Plasma levels of guanylins are reduced in patients with Crohn's disease

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Competing interests

Odd Helge Gilja has received speaker honoraria from the following companies: AbbVie, Bracco, Almirall, GE Healthcare, Takeda AS, Meda AS, Ferring AS and Allergan; and he has acted as consultant for Bracco, GE Healthcare, and Samsung.

Kim Nylund has served as speaker Takeda, MSD, MEDA AS, Ferring Pharmaceuticals

The other authors declare that they have no competing interests.

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Abstract

Background: Guanylin (GN) and uroguanylin (UGN) are endogenous ligands for the intestinal receptor guanylate cyclase C (GC-C), an important regulator of intestinal fluid homeostasis. Gene expression and protein levels of GN are suppressed in inflamed intestinal tissue from patients with inflammatory bowel disease (IBD), but knowledge about plasma levels of guanylins in these conditions is sparse. We aimed to investigate the fasting plasma levels of the prohormones proGN and proUGN in patients with Crohn's Disease (CD) and relate these to levels found in persons with other diarrheal conditions, as well as persons with normal bowel habits.

Methods: Plasma from patients with CD, patients with Familial *GUCY2C* Diarrheal Disease (FGDS), diarrhea-predominant irritable bowel syndrome (IBS-D) and healthy controls (HC) was analyzed using ELISA assays.

Results: Significantly lower fasting plasma levels of proguanylins were found in CD and FGDS patients, compared to HC. In CD patients, plasma proGN levels correlated negatively with Harvey Bradshaw Index and with number of stools / 24 h.

Conclusion: Our data indicate that diarrhea may be a determinant for levels of proGN in plasma, and should be further explored in studies of different diarrheal disorders.

Introduction

The intestinal guanylate cyclase-C receptor (GC-C) and its endogenous ligands guanylin (GN) and uroguanylin (UGN) have gained renewed interest due to their importance for intestinal hydration and homeostasis [1,2], as well as their role in intestinal inflammation [3,4]. Expression of the genes encoding GC-C (*GUCY2C*) and the guanylins (*GUCA2A* and *GUCA2B*) are reduced in inflamed colonic tissue [3,5], and the silencing of the pathway is reported to normalize when the patients experience remission [3].

Proguanylin (proGN) and prouroguanylin (proUGN) are peptides mainly produced in the intestinal epithelial cells [6] and their biologically active forms, the hormones GN and UGN represent the C-terminal parts of the biological inactive prohormones. They are cleaved from the prohormones by converting enzymes upon secretion [7,8], and elicit autocrine as well as paracrine functions [8]. After secretion into the intestinal lumen, GN and UGN bind to the guanylate cyclase-C receptor (GC-C) which in turn leads to increased cellular level of cyclic-guanosine-monophosphate (cGMP), and to phosphorylation of the cystic fibrosis transmembrane regulator (CFTR) resulting in increased secretion of Cl^-, bicarbonate and water into the lumen. GC-C activation also leads to an inhibition of the Na^+/H^+ exchanger (NHE3), resulting in reduced absorption of Na^+ [1]. ProGN and proUGN are also secreted into the circulation, and can be measured in plasma [9,10], but detailed regulation of secretion of proGN and proUGN from the gut mucosa cells into blood and into the intestinal lumen is not well investigated in humans.

In 2012 our group discovered an activating *GUCY2C* gene mutation (c.2519G>T) in a Norwegian family (n=35), named familial *GUCY2C* diarrhea syndrome (FGDS) [11,12]. In persons with this mutation the GC-C receptor is hyperactive when GN and UGN are bound to it, which in turn leads to increased intraluminal fluid in the intestine [11]. We observed that affected persons in this family had chronically loose stools, median 4 per day (range 1-10). In

addition, we found dysmotility and increased fluid in the lumen of the small bowel in the FGDS patients [12,13].

In patients with inflammatory bowel syndrome (IBD) in remission, approximately 30-40 % have symptoms resembling irritable bowel syndrome (IBS), and may therefore be difficult to separate from IBS patients [14]. IBS is a syndrome defined by long lasting, variable abdominal pain/discomfort that fluctuates in concert with changes in stool frequency or consistency. Some patients have mainly loose stools and these may be defined to have diarrhea predominant IBS (IBS-D) according to specific Rome criteria [15]. The symptoms of FGDS patients resemble IBS-D [11].

Little is known about the levels of guanylins in blood in humans with diseases where diarrhea is a predominant symptom. Therefore, the aim of the current study was to measure plasma levels of proGN and proUGN in patients with CD and compare with patients with FGDS, IBS-D and with plasma levels in a healthy control group (HC).

Subjects and Methods

Subjects

The majority of the CD patients were recruited from the hospital routine outpatient clinic. Harvey-Bradshaw Index (HBI) was recorded from all CD patients. HBI is a clinical index of Crohn's-disease activity composed of number of diarrhea last 24 hours, extra- intestinal manifestations, general condition and abdominal masses [16].

