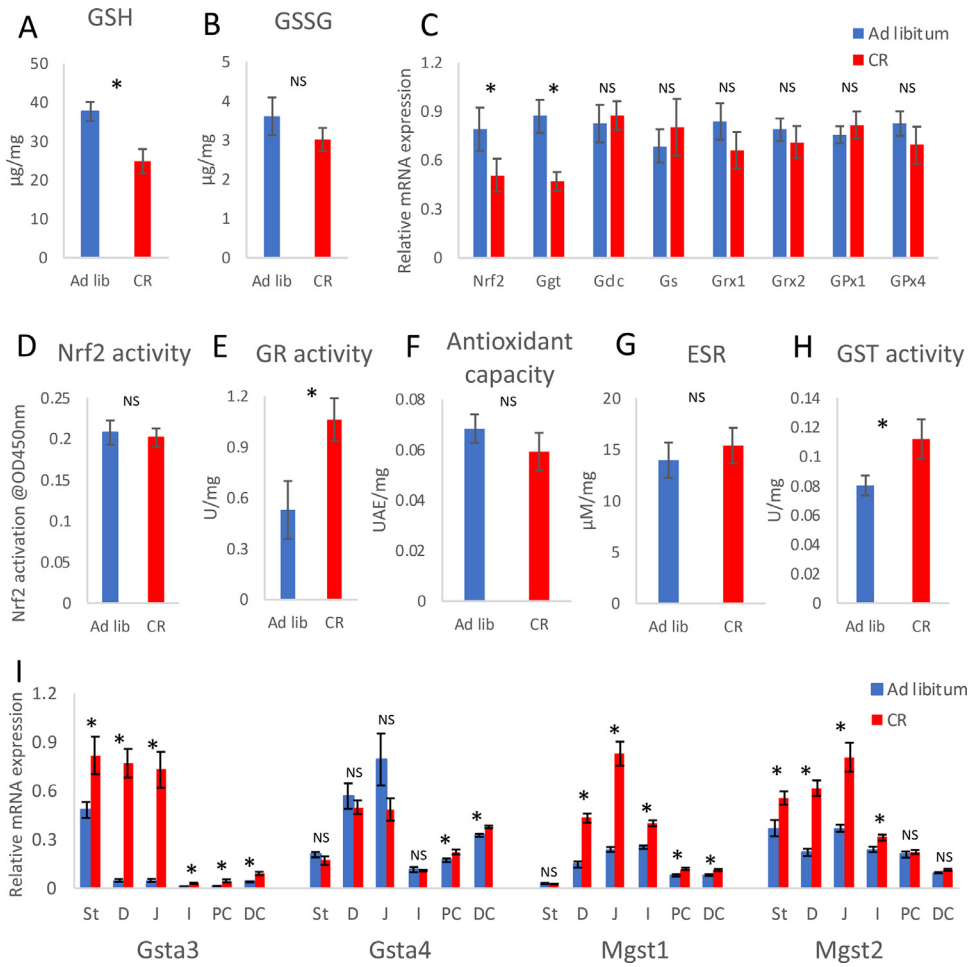

Fig. 3. CR impacts glutathione (GSH) and glutathione-S transferase (GST) in the jejunum mucosa. The levels of reduced GSH (A) and oxidized GSH (GSSG) (B) were measured in the jejunum mucosa using commercial assays. Gene expression was measured in the mucosa of the jejunum of CR and *ad libitum* mice (C). The activity of nuclear factor erythroid 2-related factor 2 (Nrf2) (D) and glutathione reductase (GR) (E) as well as antioxidant capacity (F), were measured in the mucosa of the jejunum utilizing commercial assays. Electron spin resonance (ESR) was applied to assess the levels of reactive oxygen species in the mucosa of the jejunum (G). GST activity was measured in the jejunum mucosa using commercial assays (H). Gene expression of the different GST subtypes was measured in the mucosa of six parts of the GI tract (St-stomach, D-duodenum, J-jejunum, I-ileum, PC-proximal colon, DC-distal colon) of CR and *ad libitum* fed mice (I). Nrf2: nuclear factor erythroid 2-related factor 2; Ggt: gamma-glutamyl transpeptidase; Gcl: glutamate-cysteine ligase; Gs: glutathione synthase; Grx1: glutathione reductase 1; Grx2: glutathione reductase 2; GPx1: glutathione peroxidase 1; GPx2: glutathione peroxidase; Gsta3: GSH S-transferase α 3; Gsta4: GSH S-transferase α 4; Mgst1: microsomal GSH S-transferase 1; Mgst2: microsomal GSH S-transferase 2.Two-tailed Student's t-test was applied to assess statistical differences between the groups; $^*P<.05$. Error bars indicate ±SEM. Figures in panels A-I represent the mean of eight to nine biological replicates.

