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Reduced Paneth cell antimicrobial protein levels correlate with activation of the unfolded protein response in the gut of obese individuals

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Abstract

The intestinal microbiota is increasingly acknowledged to play a crucial role in the development of obesity. A shift in intestinal microbiota composition favouring the presence of *Firmicutes* over *Bacteroidetes* has been observed in obese subjects. A similar shift has been reported in mice with deficiency of active Paneth cell α -defensins. We aimed at investigating changes in Paneth cell antimicrobial levels in the gut of obese subjects. Next, we studied activation of the unfolded protein response (UPR) as a possible mechanism involved in altered Paneth cell function. Paneth cell numbers were counted in jejunal sections of 15 severely obese (BMI > 35) and 15 normal weight subjects. Expression of Paneth cell antimicrobials human α -defensin 5 (HD5) and lysozyme were investigated using immunohistochemistry, qPCR, and western blot. Activation of the UPR was assessed with western blot. Severely obese subjects showed decreased protein levels of both HD5 and lysozyme, while Paneth cell numbers were unchanged. Lysozyme protein levels correlated inversely with BMI. Increased expression of HD5 (*DEFA5*) and lysozyme (*LYZ*) transcripts in the intestine of obese subjects prompted us to investigate a possible translational block caused by UPR activation. Binding protein (BiP) and activating transcription factor 4 (ATF4) levels were increased, confirming activation of the UPR in the gut of obese subjects. Furthermore, levels of both proteins correlated with BMI. Involvement of the UPR in the lowered antimicrobial protein levels in obese subjects was strongly suggested by a negative correlation between BiP levels and lysozyme levels. Additionally, indications of ER stress were apparent in Paneth cells of obese subjects. Our findings provide the first evidence for altered Paneth cell function in obesity, which may have important implications for the obesity-associated shift in microbiota composition. In addition, we show activation of the UPR in the intestine of obese subjects, which may underlie the observed Paneth cell compromise.

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Introduction

The intestine is colonized by a complex microbiota, which plays an important role in physiological and homeostatic functions. A close relationship between the intestinal microbiota and obesity has been revealed. First, it has been shown that germ-free animals are protected from diet-induced obesity [1,2]. Second, colonization of the gut of lean germ-free mice with the intestinal microbiota of obese mice results in a significant weight gain of these mice, while this was not observed when germ-free lean mice were inoculated with the intestinal flora of lean donor mice [3]. The underlying mechanisms explaining these observations

were suggested to be increased bacteria-mediated energy harvest from the diet and the ability of bacteria to influence host lipid metabolism, thereby affecting energy expenditure and storage [1,2]. Finally, obesity-related changes in the composition of the microbiota have been described in both man and animals [3–5]. A large shift in microbiota favouring the presence of *Firmicutes* over *Bacteroidetes* was reported in obesity [3–5], which remarkably reverted to normal after weight loss [5]. Strikingly, a similar shift in microbiota composition was observed in mice deficient in the active form of the antimicrobial peptides α -defensins [6]. On the contrary, mice overexpressing α -defensins showed a higher percentage of *Bacteroidetes* and a

lower percentage of *Firmicutes* [6,7]. In the gut, α -defensins are produced by Paneth cells. These cells, residing in the crypts of the small intestine, produce large amounts of antimicrobials including human α -defensin 5 (HD5) and lysozyme, which makes them key players in controlling the microbiota composition of both the small and the large intestine [8,11].

In view of the major impact of Paneth cell antimicrobials on intestinal microbiota composition and the striking similarity between microbiota alterations in mice defective in active α -defensins and in obesity, we hypothesized that compromised Paneth cell function might underlie the microbial shift described in obese subjects.

We report a decrease in the protein expression of both HD5 and lysozyme in the jejunum of obese subjects, compared with normal weight controls. Moreover, we show activation of the unfolded protein response (UPR) in the small intestine of obese subjects and indications of endoplasmic reticulum (ER) stress in Paneth cells, which we propose to be a putative mechanism underlying Paneth cell compromise. Our study is the first to suggest that a host factor is involved in the obesity-associated intestinal microbial shift.