FGDS patients were recruited into the study when the *GUCY2C* mutation (c.2519G >T) had been confirmed. Patients with diarrhea-predominant IBS (IBS-D) according to Rome II criteria, were recruited from a cohort of patients with post-infectious IBS during follow up around 5 years after the eliciting *Giardia* infection [17]. HCs were recruited using bulletin boards at Haukeland University Hospital and via a recruitment website. Participants in the HC

group were interviewed before inclusion in order to obtain information of their clinical status and to define them as healthy, without previous history of gastrointestinal disease.
Informed consent was obtained from all participants and the study was approved by the Regional Committee for Medical Research Ethics, Haukeland University Hospital, 2014/222, 2009/902, and performed in accordance with the declaration of Helsinki.

Samples

Plasma from all participants was obtained after an overnight fast. EDTA whole blood was centrifuged at 1800 x g, 4 °C for 10 minutes and plasma was stored in aliquots at -80 °C until analysis. Plasma proGN and proUGN concentrations were determined using sandwich enzyme-linked immunosorbent assay (ELISA) kits (BioVendor, Karasek, Czech Republic, Cat. No. RD191069200R and RD191046100R, respectively). The ELISAs were performed according to the manufacturer's instructions. The absorbance was measured by SPECTRAmax microplate reader (Molecular Devices, Sunnyvale, CA, US), and concentrations of proGN and proUGN were calculated by the SoftMaxPro Software version 5.4.5.000 (Molecular Devices, Sunnyvale, CA, US) using the four-parameter algorithm standard curve fitting.
Fecal Calprotectin (EliA^{TM} Calprotectin stool test, Thermo Scientific) was obtained from all CD patients.

Statistical analysis

Descriptive data are presented as median (range). One-way ANOVA with Bonferroni correction for adjustment was used when comparing the plasma levels of guanylins between groups, otherwise Student's t-test. For analyses of correlation, Pearson´s test was used. $P<0.05$ was considered statistically significant. Data were analysed with SPSS 22 Software

(Chicago, Illinois, USA) and Graph Pad Prism version 5.02 for Windows (Graph Pad Software Inc., La Jolla, CA, USA).

Results

Characterization of study participants

Details of study participants are shown in Table 1. There was no significant difference between age and gender in the 4 groups.

The CD patients were referred from the outpatient clinic of the Department of Medicine, Haukeland University Hospital, Bergen, Norway. They had suffered from CD for median 10 years (3-30), median age at diagnosis was 19 (10-57) and median years on TNF-α-inhibitors were 7 (0-15). None of the patients had short bowel syndrome, 25 had received bowel surgery, 4 had permanent stoma, and the majority (n=25) had colonic involvement. Thirty of the CD patients were treated with TNF-α-inhibitors (Infliximab or Adalimumab), three were treated with α4β7 integrin blockade (Vedolizumab) and 2 received no medical treatment. Thirty-one of the CD patients were scored with HBI index, 4 had stoma and HBI could not be assessed. Patients with clinical inactive (HBI $\leq$ 4) and active (HBI>4) disease were analyzed separately. No CD patients tested positive for the *GUCY2C* mutation (c.2519G>T).

Five of the FGDS patients had a concomitant diagnosis of CD, all in clinical remission, and one currently treated with adalimumab 40 mg subcutaneously every second week. Four children from the FGDS family aged 3, 5, 6 and 7 years old were included.

All IBS-D patients fulfilled the Rome II criteria for diagnosis of IBS-D [18]. The healthy volunteers had no history of chronic abdominal complaints or bowel surgery.

Fasting plasma levels of guanylins

Levels of plasma proGN were lower in all CD patients (5.1 ng/mL ± 1.6, $p<0.001$) as well as in FGDS patients (6.0 ng/mL ±1.3, $p = 0.01$) compared to the HC (7.8 ng/mL ± 1.9) (Figure 1). The five patients with FGDS combined with CD did not differ in guanylin levels from the remaining FGDS patients, but we noted that for proguanylin they clustered at the lower end of the range for the group.

Plasma proGN levels were still significantly lower compared to HC when the groups of CD patients with active inflammation (HBI > 4) or remission (HBI ≤ 4) were analyzed separately (figure 1). No difference in plasma levels of proGN between the two CD groups was found ($p=0.52$). ProGN levels differed between IBS-D (6.7 ng/ml ± 2.0 ng/ml) and all CD patients ($p=0.005$), as well as the subgroup of CD patients with active inflammation (HBI > 4) ($p<0.01$). There were no differences between IBS-D patients compared to FGDS patients or to HC.