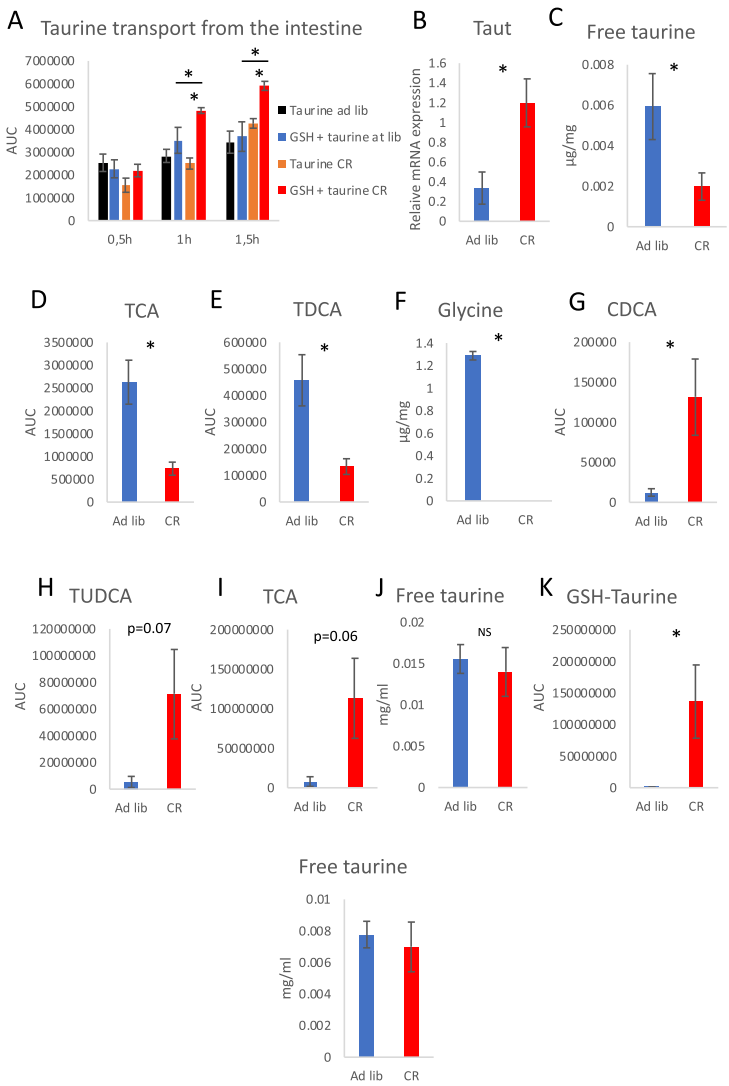

Fig 4. Both GSH and CR stimulate uptake of taurine in the intestine. Taurine uptake was assessed using *ex vivo* intestine sacs infused with a solution containing taurine or taurine and GSH. The concentration of transported taurine was measured in the surrounding medium at the indicated time points (A). The expression of *TauT* mRNA was measured in the mucosa of the ileum (B). Feces were analyzed for the levels of free taurine (C) and bile acids conjugated with taurine: TCA (D) and TDCA (E) and glycine (F). The levels of chenodeoxycholic acid (CDCA; G), TUDCA (H), TCA (I), free taurine (J), and GSH-conjugated taurine (K) were measured in mice plasma. The groups presented in panel A were compared using ANOVA. Student's t-test was applied to assess statistical differences between the groups in panels B-E; $^{*}P<.05$. Error bars indicate ±SEM. The figure in panel A represents the mean of five to seven replicates while in panels B-E eight biological replicates.