Materials and methods

Ethics

The study was approved by the Medical Ethical Committee of Maastricht University Medical Centre and conducted according to the revised version of the Declaration of Helsinki (October 2008, Seoul). Written consent of all patients was obtained.

Study population and tissue collection

A sequentially included cohort of 15 severely obese subjects (BMI > 35) undergoing gastric bypass surgery was studied retrospectively. Exclusion criteria for this study were acute or chronic inflammatory diseases, degenerative diseases, more than 10 g of alcohol consumption per day, and use of anti-inflammatory drugs. During surgery, jejunal biopsies were obtained and divided into two pieces. Control jejunal biopsies were obtained from 15 consecutive patients undergoing pancreatoduodenectomy. During this procedure, a variable length of jejunum is routinely resected in continuity with the head of the pancreas and duodenum. One piece of tissue was immediately formalin-fixed and embedded in paraffin, another piece was snap-frozen in liquid nitrogen. Snap-frozen samples were stored at -80°C until further processing for western blot or qPCR. Patient characteristics are summarized in Table 1.

Antibodies and reagents

The antibodies and reagents used are described in the Supporting information, Supplementary materials and methods.

Table 1. Patient characteristics

Characteristic	Obese ($n = 15$)	Normal weight ($n = 15$)
Sex (male : female)	2 : 13	9 : 6*
Age (years)	43.3 ± 2.7	$58.9 \pm 3.3^{\dagger}$
BMI (kg/m^2)	43.7 ± 1.6	$24.6 \pm 1.5^{\dagger}$

* $p < 0.05$. $^{\dagger}p < 0.01$.

Histology and immunohistochemistry

Tissue sections were cut at $4\text{ }\mu\text{m}$, deparaffinized in xylene, and rehydrated through graded ethanols to distilled water. For quantification of Paneth cell number, sections of all test and control subjects were stained with haematoxylin and eosin (H&E), and cells were counted in 40 crypts in representative microscopic fields ($200\times$) ($n = 15$ per group). For immunohistochemistry, endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol. After blocking non-specific antibody binding with 5% bovine serum albumin, sections were incubated with specific antibodies to human lysozyme ($1\text{ }\mu\text{g}/\text{ml}$ in 0.1% BSA/PBS) and HD5 ($1\text{ }\mu\text{g}/\text{ml}$ in 0.1% BSA/PBS) at room temperature for 60 min. Next, an appropriate biotin-conjugated secondary antibody ($1.5\text{ }\mu\text{g}/\text{ml}$ in 0.1% BSA/PBS) was applied. Binding of the primary antibody was visualized with a streptavidin–biotin HRP system and 3-amino-9-ethylcarbazole. Nuclei were stained with haematoxylin and stained sections were mounted in aqueous mounting medium (Dakocytomation, Glostrup, Denmark). To avoid inter-assay variability, immunohistochemistry was performed on all study subjects simultaneously.

Western blotting

For western blot, full-thickness tissue samples were homogenized in lysis buffer (200 mM NaCl, 10 mM Tris buffer, 5 mM EDTA, 10% glycerol, and 1% NP-40) using a Biospec mini-beadbeater and glass beads (Bartlesville, OK, USA). Samples were centrifuged at $18\,000\text{ g}$ for 15 min at 4°C . The protein concentration of supernatants was determined using a BCA protein assay kit (Pierce Thermo Fisher Scientific Inc, Rockford, IL, USA). Protein ($10\text{ }\mu\text{g}$) was heated for 5 min in reducing SDS sample buffer, separated by SDS-PAGE on a 15% polyacrylamide gel, and transferred onto polyvinylidene fluoride membrane (Immobilon P, Millipore, Bedford, MA, USA). After transfer of proteins, membranes were blocked with phosphate-buffered saline (PBS) supplemented with 5% non-fat dry milk. Next, antibody incubation was performed overnight at 4°C in PBS–0.05% Tween (PBST) supplemented with 3% non-fat dry milk, with anti-lysozyme ($1\text{ }\mu\text{g}/\text{ml}$), anti-BiP ($0.5\text{ }\mu\text{g}/\text{ml}$), anti-ATF4 ($0.2\text{ }\mu\text{g}/\text{ml}$) or anti-GADD34 ($0.8\text{ }\mu\text{g}/\text{ml}$) antibody. To assess equal protein loading, membranes were reprobed with anti- β -actin ($1\text{ }\mu\text{g}/\text{ml}$) antibodies. After washing with PBST, membranes were incubated with an appropriate HRP-conjugated secondary antibody ($0.1\text{ }\mu\text{g}/\text{ml}$) for 90 min at room temperature. Signals

