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Influence of luminal monosaccharides on secretion of glutathione conjugates from rat small intestine in vitro

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ABSTRACT

Intestinal efflux transporters can significantly reduce the absorption of the drug after peroral application. In this work we studied secretion of glutathione conjugates triggered by glucose at the luminal side of the intestine. Glucose stimulated secretion of DNPSG, NEMSG and CDNB. We used some different monosaccharides and determined that glucose, galactose and α-methylglucopyranoside trigger the secretion, while mannitol and fructose do not. We concluded that interaction with SGLT transporter is the key process necessary for this triggering. To determine which of possible glutathione conjugate transporters (MRP2, MRP4, BCRP or RLIP76) is responsible for the secretion of glutathione conjugates, we used benzbromarone, a MRP inhibitor, and sulfanitran and furosemide, two allosteric MRP2 activators. Benzbromarone inhibited glucose stimulated DNPSG secretion, while allosteric activators additionally increased the secretion. We concluded that MRP2 transporter is related to glucose stimulated DNPSG secretion. Regarding the work of Kubitz et al. we tested the effect of changed medium osmolarity on DNPSG transport and determined that hypoosmolar conditions trigger secretion of DNPSG. These findings suggest that intestinal MRP2 activity has no basal level, but can be stimulated by hypoosmolarity and SGLT transport.

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1. Introduction

MRP transporters are well known subfamily of efflux ABC transporters. The activity of these transporters can significantly influence pharmacokinetic parameters of substances, which are their substrates. MRP transporters are widely expressed in various tissues, but the extent of expression and cellular orientation are transporter specific. They are present in eliminatory organs such as the liver, the kidney and the intestine. Apical MRP transporters facilitate drug secretion into the bile, the renal tubules and into the intestinal lumen, while basolateral MRP transporters facilitate drug secretion into the blood stream. Substrates of MRP transporters include xenobiotics, i.e. anticancer and anti-HIV drugs, and endogenous substances, i.e. biliary acids and glutathione (GSH) conjugates. The latter are frequently employed as markers of MRP activity. Most commonly used are *S*-(2,4-dinitrophenyl)glutathione (DNPSG), *S*-(*N*-ethylsuccinimi-2-yl)glutathione (NEMSG) and leucotriene LTC_4. They are produced by conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), *N*-ethylmaleimide (NEM) and endogenous arachidonic acid, respectively (Chan et al., 2004). GSH conjugates are also substrates for another efflux ABC transporter, BCRP, and a non-ABC transporter, RLIP76. Both transporters share tissue distribution and substrate specificity of MRP transporter family (Awasthi et al., 2002; Chan et al., 2004).

Several researchers have studied regulatory strategies of MRP transporters. Their activity is under constant translational, transcriptional and posttranscriptional control. Posttranscriptional level includes allosteric regulation and vesicular trafficking (Gerk and Vore, 2002). Zelcer and coworkers studied the mechanism of allosteric regulation of MRP2 transporter and suggested that the substrate and the allosteric binding site are very similar. Therefore, a drug can bind to one or to both sites, complex drug–drug interactions emerge. This kind of regulation is also pertaining to other MRP transporters, but affinities for substrates and allosteric activators are different (Zelcer et al., 2003). Cellular trafficking of MRP transporters containing vesicles is well documented in the liver and in the kidneys. It affects the amount of transporters in the cellular membrane by regulating rates of exocytotic incorporation of vesicles containing transporters into the apical or basolateral membrane and of their endocytotic retrieval into the intracellular pool (Gerk and Vore, 2002). The described regulatory strategies differ in stimuli and in response time (from days at transcriptional regulation to minutes and seconds in vesicular and allosteric regulation, respectively). Some stimuli can regulate MRP activity on multiple levels. For example expression of MRP2 transporters in hepatic cell culture can be increased by dexamethasone and by

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hypoosmolar medium on transcriptional level (Kubitz et al., 1999), but only the hypoosmolar medium triggers also the response on the vesicular level (Dombrowski et al., 2000). Different transporters and organs can also respond differently to the same stimuli. For example, cholestasis decreases hepatic MRP2 activity, while it increases the hepatic MRP3 and renal MRP2 activity (Tanaka et al., 2002).

