Small Intestinal Alterations in Severely Obese Hyperglycemic Subjects

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Context: Type 2 diabetes mellitus (DM2) is associated with small intestinal hyperplasia and hypertrophy in rodents. Moreover, the small intestine is increasingly acknowledged to play a role in the pathophysiology of DM2.

Objective: The objective of the study was to investigate the relation between plasma markers of small intestinal function and chronic hyperglycemia in man.

Design, Setting, and Participants: We conducted a cross-sectional observational study of 40 severely obese subjects with chronic hyperglycemia and 30 severely obese subjects without chronic hyperglycemia who were indicated for bariatric surgery.

Main Outcome Measures: We assessed plasma levels of citrulline, representing small intestinal enterocyte mass, intestinal fatty acid binding protein (I-FABP), a marker of enterocyte loss, and glucagon-like peptide-2, an intestinotrophic factor, and related them to glycated hemoglobin (HbA_{1c}) levels.

Results: Plasma citrulline and I-FABP levels were both significantly elevated in subjects with chronic hyperglycemia ($HbA_{1c} > 6.0\%$) compared with subjects with a normal HbA_{1c} ($\leq 6.0\%$) (citrulline, 35 ± 2.1 μM *vs.* 26 ± 1.4 μM, $P = 0.001$; I-FABP, 140 ± 22 pg/ml *vs.* 69 ± 14 pg/ml, $P = 0.001$). Moreover, plasma citrulline and I-FABP levels correlated with HbA_{1c} levels (citrulline, $r_s = 0.30$, $P = 0.02$; I-FABP, $r_s = 0.33$, $P = 0.005$). The I-FABP to citrulline ratio was higher in subjects with an elevated HbA_{1c} (4.0 *vs.* 3.1, $P = 0.03$). Plasma glucagon-like peptide-2 levels were not related to citrulline or I-FABP levels ($r_s = 0.06$, $P = 0.67$; r_s 0.08, $P = 0.54$, respectively).

Conclusion: Chronically elevated glucose levels in obese individuals are associated with increased small intestinal enterocyte mass and increased enterocyte loss. These findings argue for the further exploration of the role of the intestine in the pathophysiology of DM2. **(*J Clin Endocrinol Metab* 96: E379–E383, 2011)**

Type 2 diabetes mellitus (DM2) affects more than 285 million people worldwide, a number rapidly increasing in this era of obesity (1). Traditionally, studies on the pathogenesis of DM2 have mainly focused on insulin sensitivity of liver, muscle, and adipose tissue. However, there is increasing evidence for an important role of the small intestine in the control of glucose homeostasis and in the pathogenesis of DM2. For example, the release of various small intestinal peptides that control glucose homeostasis, such as glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1, is disturbed in subjects with DM2 (2). In addition, gluconeogenesis in the small intestine was recently shown to regulate hepatic glucose metabolism and to contribute to the rapid beneficial effects of Roux-en-Y gastric bypass surgery on DM2 in mice (3). Importantly, bypassing the proximal intestine is associ-

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Abbreviations: DM2, type 2 diabetes; GLP-2, glucagon-like peptide-2; HbA_{1c}, glycated hemoglobin; I-FABP, intestinal fatty acid binding protein.

ated with a rapid resolution of DM2 in man (4), supporting an important role for intestinal factors in the regulation of glucose homeostasis.

Interestingly, animals with DM2 display alterations of the small intestine such as generalized small intestinal hyperplasia and mucosal hypertrophy (5). More specifically, elevated glycated hemoglobin (HbA_{1c}), an indicator of chronic hyperglycemia, was found to coincide with longer intestinal villi and increased intestinal proliferation in rats with DM2. Besides morphological changes, functional alterations such as increased activity of disaccharidases and enhanced nutrient absorption were reported. Importantly, the combination of small intestinal hyperplasia and increased disaccharidase activity may contribute to the postprandial hyperglycemia that characterizes DM2.