were detected using the chemiluminescent substrate Supersignal West Pico (Pierce Thermo Fisher Scientific Inc) on blue X-ray film (Fuji, SuperRX, Tokyo, Japan). Band intensity was semi-quantitatively analyzed using Quantity One (Bio-Rad, Hercules, CA, USA).

Quantitative polymerase chain reaction (qPCR)

RNA was extracted from full-thickness jejunal tissue using TRI reagent according to the manufacturer's protocol. RNA samples were treated with DNase (Promega, Madison, WI, USA) to ensure the removal of contaminating genomic DNA. RNA concentration was determined by Nanodrop (Nanodrop, Wilmington, DE, USA) and 750 ng of RNA was used as the template for reverse transcription in a cDNA synthesis reaction using iScript cDNA synthesis kit (Bio-Rad). qPCR reactions were conducted in a volume of 20 μ l containing 10 ng of cDNA, 1 $\times$ Absolute qPCR SYBR Green Fluorescein Mix (Westburg, Leusden, The Netherlands), and 150 nM of gene-specific forward and reverse primers. The sequences of the primers used are provided in the Supporting information, Supplementary Table 1. Gene expression levels of lysozyme (*LYZ*) and HD5 (*DEFA5*) were determined with iQ5 software (Bio-Rad) using a Δ -Ct relative quantification model. The geometric mean of the expression levels of two reference genes, cyclophilin A (*PPIA*) and β 2-microglobulin (*B2M*) was calculated and used as a normalization factor.

Electron microscopy (EM)

For electron microscopy scanning, jejunal tissue ($n = 4$ per group) was immersed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4. Next, samples were rinsed in cacodylate buffer and postfixed in 1% OsO₄ in 0.067 M cacodylate buffer (pH 7.4) supplemented with 1.5% potassium ferrocyanide at 4 °C for 1 hour. After a short rinse in cacodylate buffer, dehydration was carried out in graded ethanol series, followed by embedding in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM 100 electron microscope at an accelerating voltage of 80 KV.

Statistical analysis

Statistical analysis was performed using Prism 5.02 for Windows (GraphPad Software Inc, San Diego, CA, USA). A two-tailed Mann–Whitney *U*-test was used to detect differences between groups. Data are presented as mean $\pm$ standard error of the mean (SEM). Spearman's correlation coefficient was determined to study associations between variables. Differences were considered statistically significant at $p < 0.05$. Multivariate linear regression analysis was used to test for confounding in the data analysis.

Results

Reduced levels of Paneth cell antimicrobials in obese subjects

To study potential Paneth cell alterations in obesity, we first investigated HD5 expression in the jejunum of obese and normal weight control subjects. Immunohistochemical staining for HD5 showed intense staining in Paneth cell granules of normal weight subjects, whereas staining for this antimicrobial protein was substantially reduced in the jejunum of obese individuals (Figure 1A).

Next, to investigate whether the reduction in HD5 expression in obese subjects was a result of generalized Paneth cell compromise, we studied lysozyme levels, another highly expressed antimicrobial protein in Paneth cells. Similar to HD5, lysozyme was abundantly present in granules of Paneth cells of normal weight control subjects, while staining was strongly diminished in obesity (Figure 1B). Semi-quantitative analysis by western blot confirmed a reduced amount of lysozyme in the small intestine of obese patients compared with intestinal samples from normal weight subjects (Figure 1C; $p < 0.05$). A relationship between obesity and antimicrobial protein expression in the gut of obese subjects was demonstrated by an inverse correlation between BMI and lysozyme protein levels (Figure 1D; $r_s = -0.42$, $p < 0.05$).