Although MRP transporters are also expressed in the small intestine, no research has been done on cellular trafficking of MRP transporters in the intestinal mucosa. The aim of the present study was thus to investigate the regulation of MRP activity in the rat small intestine *in vitro* using DNPSG, NEMSG and CDNB as marker substrates.

2. Materials and methods

2.1. Materials

DNPSG was synthesized according to Hinchman (Hinchman et al., 1991). Product was dried for 24 h and its identification was performed on a Varian LC/MS-MS 1200L system. All other chemicals were purchased from Sigma and were of highest purity grade available.

2.2. Experimental methods

Rat intestine from male Wistar rats (250–320 g) was obtained, prepared and mounted in easy mount side-by-side diffusion chambers (Physiologic Instruments, San Diego, CA, USA) as described previously (Žakelj et al., 2004). The experiments conformed to the law for the protection of animals (Republic of Slovenia, EU) and are registered at the Veterinary Administration of the Republic of Slovenia.

Normoosmolar (300 mOsm) and hypoosmolar (200 mOsm) Ringer buffer differed in concentration of NaCl used. For hyperosmolar (400 mOsm) Ringer buffer, osmolarity was increased using mannitol or glucose at mucosal and mannitol at serosal side of the tissue. Mucosal acceptor solution additionally contained 10 mM of appropriate monosaccharides in Ringer buffer depending on experiment and 1 mM acivicin, irreversible gama-glutamyltransferase inhibitor, to prevent the metabolism of GSH conjugates (Hinchman et al., 1998). Serosal solutions contained 10 mM glucose in Ringer buffer. Unless otherwise stated, the concentrations of DNPSG and NEMSG in the donor solutions were 1 mM, while CDNB was used in 0.1 mM concentration because of its toxicity. The solutions of NEMSG were prepared by the in-situ reaction (Fig. 1) simply by dissolving appropriate amounts of GSH and NEM in the Ringer buffer. In experiments with allosteric MRP2 activators, sulfanitran (0.25 mM) and furosemide (0.5 mM) in Ringer buffer were present in mucosal solutions during the entire experiment.

Experiments were performed in serosal to mucosal (S–M) and in mucosal to serosal (M–S) direction. Tissue viability and integrity were controlled throughout the experiments by monitoring the trans-tissue potential difference, the short circuit current and the trans-tissue electrical resistance with a multichannel voltage–current clamp (model VCC MC8, Physiologic Instruments) as described previously (Žakelj et al., 2004). The trans-tissue potential difference is a highly reliable parameter for the determination of the tissue viability (Polentarutti et al., 1999). At the end of the experiments, the trans-tissue potential difference after the addition of glucose to the mucosal compartment (final concentration was 25 mM) was also measured. Segments with inappropriate viability were excluded from the statistical analysis.

2.3. Analytical procedures

All analytes (CDNB, DNPSG and NEMSG) from the transport experiments were analyzed by HPLC system (Series 1100, Hewlett Packard, Waldbron, Germany). For chromatographic separation of samples with CDNB and DNPSG Phenomenex Onyx Monolithic C18 (100 mm × 3 mm) column was used, while separation of samples with NEMSG was performed on Phenomenex Gemini C18 (50 mm × 4.6 mm) column. For determination of DNPSG and CDNB temperature of 35 °C and flow rate of 4 mL/min were applied. Gradient elution with acetonitrile and 10 mM phosphate buffer (pH 3.5) was used. The analysis started with 5% acetonitrile, which was raised linearly to 45% in 3 min. Wavelengths of 250 nm and 335 nm were used for the detection of CDNB (retention time 1.46 min) and DNPSG (retention time 2.63 min), respectively. NEMSG was analyzed at 40 °C, flow rate 3 mL/min. Elution was isocratic and mobile phase consisted of 5% acetonitrile and 95% 10 mM phosphate buffer (pH 2.0). Detection wavelength was 200 nm. The analyte produced two peaks at 0.83 and 0.95 min with a resolution of 1.8. The dual peak phenomenon was already described (Kuninori and Nishiyama, 1991) and is caused by the resolution of two NEMSG diastereomeres. Volume of injection was 100 μL for all analyses.