To investigate whether chronic hyperglycemia in man is associated with similar intestinal alterations as previously described in animal models, we determined the plasma levels of citrulline, intestinal fatty acid-binding protein (I-FABP), and glucagon-like peptide-2 (GLP-2), parameters indicating intestinal mass, enterocyte loss, and enterocyte proliferation (6–8), in a population of severely obese patients with and without chronic hyperglycemia. Our results indicate that chronic hyperglycemia is associated with a higher intestinal mass and increased enterocyte loss, supporting a role for the intestine in the pathophysiology of DM2 in man.

Subjects and Methods

Subjects

From June 2006 to November 2008, 70 severely obese subjects indicated for bariatric surgery were sequentially included at the Department of General Surgery of the Maastricht University Medical Center. Patient characteristics are summarized in Table 1. Patients with type 1 diabetes, other acute or chronic inflammatory diseases (*e.g.* M. Crohn, colitis, hepatitis, autoimmune diseases) and patients using antiinflammatory drugs and/or reported alcohol consumption (more than 10 g/d) were excluded from the study. All severely obese subjects were screened for diabetes and used appropriate medication for their diabetic status. Eleven subjects used metformin, five subjects used insulin, 11 patients were treated for hyperlipidemia, and 31 subjects used antihypertensive medication. Subjects did not follow a specific diet and were not under dietary counseling at the time of sampling. This study was approved by the Medical Ethical Board of the Maastricht University Medical Center in line with the ethical guidelines of the revised version of the Declaration of Helsinki (October 2008, Seoul), and informed consent in writing was obtained from each subject.

Blood sampling

Venous blood samples were obtained after a minimum of 8 h fasting before bariatric surgery. All samples were collected in prechilled EDTA tubes and centrifuged at 4 C for 10 min at $2000 \times g$. The plasma was centrifuged again for 10 min at $3500 \times g$ and stored in aliquots at −80 C until analysis.

Biopsy sampling and quantitative PCR

Small intestinal biopsies were obtained from all patients undergoing gastric bypass surgery (n = 22). Biopsies were immediately snap frozen. Total RNA was isolated from 50 mg tissue by homogenization in Tri reagent (Sigma, St. Louis, MO) according to the manufacturer's instructions. RNA (750 ng) was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed with the ABsolute SYBR Green master mix (ABgene, Leusden, The Netherlands), and the iQ5 iCycler (Bio-Rad) using the following primers: I-FABP forward, 5′-TAGCAGACGGAACTGAACTC-3′, I-FABP reverse, 5′-CATAAGTCTGGACTAGTTCATCAC-3′; β_2-microglobulin forward, 5′-TCCATCCGACATTGAAGTTG-3′, β_2-microglobulin reverse, 5′-CGGCAGGCATACTCATCTT-3′. Relative I-FABP expression was assessed in duplicate by the Δ cycle threshold method after normalization for β_2-microglobulin expression.

Measurements

HbA_{1c} levels were measured at the Department of Clinical Chemistry according to the protocol of the University Medical Center. Citrulline levels were measured as previously described (9). Plasma I-FABP levels were determined with an ELISA according to the manufacturer's instructions (Hycult Biotech, Uden, The Netherlands). Samples were analyzed in the same run, and the interassay and intraassay coefficients of variation were less than 10%. The detection limit was 40 pg/ml. Plasma GLP-2 levels were determined with an ELISA according to the manufacturer's instructions (Biovendor, Modrice, Czech Republic). Samples were analyzed in two runs, and the intra- and interassay coefficient variation was less than 15%.