Quantification of Paneth cells revealed equal numbers in the jejunum of both study groups, excluding the possibility that the observed decrease in antimicrobial protein expression in Paneth cells was caused by reduced cell numbers (Figure 2). Taken together, these data indicate that antimicrobial protein levels are reduced in the Paneth cells of obese subjects.

Enhanced expression of Paneth cell antimicrobial genes in obesity

To investigate whether the reduced antimicrobial protein levels in obese subjects were due to reduced gene expression, we quantified the mRNA expression of HD5 (*DEFA5*) and lysozyme (*LYZ*). Surprisingly, qPCR data showed 2.6-fold increased expression of HD5 (Figure 3A; $p < 0.01$) and 1.6-fold increased expression of lysozyme (Figure 3B; $p = 0.15$) in the jejunal tissue of obese subjects compared with normal weight controls. Since the discrepancy seen in protein expression and mRNA expression of HD5 and lysozyme could point towards a translational arrest due to ER stress, we next assessed activation of the UPR.

Activation of the UPR and indications of ER stress in the small intestine of obese subjects

ER stress arises from circumstances resulting in the accumulation of misfolded or unfolded proteins in the ER. Upon ER stress, the UPR is activated, which is aimed at restoring ER homeostasis. To investigate the putative involvement of ER stress in the reduced

Figure 1. HD5 and lysozyme levels are decreased in the Paneth cells of obese subjects. (A) Immunohistochemistry for HD5 in the jejunum of normal weight (upper panel) versus obese (lower panel) subjects showed strongly reduced staining in the Paneth cells of obese subjects. (B) Immunohistochemistry for lysozyme in the jejunum of normal weight (upper panel) versus obese subjects (lower panel) showed markedly reduced expression in the Paneth cells from obese subjects. (C) Western blot analysis revealed a significant decrease in the jejunal levels of lysozyme in obese subjects compared with normal weight subjects, confirming the staining results ($p < 0.05$). β -Actin was used to assess equal protein loading. (D) Jejunal lysozyme content was inversely correlated with BMI ($r_s = -0.42$, $p < 0.05$). The histology shown is representative for all tissue samples studied (original magnification 200 $\times$).

Figure 2. Equal Paneth cell numbers in the jejunum of obese and normal weight subjects. (A) H&E staining demonstrated equal numbers of Paneth cells in the jejunal crypts of normal weight and obese subjects. (B) Quantification of the number of Paneth cells present in the jejunum of obese subjects showed no difference in Paneth cell count compared with control tissues. The histology shown is representative for all tissue samples studied (original magnification 200 $\times$).

antimicrobial protein expression by Paneth cells in obesity, activation of the protein kinase RNA-like endoplasmic reticulum kinase (PERK) pathway was assessed. This branch of the UPR is responsible for a translational arrest which reduces protein load to the ER and thus alleviates ER stress [12]. We first studied binding protein (BiP), an important player in the initiation and maintenance of the UPR [13]. BiP levels

were significantly increased in jejunal samples of obese patients compared with normal weight subjects, signifying activation of the UPR (Figures 4A and 4B; $p < 0.05$). A correlation between BMI and BiP levels was found and further indicated a relationship between obesity and activation of the UPR in the gut (Figure 4B, right panel; $r_s = 0.45$, $p < 0.05$). In addition, protein levels of activating transcription factor 4 (ATF4), which

Figure 3. Increased HD5 and lysozyme gene expression in the jejunum of obese subjects. (A) qPCR analysis showed a 2.6-fold increase in HD5 (*DEFA5*) mRNA ($p < 0.01$) and (B) a 1.6-fold increase in the levels of lysozyme (*LYZ*) mRNA ($p = 0.15$, ns).

induces transcription of downstream mediators in the PERK pathway, were also significantly increased in obese subjects (Figures 4A and 4C; $p < 0.05$). ATF4 protein levels correlated with BMI as well, providing additional evidence for a relationship between obesity and UPR activation (Figure 4C, right panel; $r_s = 0.41$, $p < 0.05$). We next analyzed the protein expression of growth arrest and DNA damage-inducible protein 34 (GADD34), a downstream target of ATF4 that provides a negative feedback loop to the PERK pathway to reinitiate protein translation. GADD34 levels were similar in both study groups (Figures 4A and 4D). ER compromise was studied using EM to assess the contribution of Paneth cells to the results obtained by western blot on UPR activation. Indeed, Paneth cells of obese subjects showed an enlarged ER with vacuoles (Figure 5B), indicative of ER stress, whereas a normally structured ER was found in Paneth cells of normal weight subjects (Figure 5A).