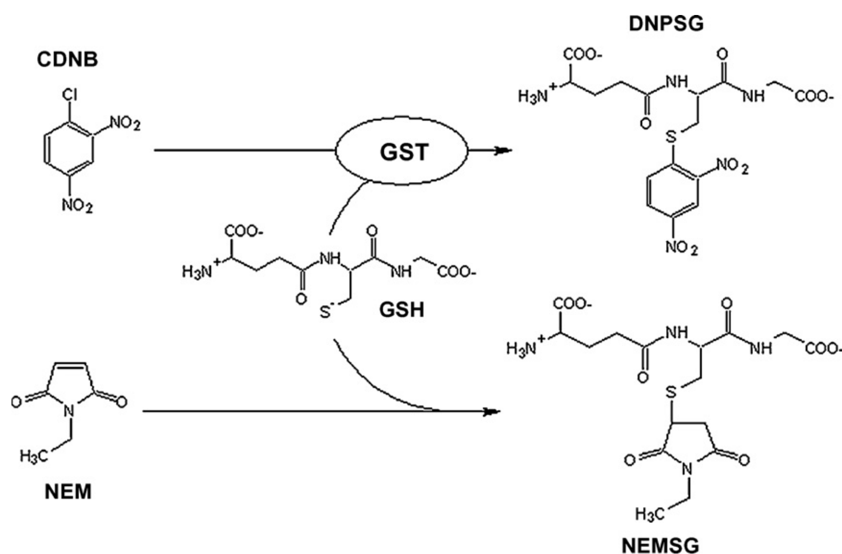

Fig. 1. GSH conjugates and their precursors. CDNB and NEM are precursors of GSH conjugates DNPSG and NEMSG, respectively. While NEM and GSH react quickly (Gregory, 1955), conjugation of CDNB with GSH must be catalyzed by glutathione-*S*-transferase (GST).

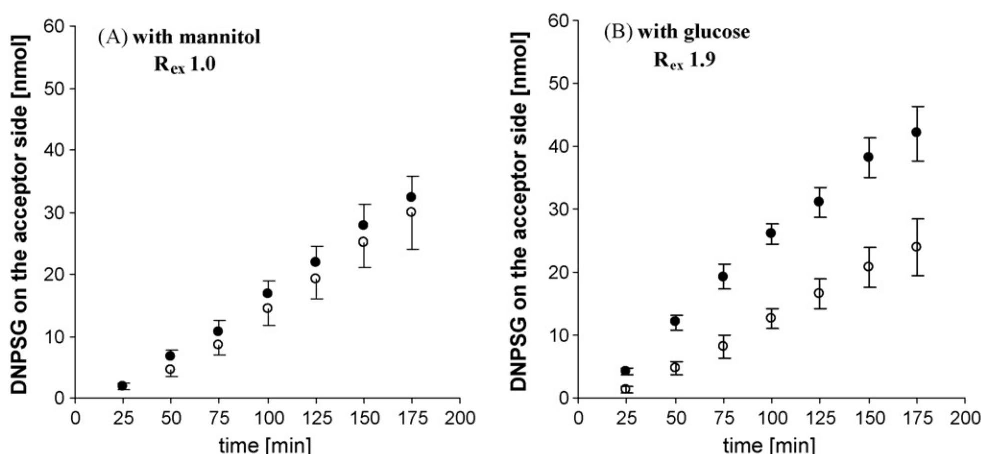

Fig. 2. The results of permeability experiments with mannitol (A) and glucose (B) at the mucosal side. Permeability of DNPSG (C_0 = 1 mM) in S–M and M–S directions was measured by taking samples from mucosal (open circles) and serosal (closed circles) acceptor solutions, respectively. The amounts of the analyte at the acceptor side plotted against time with SEM are given.