TABLE 1. Patient characteristics

	HbA_{1c} ≤6.0 (SEM)	HbA_{1c} >6.0 (SEM)	*P* value
Number of patients	30	40	
Sex (male:female)	8:22	12:28	0.76
Age (yr)	42 (1.4)	48 (1.3)	<0.01
Body mass index (kg/m^2)	46.2 (1.7)	45.7 (1.5)	0.72
HbA_{1c} (%)	5.7 (0.1)	7.3 (0.2)	<0.01
Citrulline (μmol/liter)	26 (1.4)	35 (2.1)	<0.01
I-FABP (pg/ml)	69 (14)	140 (22)	<0.01
Citrulline to I-FABP ratio	3.1 (0.7)	4.0 (0.6)	0.03
GLP-2 (ng/ml)	14 (1.6)	18 (2.1)	0.29

Statistical analysis

Statistical analysis was performed using Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). Data are presented as mean ± SEM. Correlations were calculated using Spearman's rank correlation coefficient. Differences between groups were analyzed by the Mann-Whitney test or the χ^2 test. A $P < 0.05$ was considered statistically significant.

Results

Increased plasma citrulline levels in subjects with elevated HbA_{1c}

The functional small intestinal mass has previously been shown to be accurately reflected by plasma levels of citrulline, an amino acid that is not incorporated into proteins, and produced by differentiated small intestinal enterocytes from glutamine (6). Therefore, to study the relation between small intestinal mass and hyperglycemia, we first assessed citrulline plasma levels of 30 severely obese subjects with HbA_{1c} of 6.0% or less [the upper limit of normal (10)] and 40 severely obese subjects with HbA_{1c} greater than 6.0%. In the total population, plasma citrulline levels ranged from 13 to 78 μM, with a mean of 31 ± 1.4 μM, and a median of 30 μM. Interestingly, plasma citrulline levels were significantly higher in patients with an elevated HbA_{1c} ($HbA_{1c} > 6.0$: 35 ± 2.1 μM *vs.* $HbA_{1c} \leq 6.0$: 26 ± 1.4 μM, $P = 0.001$, Fig. 1A). Furthermore, citrulline levels correlated with HbA_{1c} levels ($r_s = 0.30$, $P = 0.02$, Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). These data indicate that severely obese patients with chronic hyperglycemia have an increased functional small intestinal enterocyte mass.

High HbA_{1c} is associated with increased plasma I-FABP in severely obese subjects

To obtain additional evidence for the increased small bowel mass in subjects with chronic hyperglycemia, we next investigated small intestinal enterocyte turnover by measuring plasma I-FABP levels. I-FABP is a gut-associated cytosolic carrier protein involved in the intracellular buffering and transport of fatty acids, which plasma levels have been shown to indicate small intestinal enterocyte loss (8, 11). Assuming steady state, circulating levels of I-FABP also represent the production of enterocytes, or in other words enterocyte turnover. Like citrulline, plasma I-FABP levels were significantly higher in severely obese patients with an elevated HbA_{1c} ($HbA_{1c} > 6.0$: 140 ± 22 pg/ml *vs.* $HbA_{1c} \leq 6.0$: 69 ± 14 pg/ml, $P = 0.001$, Fig. 1B). Importantly, quantitative PCR analysis showed that there was no difference in relative I-FABP expression between subjects with and without chronic hyperglycemia (0.63 ± 0.09 *vs.* 0.60 ± 0.14, $P = 0.55$, Supplemental Fig. 2). This indicates that the increased plasma I-FABP levels in subjects with elevated HbA_{1c} cannot be attributed to increased expression of I-FABP. Furthermore, I-FABP levels also significantly correlated with HbA_{1c} ($r_s = 0.33$, $P = 0.005$). Circulating plasma I-FABP levels showed a significant positive correlation to plasma citrulline levels as well