Importantly, involvement of the UPR in the lowered antimicrobial protein levels in obese subjects was strongly suggested by a negative correlation between BiP levels and lysozyme levels (Figure 6; $r_s = -0.39$, $p < 0.05$).

The age- and gender-differences between the study groups do not confound the results on UPR activation and Paneth cell products, as assessed by multivariate analysis ($p > 0.05$ for all variables tested).

All in all, these results suggest that activation of the UPR contributes to the diminished expression of antimicrobial proteins in the jejunum of obese individuals.

Discussion

Over the past decade, the influence of Paneth cells on controlling intestinal microbiota composition and limiting bacterial translocation has become increasingly clear [6,10,14]. Intestinal microbiota on its part has been shown to play an important role in obesity. In this study, we provide the first evidence for altered Paneth cell properties in human obesity. We show decreased levels of the crucial Paneth cell antimicrobials HD5 and lysozyme in the jejunum of obese subjects compared

with normal weight subjects. In addition, we provide new insight into the mechanisms that could account for this phenomenon showing activation of the UPR in the gut of obese subjects for the first time.

Changes in α -defensin levels, as also described in Crohn's disease [8], have been shown to influence the composition of the intestinal microbiota in mice [6,8]. A reduction in α -defensin levels has been shown to result in a higher percentage of *Firmicutes* and a lower percentage of *Bacteroidetes* in the small intestine [6]. Moreover, Paneth cell-produced antimicrobial proteins were proven to act in the colon [11]. Interestingly, a similar *Firmicutes*–*Bacteroidetes* shift has been observed in the colon of obese subjects [5]. The decreased HD5 expression in the gut of obese subjects that we observed could therefore very well explain the reported shift in bacterial composition. However, the composition of the intestinal microbiota in our study groups could not be investigated since the clinical study from which the tissue samples were derived did not include faecal samples and this remains subject for future studies. Alternatively, it should be noted that changes in the expression of antimicrobial peptides could occur secondary to alterations in the composition of the intestinal microbiota.

Since changes in the gut microbiota have been implicated in the obesity-associated increase in intestinal permeability and metabolic endotoxaemia, we hypothesize that disturbed Paneth cell function may underlie these phenomena [15,16]. Furthermore, increased intestinal permeability in obesity has been suggested to lead to enterogenous endotoxaemia contributing to non-alcoholic steatohepatitis [17–20].

In addition to the change in microbiota composition, a generalized Paneth cell malfunction might contribute to these obesity-related complications in other ways. For example, Paneth cells prevent bacterial translocation in the healthy intestine and act as a second line of defence in limiting bacterial translocation in situations of physical intestinal barrier loss [10,21]. An overall reduction in antimicrobial protein production by Paneth cells might therefore play an important role in facilitating bacterial translocation, as has been suggested to occur in obesity [15,22,23], thereby adding to the role of an increased intestinal permeability.

Figure 4. The UPR is activated in the jejunum of obese subjects. (A) Western blot analysis showed increased band density for BiP and ATF4 in obese samples (representative bands of three subjects per group). GADD34 levels did not differ between the study groups. Quantification of western blots demonstrated a significant ($p < 0.05$) up-regulation of both BiP (B, left panel) and ATF4 (C, left panel). No difference in GADD34 expression was observed (D). Both BiP (B, right panel) and ATF4 (C, right panel) concentrations correlated with BMI ($r_s = 0.45$, $p < 0.05$ and $r_s = 0.41$, $p < 0.05$, respectively).