2.4. Data analysis and statistics

The flux (J) and apparent permeability coefficient (P_{app}) values of the investigated substances were calculated from the following equations:

$$J = \frac{1}{A}\frac{dQ}{dt}\left(\frac{1}{\text{cm}^2}\frac{\text{mol/L}}{\text{s}}\right), \quad P_{app} = \frac{J}{C_0}(\text{cm/s})$$

where dQ/dt is the steady-state appearance rate of the examined substance at the acceptor side of the tissue, A is the exposed area of the tissue and C_0 is the initial concentration of the investigated substance in the donor compartment. Excretion ratio (R_{ex}) is defined as a quotient of S–M and M–S P_{app}. When CDNB was present in the donor solution, DNPSG formed intracellularly and was therefore determined both in the serosal and in the mucosal solution. Since C_0 of DNPSG was an unknown intracellular concentration, R_{ex} was calculated from fluxes. All data are presented as means ± SEM of four measurements (unless otherwise stated). To investigate the effects of various stimuli on the values of P_{app}, and R_{ex} two-sided Student's t-tests and planned multiple comparisons with false discovery rate method were used (Benjamini and Hochberg, 2000). Probability of type 1 errors (α) was set on 0.05.

3. Results and discussion

We measured the S–M and M–S permeability of GSH conjugate DNPSG with mannitol or glucose at the mucosal side of the rat intestine. Fig. 2A presents the transport of DNPSG when mannitol was at the mucosal side of the tissue. Slopes in both (M–S and S–M) directions are similar, comparison of P_{app} does not reveal significant difference ($p > 0.05$) and R_{ex} is close to unity. Fig. 2B shows the results obtained when glucose was present at the mucosal side. The distinction between M–S and S–M experiment is evident ($p < 0.05$); R_{ex} increased by 1.9. These experiments indicated that glucose at the mucosal side of the intestinal tissue triggers a change in DNPSG permeability.

When glucose is present in the intestinal lumen, it triggers a variety of processes in enterocyte (Rolland et al., 2001). The most known process is opening of tight junctions between enterocytes. The opening can be noticed as decrease of transepithelial electrical resistance and increase in permeability of paracellularly permeating compounds (Turner et al., 2000). To evaluate the effect of tight junctions opening, we performed experiments with additional markers (NEMSG and CDNB). These two compounds have different physiochemical properties and can therefore permeate differently through the tight junctions.

DNPSG and NEMSG are GSH conjugates with similar physiochemical properties. They share the tripeptide GSH moiety which is zwitterionic and bears two carboxylic groups and one amino group (Fig. 1). On the other hand, CDNB is a DNPSG precursor. It is uncharged at all physiological conditions, smaller then conjugates and lipophilic. Metabolism of CDNB in the enterocyte is shown in Fig. 3. It readily passes through the cellular membrane and when it is inside the enterocyte, it undergoes GSH conjugation by glutathione-S-transferase resulting in an intracellular pool of DNPSG. The results of NEMSG and CDNB are similar to those obtained with DNPSG. The asymmetric transport ($p < 0.05$) of markers was observed only when glucose was present at the mucosal side of the tissue (Fig. 4). It is also evident that the secretion of GSH conjugates is triggered by glucose regardless of marker used. The opening of tight junctions or any other process that change extracellular conditions is most probably not the underlying mechanism of the asymmetry in GSH conjugate transport.

To confirm the active nature of GSH conjugate transport, a lower (0.1 mM) donor concentration of DNPSG was used. Since all active processes are saturable, their responses do not increase proportionally with increasing concentration. On the other hand, passive

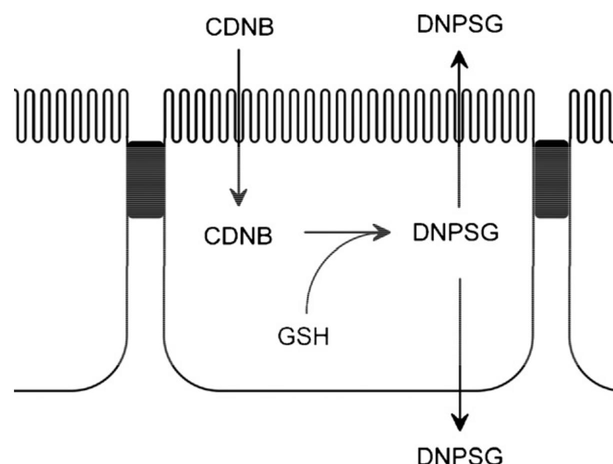

Fig. 3. The metabolism of CDNB in the enterocyte. CDNB enters enterocyte and is conjugated with GSH, forming DNPSG. DNPSG can be transported to apical or basolateral side of the enterocyte. In the figure, CDNB enters from the apical side, but the process is the same regardless of direction of its entering.