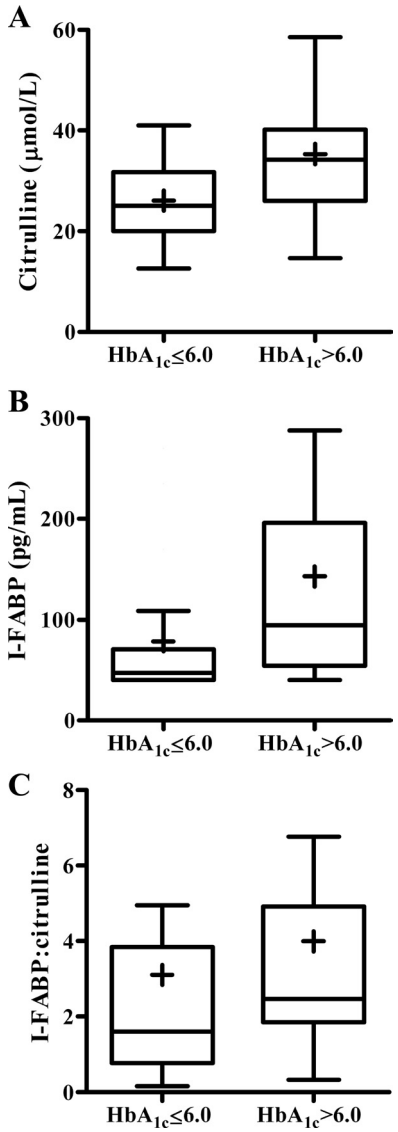

FIG. 1. Increased levels of plasma parameters reflecting enterocyte mass and enterocyte loss in severely obese subjects with chronic hyperglycemia. A, Tukey box and whiskers plot showing that severely obese subjects with an elevated HbA_{1c} (>6.0) display significantly increased plasma levels of citrulline as compared with severely obese subjects with a normal HbA_{1c} (≤ 6.0) ($P = 0.001$). The *horizontal line* corresponds with the mean, whereas the *outer boxes* represent the 25th and the 75th percentiles. Whiskers show the nonoutlier range. A value was defined as an outlier if it was more than 1.5 times the box height above or below the box. The + symbol indicates the mean value. B, Plasma I-FABP levels of severely obese subjects with an $HbA_{1c} > 6.0$ are significantly higher in comparison with I-FABP levels of severely obese subjects with a normal HbA_{1c} ($P = 0.001$). C, Significantly higher I-FABP to citrulline ratio in severely obese subjects with HbA_{1c} greater than 6.0 as compared with severely obese subjects with a normal HbA_{1c} ($P = 0.03$).

($r_s = 0.26$, $P = 0.03$, Supplemental Fig. 1B). Taken together, these data suggest an increased enterocyte turnover in severely obese patients with an elevated HbA_{1c}.

Plasma I-FABP to citrulline ratio indicates disproportionally increased enterocyte loss in subjects with elevated HbA_{1c}

To further explore the relation between enterocyte mass, enterocyte loss, and enterocyte turnover, we calculated the ratio of plasma values of I-FABP and citrulline for every subject. The I-FABP to citrulline ratio correlated significantly with HbA_{1c} levels ($r_s = 0.30$, $P = 0.04$, Supplemental Fig. 1C). Whereas the I-FABP to citrulline ratio was 3.1 for subjects with a normal HbA_{1c}, subjects with an HbA_{1c} greater than 6 displayed a significantly higher ratio of 4.0 ($P = 0.03$, Fig. 1C). This suggests that the increased enterocyte loss in chronically hyperglycemic subjects as indicated by I-FABP plasma levels cannot merely be explained by their relatively higher enterocyte mass. Moreover, the higher enterocyte mass despite the increased enterocyte loss suggests that severely obese subjects with an elevated HbA_{1c} have an increased enterocyte proliferation and turnover.