Figure 5. Expansion of the ER indicates ER stress in the Paneth cells of obese subjects. (A) Representative EM images of Paneth cells in the jejunum of normal weight subjects revealed a normally structured ER (arrowheads), while the ER in the Paneth cells of obese subjects displayed vacuoles (V) and was expanded (B), indicative of ER stress. L = lumen; N = nucleus; G = granule.

Figure 6. Activation of the UPR is associated with a reduction in lysozyme expression in the gut of obese subjects. BiP protein levels were correlated inversely with lysozyme protein levels ($r_s = -0.39$, $p < 0.05$).

The diminished antimicrobial protein expression and increased mRNA levels of their corresponding genes could be explained by a Paneth cell-depleting hypersecretory response to obesity. However, this seems improbable considering that during a chronic steady state, both enhanced mRNA and protein levels are expected. We therefore hypothesized the discordance between antimicrobial protein expression and mRNA expression in the Paneth cells of obese subjects to be a consequence of a translational block caused by ER stress. Paneth cells have been shown to be susceptible to ER stress, due to their highly secretory nature [24]. In addition, excessive nutrient intake, increased need for protein synthesis, and excess lipid accumulation associated with obesity are known to be chronic stimuli in causing ER stress [25–27]. The latter fact could, via its influence on intestinal microbiota, result in a vicious circle. In this regard, the UPR has been shown to be activated in both the liver and adipose tissue of obese subjects. Alternatively, it could be envisaged that the altered microbiota is causative for ER stress resulting in reduced Paneth cell antimicrobial expression. Here, we provide the first evidence for UPR activation in the gut of obese subjects. We show that obese subjects display increased protein levels of BiP, an important initiator of the UPR pathway, and of ATF4, a player in the PERK axis of the UPR, which is required for the induction of a translational

block resulting in overall reduced protein synthesis [12]. It should be noted that goblet cells might contribute to these results since they are also susceptible to ER stress, although to a lesser extent than Paneth cells [24]. In addition, using EM, we observed indications of ER stress in the Paneth cells of obese subjects. Although we show clear activation of the UPR in the gut of obese subjects, downstream elements in the UPR pathway, including CHOP (data not shown) and GADD34, were unaltered. In support of our findings, it has been shown that adaptation to chronic ER stress induces persistent expression of BiP and other proteins concerned with alleviating protein folding stress, whereas the expression of both pro-apoptotic CHOP and GADD34 is unchanged [28]. These findings represent a fascinating aspect of the UPR: the ability to either facilitate adaptation to stress or induce apoptosis, depending on the nature and severity of the stressor. In obesity, a chronic condition, cells chronically exposed to ER stress must survive and adapt. Recent studies have provided insight into the mechanisms by which cells translate acute or chronic stress signals from the ER into a life-or-death response [28,29]. The involvement of such mechanisms in the gut of obese subjects is suggested by our findings that upstream players of the PERK pathway, constituting an adaptive response concerned with alleviating protein folding stress, are up-regulated, while pro-apoptotic CHOP and GADD34 are unchanged.

Our results, indicating a relationship between activation of the UPR in the small intestine and altered Paneth cell function, are supported by Kaser *et al*, who showed diminished antimicrobial protein secretion by Paneth cells in a mouse model displaying ER stress [24].

In conclusion, our findings provide important new insight into the involvement of Paneth cells in obesity. We identified these cells as a conceivable host factor responsible for the shift in microbiota composition accountable for many obesity-associated disorders. In addition, we show for the first time activation of the UPR in the intestine of obese subjects and indications of ER stress in Paneth cells, which may cause the observed Paneth cell compromise. Further studies on the role of altered Paneth cell function in relation to

the obesity-associated intestinal microbiota shift are warranted.

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Author contribution statement

The authors contributed in the following way: CMH, JG, SSR, WAB, JWG, and KL: study concept and design; FJV, JG, CHCD, and JWG: collection of clinical samples; CMH and FKV: acquisition of data; CMH, FJV, JG, SSR, FKV, WAB, and KL: analysis and interpretation of data; CH: drafting of the manuscript; FJV, JG, SSR, CHCD, WAB, JWG, and KL: critical revision of the manuscript for important intellectual content.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Supplementary materials and methods.

Table S1. Primers used for qPCR assays.