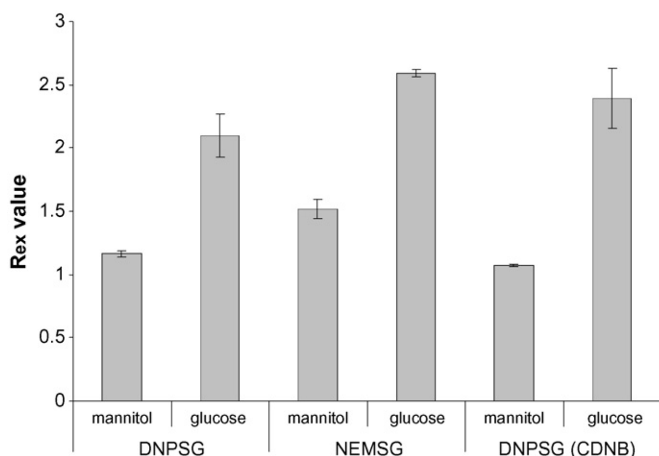

Fig. 4. The results of permeability experiments (R_{ex} values) of different markers used with mannitol and glucose. Donor concentrations of DNPSG and NEMSG were 1 mM, while CDNB was applied in 0.1 mM concentration. In experiment with CDNB, the precursor (CDNB) was used and DNPSG was analyzed.

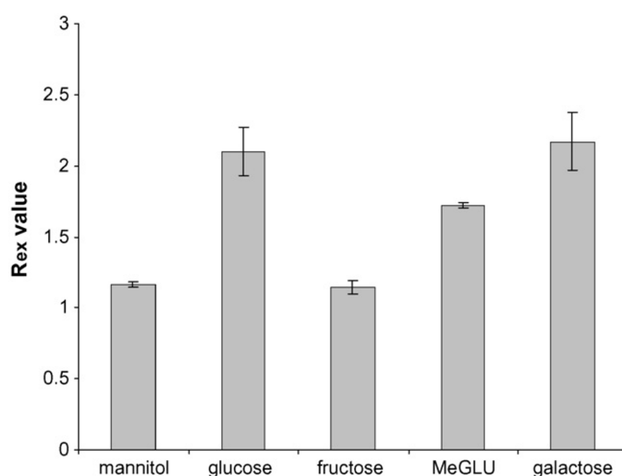

Fig. 6. The permeability results of DNPSG transport (R_{ex} values) with different monosaccharides at the mucosal side. Additionally to mannitol and glucose, fructose, α-methylglucopyranoside (MeGLU) and galactose were used.

processes tend to have linear dependence over a broad range of concentrations (Swaan et al., 1995). Therefore, when active and passive processes take place simultaneously, relative contribution of active processes decreases with increasing concentration and vice versa. The active DNPSG secretion is evident from our result in Fig. 5. While the donor concentration of DNPSG was lowered from 1 mM to 0.1 mM, R_{ex} increased significantly from 1.9 to 2.6, respectively ($p > 0.05$). This confirmed the active nature of asymmetry in DNPSG permeability.

To further investigate the way by which glucose influences GSH conjugate permeability we focused on the cellular processes that occur when specific monosaccharide comes in contact with enterocyte and enters it. Therefore, we tested the influence of three other monosaccharides (fructose, galactose and α-methylglucopyranoside), which share some metabolic/transport processes with glucose, on DNPSG transport. These monosaccharides were added to the mucosal side of the intestine. R_{ex} values for these experiments are shown in Fig. 6.