GLP-2 plasma levels do not correlate with plasma citrulline and plasma I-FABP

To study a potential mechanistic factor underlying the increased enterocyte mass and turnover in subjects with DM2, we next measured plasma levels of the incretin GLP-2. This peptide is a potent intestinal proliferative factor (7). However, fasting GLP-2 levels did not correlate with either plasma citrulline or I-FABP levels in the severely obese population ($r_s = 0.06$, $P = 0.67$ and $r_s = 0.08$, $P = 0.54$, respectively) or the I-FABP to citrulline ratio ($r_s = 0.09$, $P = 0.50$). On the other hand, plasma GLP-2 levels did significantly correlate with HbA_{1c} levels ($r_s = 0.25$, $P < 0.05$), in line with the known glucose-stimulated release of GLP-2 from L cells in the small intestine (7). These findings suggest that the increased small intestinal enterocyte mass and turnover in severely obese subjects with elevated HbA_{1c} are not mediated by GLP-2.

Discussion

In the present study, we obtained the first evidence that chronic hyperglycemia in man is associated with both an increased small intestinal enterocyte mass and increased enterocyte loss. The increase in small intestinal enterocyte mass is in line with findings in rat models of DM2 which show increased intestinal proliferation and longer villi (5). Moreover, streptozotocin-induced hyperglycemia was also found to be associated with small intestinal hyperplasia and hypertrophy in rats (12).

An increased small intestinal enterocyte mass can have important implications in the context of diabetes. First of all, it may account for the enhanced intestinal capacity to absorb glucose, as observed in DM2 (13). This enhanced carbohydrate absorption is likely to be involved in the postprandial hyperglycemia and elevated HbA_{1c} levels that characterize DM2. Moreover, a higher number of small intestinal enterocytes results in an increased potential for intestinal gluconeogenesis, a phenomenon that has been shown to contribute up to one third of the total glucose production in diabetic rats (14).

The mechanisms responsible for the higher small intestinal mass in subjects with chronic hyperglycemia remain unknown, although their relatively increased I-FABP to citrulline ratio indicates that intestinal epithelial proliferation must be augmented in these subjects. It was previously shown that hyperphagia is one of the factors promoting intestinal hyperplasia in streptozotocin-induced diabetic rats (12). However, it seems unlikely that hyperphagia is responsible for the increased intestinal proliferation observed in this study because both study groups had a similar body mass index. Nevertheless, it would be of interest to relate enterocyte mass and turnover to appetite and caloric intake in future studies.

Next to hyperphagia, enhanced secretion of the intestinotrophic peptide GLP-2 by enteroendocrine cells could be a factor driving the increased enterocyte proliferation in chronically hyperglycemic patients. However, GLP-2 levels were comparable between both study groups, indicating that the observed proliferative alterations cannot be attributed to GLP-2 either. The similar GLP-2 levels may also suggest that, in contrast to the enterocytes, enteroendocrine cells are not affected in DM2. Further studies are required to unravel potential alterations in enterocytes and enteroendocrine cells in DM2 in more detail.

Interestingly, next to the increased intestinal mass in subjects with elevated HbA_{1c} levels, both their small intestinal enterocyte loss, as reflected by plasma I-FABP levels, and their I-FABP to citrulline ratio were also higher. Recently it was shown that DM2 is accompanied by significant alterations in tight junction distribution and intestinal permeability in mice, thereby promoting endotoxin-induced low-grade inflammation (15, 16). Subjects with DM2 have also been shown to display elevated plasma endotoxin levels that may be related to increased intestinal permeability (17). It is tempting to speculate that the increased enterocyte loss that we observed in subjects with chronic hyperglycemia may contribute to the impaired intestinal barrier function in DM2. However, we cannot rule out that the elevated plasma I-FABP levels in obese sub-

jects with DM2 reflect increased expression by enterocytes rather than increased enterocyte loss. In fact, some evidence was recently reported for an association between body weight and increased I-FABP expression after small bowel resection in pigs (18).

In summary, we presented evidence for both an increased functional enterocyte mass and increased enterocyte loss and turnover in severely obese subjects with elevated HbA_{1c} levels. Our findings indicate for the first time significant alterations in the pathophysiology of the intestine in human obesity-induced DM2.

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