Fasting plasma levels of proUGN showed a similar pattern, with significantly reduced levels in all CD patients (2.1 ng/ml ± 0.8, $p<0.05$), and FGDS patients (2.1 ng/ml, SD ± 0.5, $p<0.05$) compared to HC (2.6 ng/mL ±0.8). CD patients with HBI ≤ 4 (2.0 ng/mL ± 1.4) had significantly lower proUGN levels than HC ($p<0.05$). However, in CD patients with HBI >4, plasma proUGN levels (2.4 ng/mL ± 1) were not significantly different from those observed in HC. No significant differences between IBS-D and HC were found in plasma guanylins. There was no correlation between CRP and guanylins, or F-Calprotectin and guanylines in the CD patients.

Number of loose stools in CD patients

CD patients in remission (HBI ≤ 4) had median 0 stool/24 h (range 0-4), while CD with inflammatory activity (HBI >4) had median 6 stools /24 h (5-15) (Table 1).

Looking at all CD patients together we found a negative correlation between plasma proGN and number of loose stools/24 hours, ($r = -0.463$, $p=0.009$, figure 2) as well as a negative correlation between plasma proGN and HBI ($r = - 0.488$, $p= 0.01$, figure 2). This remained significant also when the outlier (number of loose stools 15) was removed.

There was no similar correlation between plasma proUGN and HBI or number of loose stools, or between any of the plasma guanylins and calprotectin in CD patients.

Discussion

In this study, we show that fasting plasma levels of guanylins are significantly lower in patients with CD and FGDS compared to HC. The changes were more marked for proGN in CD patients as plasma proGN correlated negatively with HBI and number of stools. In FGDS patients, we have previously reported negative correlation between plasma proGN and stool consistency score (Bristol stool chart) [13]. These data taken together suggest that frequency and/or consistency of stools may be important determinants for fasting plasma levels of proGN.

In FGDS patients, diarrhea is caused by increased secretion of Cl^- through the CFTR and reduced reabsorption of Na^+ through NHE3 [11]. In CD patients, however, diarrhea is mainly caused by reduced electroneutral reabsorption of Na^+ through NHE3 and Cl^- through the Cl/HCO_3^- exchanger [19]. Regardless of mechanism, the net result is loss of fluid and NaCl into the intestinal lumen in both conditions.

Previous studies of guanylins in patients with IBD have focused on measurements in intestinal tissue, and only a small number of studies have measured guanylins in plasma. In 1995, Kuhn *et al* found no significant differences in circulating proGN levels between IBD patients (both CD and ulcerative colitis (UC)) and HC, and no correlation between plasma proGN and disease activity scores [20]. However, a recent proteomic study reported reduced

plasma proGN in CD patients compared to HC (in line with our data) as well as to UC [10]. The authors suggested that proGN could discriminate between CD and UC. The reason for the conflicting results in the literature on plasma GN in CD patients is not clear, but could be due to methodological issues.

Active inflammation in colonic mucosa seems to lead to reduced GC-C signaling in this tissue, as gene expression and cellular protein content of both GC-C and guanylins are reduced compared to levels in HC and to non-inflamed colonic tissue in IBD patients (normal levels) [3,5,21]. Such reduction in guanylin formation could be reflected in a lower secretion of guanylins to the bloodstream, but this was not measured in the studies referred to above. Interestingly, our data show that CD patients with little inflammation (low HBI) compared to those with higher levels of inflammation (high HBI) had reduced plasma guanylin levels compared to HC. This may reflect additional unknown mechanisms for reduced signaling of guanylins in CD patients.

The mechanism for diarrhea in IBS-D patients is unknown, but in this study, we found a trend towards lower plasma levels of both guanylins in patients with IBS-D compared to HC, although the differences were not statistically significant. Further studies in different defined subpopulations of individuals with CD, ulcerative colitis (UC) and other diarrheal disorders are warranted, in order to evaluate whether fasting plasma proGN may be an indicator of intestinal hydration, or a consequence of low grade intestinal inflammation.

Plasma proUGN showed less pronounced differences between patient groups and HCs than proGN in this study. There was no correlation between proUGN and number of stools or HBI in CD patients, nor with stool consistency score in our previous study [13]. Plasma levels of

UGN could be more tightly regulated and is influenced by several additional factors compared to GN, as UGN is expressed in a wider range of tissues and may be important for regulating satiety and body weight as part of the gut-brain axis [9,22]. Thus, it might be less interesting to further pursue the relationship between UGN and intestinal hydration compared to GN.

In this study, we did not have information about number of stools in the FGDS group nor in the IBS-D group at the time of plasma sampling. However, later studies obtained from the same FGDS patients have shown that they all have loose stools, lower plasma proGN and proUGN as well as increased number of fluid- filled small bowel loops compared to HC [13].

In conclusion, fasting plasma levels of guanylins, particularly proGN, were significantly reduced in both CD and FGDS patients compared to HC. Plasma guanylins in the IBS-D patients showed a trend towards lower levels. Our data indicate intestinal fluid loss as a determinant of plasma guanylins, especially proGN, but call for further studies regarding interactions between guanylin metabolism and intestinal fluid homeostasis in different diarrheal disorders.

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