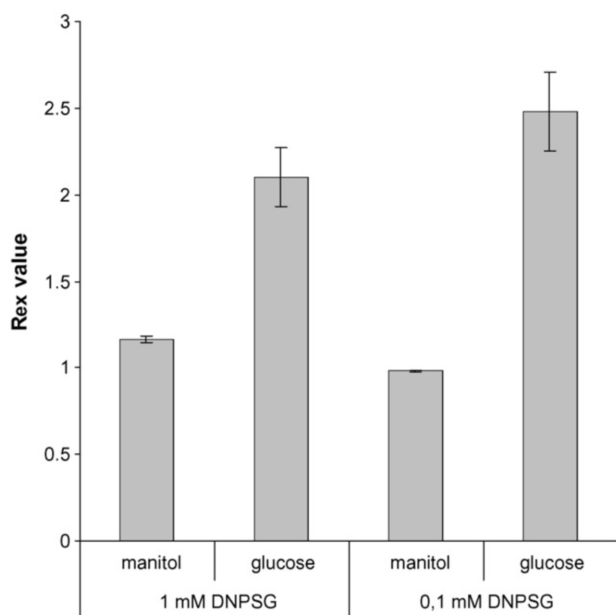

Fig. 5. The permeability results of DNPSG transport (R_{ex} values) with different donor concentrations of DNPSG (C_0 = 1 mM). Additionally to 1 mM donor concentration of DNPSG, experiments with mannitol and glucose were performed also with 0.1 mM concentration.

Glucose is transported by an apical SGLT (active, sodium coupled) transporter and a GLUT (passive facilitated) transporter into enterocyte and it exits through basolateral GLUT transporters. Inside the cells it can also be phosphorylated and further metabolized to yield energy (Kellett and Brot-Laroche, 2005; Nilsson et al., 1996). We see that mannitol, an inert monosaccharide, and fructose, a GLUT substrate, do not trigger the secretion of DNPSG ($p > 0.05$), while the other three monosaccharides produce significant ($p < 0.05$) and comparable DNPSG secretion (Fig. 6).

Metabolic/transport properties of tested monosaccharides and the results of experiments (R_{ex} values) with DNPSG are given in the Table 1. One can see that secretion of DNPSG ($p > 0.05$) occurs only when monosaccharide is able to interact with SGLT transporter. Therefore, binding to or transport with a SGLT transporter is most probably the key metabolic/transport process responsible for triggering the secretion of GSH conjugates.

GSH conjugates are substrates for RLIP76 and for some members of MRP transporter family (MPR1-5) (Chan et al., 2004). MRP1, MRP3 and MRP5 are located at basolateral side of the enterocyte and MRP2 and MRP4, BCRP and RLIP76 at the apical side. Since glucose stimulates secretion of GSH conjugates to the mucosal side of the intestine, only apically located transporters can be involved in GSH secretion. Therefore, the influence on GSH conjugate permeability is most probably related to the increase in MRP2, MRP4, BCRP or RLIP76 activity.

To determine, which of the apical GSH conjugate transporters is involved, we used two allosteric MRP2 activators (sulfanitran and

Table 1
Metabolic/transport process of tested monosaccharides (Bell et al., 1993; Kellett and Brot-Laroche, 2005; Nilsson et al., 1996; Rolland et al., 2001; Wright et al., 2004). Each property of a specific monosaccharide is denoted as YES/NO. The results of permeability experiments are given as R_{ex} values.

Metabolic/transport process	Mannitol	Fructose	MeGLU	Galactose	Glucose
Transport with SGLT	NO	NO	YES	YES	YES
Transport with GLUT	NO	YES	NO	NO	YES
Forms metabolites and ATP	NO	YES	NO	YES	YES
R_{ex}	1.0	1.0	1.5*	1.9*	1.9*

* Denotes statistically significant value ($p < 0.05$).