3.3. GSH and CR stimulate uptake of taurine in the intestine

The strongest difference between CR and ad libitum mice in the expression of GSTs was observed in the duodenum and jejunum (Fig. 3I), however, the difference in taurine and its conjugates increased along the small intestine and peaked in the ileum (Fig. 1). Therefore, for the next experiments, ileum was applied. To verify the functionality of the occurrence of GSH-taurine conjugates, we incubated ex vivo intestinal sacs of CR and ad libitum fed mice which were infused with taurine or a mix of taurine and GSH solutions to measure uptake of taurine. The levels of taurine in the surrounding medium were measured at three time points over 1.5 h of incubation. The uptake of taurine did not change between CR and ad libitum fed mice when sacs were infused with a taurine solution (Fig. 4A). Similarly, a solution containing taurine and GSH did not impact taurine uptake in ad libitum fed mice. However, incubation of the intestine of CR mice with taurine and GSH solution strongly increased the concentration of taurine in the surrounding medium. Therefore, CR and GSH jointly increased the uptake of taurine. Correspondingly, the mRNA expression of TauT, the main intestinal transporter responsible for capturing taurine between the meals [46], was higher in ileum mucosa of CR compared to ad libitum fed mice (Fig. 4B). In accordance, the levels of free taurine and taurine-conjugated BA (TCA and TDCA) were lower in the feces of CR compared to ad libitum fed mice (Fig. 4C-E). CR completely removed glycine from feces (Fig. 4F). Furthermore, the levels of chenodeoxycholic acid (CDCA; Fig. 4G) and taurine conjugated BAs TUDCA and TCA (Fig. 4H-I) were higher in the plasma of CR compared to ad libitum mice corresponding well the suggested increased BA synthesis in the liver and enhanced intestinal uptake. Despite TUDCA and TCA showing a very strong trend towards increased levels in CR mice the results were not statistically significant ($P=.07$ and $P=.06$ respectively). Importantly, CR did not affect the plasma levels of free taurine (Fig. 4J) but strongly increased the levels of GSH-taurine (Fig. 4K) confirming that the conjugation by GSH is necessary for the uptake of taurine.

4. Discussion

To contribute to building the full picture of the molecular mechanism of intestinal response to CR we focused on one of the previously reported by us phenotypes [41] connected with increased levels of taurine-conjugated BAs in the intestinal contents. In the current study, we prove that CR is associated with increased levels of free and conjugated taurine in the intestinal mucosa. The increased level of intestinal and plasma BAs matches well with elevated expression of BA synthesis- and transport-related genes in the liver of CR mice presented in this study. Correspondingly, based on gene expression data, the synthesis of taurine is likely to increase in the cysteine dioxygenase-associated cysteine metabolic pathways in the liver but not in the intestinal epithelium. However, the expression of the TauT in the liver was not affected, suggesting that taurine is not exported from the liver as free taurine but as bile conjugate. Further, we report that during CR, the levels of taurine increase along the small intestine but not in the liver. Our results show that the amount of BA-derived taurine increases in the CR intestine and various types of conjugates are created to bind taurine. Conjugation of taurine to GSH enhances taurine reuptake from the intestine and CR affects plasma levels of GSH-conjugated but not free taurine. Importantly, the impact of GSH on taurine uptake has never been described before and the molecular background of this interaction is not known. Correspondingly, we showed that the level of taurine bound to BAs as well as free taurine is lower in the feces and higher in plasma of CR compared to *ad libitum* mice indicating increased efficiency of uptake in the small intestine.

The BAs are secreted to the proximal intestine upon lipid consumption and travel along the GI tract to solubilize lipids aiding digestion and absorption. However, the secretion of motilin, a hormone stimulating BAs secretion, increases also during fasting [47], which likely triggers the observed phenotype. Once reaching the distal intestine, 95% of BAs are reabsorbed and recirculated to the liver [48]. The amount of free taurine, GSH-taurine, and other taurine conjugates are increased in all parts of the intestinal mucosa in CR compared to *ad libitum* mice. However, in this study, the differences between CR and *ad libitum* fed mice are stronger in the distal intestine compared to the duodenum. Most likely, this is due to increased BA reabsorption and high expression of TauT in the ileum [46].