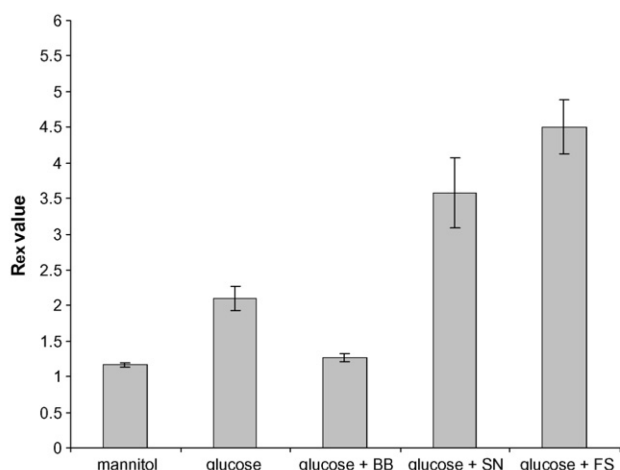

Fig. 7. The effect of MRP modulators (benzbromarone – BB, sulfanitran – SN, furosemide – FS) on DNPSG (C_0 = 1 mM) transport (R_{ex} values) in the presence of glucose. The modulators and glucose (10 mM) were added to the mucosal side of the intestine. Benzbromarone was applied apically in 0.1 mM concentration, while furosemide and sulfanitran were present on both sides of the intestine in 0.5 mM and 0.25 mM concentrations, respectively. Mannitol – reference experiment with mannitol (no glucose and no modulator) at the mucosal side.

furosemide) and MRP inhibitor (benzbromarone). Zelcer et al. have proved that allosteric activation of sulfanitran is proportional to its concentration, while furosemide gives maximal allosteric activation at 0.5 mM concentration (Zelcer, 2003). Therefore, to achieve maximal attainable allosteric activation, we used 0.25 mM (saturated) and 0.5 mM solutions of sulfanitran and furosemide.

The secretion of DNPSG was inhibited by benzbromarone and was comparable to the unstimulated reference experiment with mannitol (Fig. 7; $p > 0.05$). Assuming that benzbromarone is a specific inhibitor of MRP transport, these results indicate that the change in permeability of GSH conjugates is MRP mediated and not caused by a change in BCRP or RLIP76 activity. On the other hand, since BCRP and RLIP76 share MRP transporter family substrate specificity (Zamek-Gliszczynski et al., 2006; Awasthi et al., 2002) we cannot unambiguously conclude that GSH conjugate secretion is MRP mediated.

Furosemide and sulfanitran are structurally and pharmacologically unrelated allosteric MRP2 activators. They are not MRP2 substrates, but they increase DNPSG transport by binding to the allosteric site of MRP2. Additionally, sulfanitran produced no allosteric activation of MRP4 transport (Zelcer et al., 2003), while furosemide is a MRP4 substrate and can therefore inhibit transport of other MRP4 substrates. Regarding BCRP, no reports can be found on interactions with sulfanitran, while furosemide is a BCRP substrate (Hasegawa et al., 2007). Since both MRP2 activators additionally increased glucose stimulated DNPSG secretion ($p < 0.05$), involvement of MRP4 or BCRP in the transport of GSH conjugates is less likely. On the basis of our results we can assume that glucose stimulated GSH conjugate secretion is mediated by an increase in the MRP2 activity.

Regulation of MRP transporters was already researched in the liver and in the kidney. Kubitz et al. (1999) demonstrated that MRP2 activity in hepatocytes is under constant vesicular control. Canalicular membrane of the hepatocytes has a basal MRP2 activity, which can be regulated by intracellular trafficking of MRP2 containing vesicles. Membrane activity of MRP2 transporters can be increased by insertion of these vesicles into the membrane or decreased by their retrieval into the pericanalicular pool (Dombrowski et al., 2000). These two processes are commonly referred to as inhibition and activation of the membrane MRP2 activity, respectively. In the liver, activation is triggered by taurocholate and dibutyryl-cAMP, while inhibition is triggered by bile duct ligation (experimental model of cholestasis) and presence of lipopolysaccharides (Gerk and Vore, 2002). Changed medium osmolarity can act both stimulatory (triggering insertion) by hypoosmolarity and inhibitory (triggering retrieval) by hyperosmolarity on the membrane MRP2 activity (Dombrowski et al., 2000; Kubitz et al., 1999). Following the work of Kubitz et al., we tested if hypoosmolar medium that up-regulates canalicular MRP2 activity in the liver can also trigger secretion of DNPSG in the intestine.

Our results in Fig. 8 show that hyperosmolar conditions do not influence the secretion of DNPSG (hyperosmolar buffer – no glucose) and do not decrease the secretion triggered by glucose

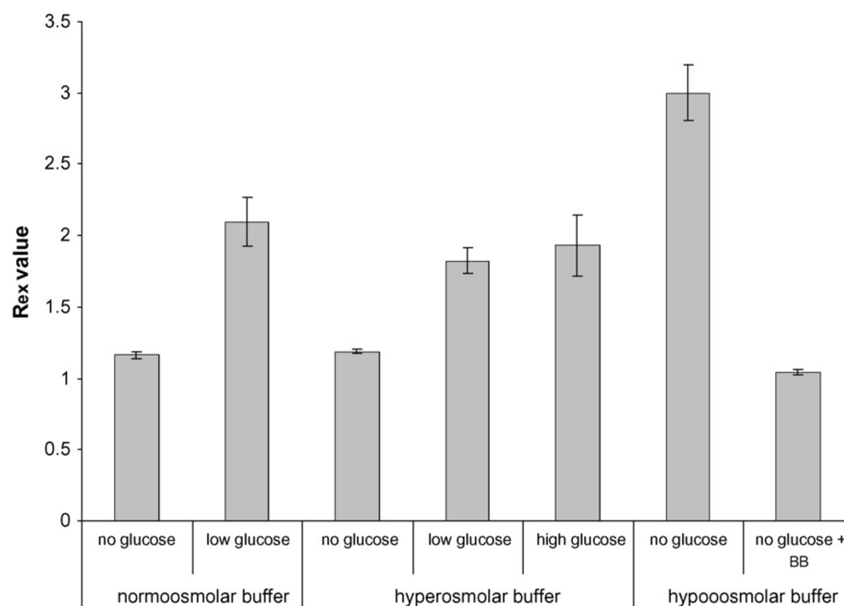

Fig. 8. The results of DNPSG (C_0 = 1 mM) permeability experiments (R_{ex} values) with different medium osmolarity and glucose concentrations and benzbromarone (BB). Glucose (10 mM) was present at the serosal side in all experiments. Low (10 mM) and high (100 mM) glucose concentrations at the mucosal side were used with normoosmolar and hyperosmolar media. Total osmolarity of the solutions at the mucosal and serosal side in the experiments was changed from normoosmolar (305 mOsm) to hypoosmolar (205 mOsm) and hyperosmolar (405 mOsm) by lowering NaCl and adding mannitol, respectively. Benzbromarone was applied apically in 0.1 mM concentration.

(hyperosmolar buffer − low glucose). Furthermore, the results also show that additional amounts of glucose (hyperosmolar buffer − high glucose) cannot increase the secretion. On the other hand, hypoosmolar medium (hypoosmolar buffer − no glucose) raised DNPSG secretion much more then glucose (normoosmolar buffer − low glucose) ($p < 0.05$). Similarly to the inhibition of glucose stimulated DNPSG secretion, benzbromarone inhibits also the secretion of DNPSG stimulated by hypoosmolar conditions (hypoosmolar buffer − no glucose + BB).

Our results thus suggest that intestinal secretion by MRP2 transporters is activated by intraluminal conditions (hypoosmolarity, presence of glucose), while apical membrane of enterocytes has no basal level of MRP2 activity (in contrast to canalicular membrane of hepatocytes), since no secretion of GSH conjugates is present in the reference (unstimulated - normoosmolar conditions with mannitol) conditions.

4. Conclusion

Using isolated rat small intestine as a model, we established that glucose in intestinal lumen triggers secretion of GSH conjugate transport. With the application of different monosaccharides we showed that interaction with SGLT transporter is needed for this triggering. Since GSH conjugates are substrates of BCRP, RLIP76 and MRP transporter family, we used modulators (inhibitor and two activators) to determine that MRP2 is most probably the key transporter responsible for the secretion of GSH conjugates. Additionally, we established that hypoosmolar medium, a known stimulus for hepatic MRP2 activation, can stimulate the intestinal MRP2 activity, too. We also showed that intestinal MRP2 activity has no basal (unstimulated) activity in contrast to basal canalicular MRP2 activity in the liver.

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