While, as we showed previously, CR results in the reduction of levels of multiple metabolites in the GI tract [41], taurine is an intriguing example of a molecule with an increased concentration, suggesting a physiological role. The role of enterohepatic circulation or accumulation of taurine during CR has never been investigated. However, we hypothesize that taurine could serve as a regulator of metabolic adjustment as it had been shown to reduce the hepatic secretion of lipids while enhancing fatty acid oxidation and ketogenesis which are processes characteristic to fasting and CR [49]. Additionally, as published before [41], CR results in the downregulation of the immune and anti-microbial genes in the intestine. Fittingly, taurine [24-27, 34], as well as BAs [50-52] are known for their anti-inflammatory role and impact on microbiota. Therefore, the increased levels of BAs and taurine in the intestine during CR could contribute to the previously described phenotype. Importantly, taurine supplementation, similarly to CR has been shown to reduce endoplasmic reticulum stress and extend lifespan in *C. elegans* [53]. Consequently, taurine has already been suggested as a supplement mimicking the beneficial outcomes of CR [54]. An important aspect of mimicking CR is the enhancement of mitochondrial function. Taurine reduces the generation of superoxide in mitochondria and is required for normal mitochondrial structure and metabolism. This is likely a consequence of creating 5-taurinomethyluridine-$tRNA^{Leu}$ conjugate which is vital for mitochondrial protein expression [55-58]. Therefore, compartmentalization of taurine and organelle-specific biological activity may be a key to unveiling its contribution to the beneficial outcomes of CR.

Seeing the changes of GSH levels in the context of CR we, first, hypothesized changes in the REDOX response, which are often associated with CR. However, contradictory data concerning oxidative stress in CR have been reported [59-63]. Our results show that intestinal mucosa does not respond to CR by modulating the levels of reactive oxygen species or anti-oxidative capacity. Surprisingly, the shifted ratio of GSH/GSSG was not reflected in the total oxidative stress. Assuming diminished oxidative stress during CR, as reflected by MnSOD, Cat, and Trx expression, the requirement for GSH is reduced, and therefore, the level of GSH would not influence the oxidative stress balance. We conclude that it is not the oxidative but the conjugative activity of GSH that is involved in the intestinal response to CR and that GST is activated during CR to conjugate taurine which is likely released from BAs. We show that GST gene expression increases and accordingly, the activity of GST and the occurrence of GSH conjugate are elevated in the mucosa of CR mice. Due to the higher demand from the GST side, the level of free GSH decreases. Consequently, the GSSG/GSH ratio is elevated, and this activates GR which converts GSSG to GSH and reestablishes the basic state ratio. It is, however, possible that the decreased level of GSH could also partly result from the CR-accompanied shortage of nutrients, substrates to synthesize GSH, and food-derived GSH. The reduced expression of *Ggt*, coding for GSH hydroxylase which digests external GSH in order to deliver amino acids for absorption and intracellular GSH synthesis, may reflect the reduced availability of the extracellular GSH.

In summary, we describe a novel role of GSH in the handling of CR-associated abundance of taurine in the small intestine. Interestingly, the role of BAs secreted not in the context of food consumption is not known. Importantly, the here described CR-triggered upregulation of GST expression and activity, a link between intestinal GSH and BA-derived taurine as well as the impact of GSH on taurine absorption has never been established before. With this and future studies, we build upon our previous publications, focusing on a specific novel piece of regulation and aiming at the development of a more detailed molecular picture of the response of the intestine to CR. Currently, the mechanism behind the remarkable health benefits of CR is largely undetermined, particularly in the GI tract. Based on our previous work we speculate that BA and taurine could contribute to the CR-triggered reduction of intestinal inflammation or increased efficiency of nutrient uptake. However, the exact physiological purpose and consequences of this regulation and its relevance to human physiology need to be further investigated.

Author Contributions

AG: data curation, investigation, writing-review and editing; MP: methodology, writing-review and editing; CF: methodology; SB: methodology; VS: conceptualization, writing-review and editing; JK conceptualization, writing-review and editing; KD conceptualization, data curation, investigation, supervision, visualization, writing-original draft. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors have no competing interest to declare.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Supplementary materials

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