EBM

Experimental Biology and Medicine

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Experimental Biology and Medicine 2013, 238:90-97.
doi: 10.1258/ebm.2012.012232

Original Research

Intestinal and renal guanylin peptides system in hypertensive obese mice

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Abstract

Guanylin (GN), uroguanylin (UGN) and the GC-C receptor have been associated with two endocrine axes: the salt and water homeostasis regulating enterorenal axis and the recently described appetite-regulating UGN/GC-C extraintestinal axis. The present work assessed the mRNA expression levels of GN peptides system (GPS) in a model of diet-induced obesity. Male C57BL/6J mice were submitted to either a high-fat high-simple carbohydrate diet (obese) or a normal diet (control). The renal and intestinal GN, UGN and GC-C receptor mRNA expression were evaluated by reverse transcriptase quantitative polymerase chain reaction in both groups, during normo-saline (NS) and high-saline (HS) diet. The diet-induced obesity was accompanied by glucose intolerance and insulin resistance as well as by a significant increase in blood pressure. During NS diet, obese mice presented reduced mRNA expression of GN in ileum and colon, UGN in duodenum, ileum and colon and GC-C in duodenum, jejunum, ileum and colon. This was accompanied by increased UGN mRNA expression in renal cortex. During HS diet, obese mice presented reduced mRNA expression of GN in jejunum as well as reduced mRNA expression of UGN and GC-C in duodenum, jejunum and colon. The data obtained suggest that, in a mouse model of diet-induced obesity, a down-regulation of intestinal mRNA expression of GN, UGN and its GC-C receptor is accompanied by a compensatory increase of renal UGN mRNA expression. We hypothesize that the decrease in gene expression levels of intestinal GPS may contribute to the development of hypertension and obesity during hypercaloric diet intake.

Keywords: GC-C receptor, guanylin, sodium load, obesity, uroguanylin

Experimental Biology and Medicine 2013; **238**: 90–97. DOI: 10.1258/ebm.2012.012232

Introduction

Currently, more than 1.6 billion adults worldwide are overweight and over 400 million are obese.[1,2] Obesity is associated to an increased incidence of hypertension, diabetes and metabolic syndrome.[3,4] However, the pathophysiological link between obesity and hypertension is still not fully understood.

Guanylin (GN, also named GUCA2A) and uroguanylin (UGN, also named GUCA2B) are closely related peptides, regulating electrolyte and water transport in intestinal and renal epithelia.[5] GN and UGN act via activation of guanylyl cyclase C (GC-C, also named GUCY2C) receptor in the intestine and other GC-C like receptors in the kidney.[6] The gastrointestinal tract is the main source of GN and UGN and contains the highest levels of its respective mRNAs and mature proteins among all tissues.[7] Small intestine has abundant UGN mRNAs levels whereas the large intestine is rich in GN mRNAs.[8] The circulating GN peptides can have origin, besides the intestine, in the kidneys and other tissues like adrenal glands, reproductive system, lung and pancreas.[9–15] The hypothesis that UGN could serve as an enterorenal endocrine axis connecting the gastrointestinal tract to the kidney for regulation of sodium excretion was suggested by the fact that oral administration of sodium induces a natriuresis that greatly exceeds the increase in urine sodium excretion elicited by intravenous NaCl infusion.[16–18] Also, mice deficient in UGN have blunted urinary sodium excretion responses to oral sodium loads but are able to excrete sodium normally after intravenous administration of NaCl.[19]

More recently, it was suggested by Valentino *et al.*[20] that GN peptides system (GPS) could also be involved in satiation control. The presence of an appetite-regulating UGN/GC-C extraintestinal endocrine axis was suggested in GC-C knockout mice, which presented disrupted satiation resulting in hyperphagia and subsequent obesity and metabolic syndrome.[20] In this context, the GPS may represent an excellent target to further explore the link between obesity and hypertension.

Given the above stated, the aim of the present work was to evaluate renal and intestinal GPS mRNA expression in an animal model of obesity, induced by ingestion of high-caloric diet during conditions of normal and high sodium oral intake.

Material and methods

Animal model

Animal experiments were performed in accordance with the European Directive number 86/609, transposed to the Portuguese Law by DL 129/92 and by Portaria 1005/92 and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.[21] Five-week-old male C57BL/6J mice were purchased from Charles River (Barcelona, Spain). Animals were kept in a controlled environment under 12:12 h light–dark cycle, at a room temperature of $22 \pm 2^{\circ}C$. At six weeks of age C57BL/6J mice were randomly assigned for 12 weeks to either a normal diet (Teklad LM-485 Mouse/Rat Sterilizable Diet; Madison, WI, USA), with 4.1 kcal/g composed of 5% fat, 54% carbohydrate (ground corn), 19% protein, 5% fiber, 0.31% sodium and 0.85% potassium – control mice, or a high-fat and high-simple carbohydrate diet (HFHSC; Diet F2685; BioServ, Frenchtown, NJ, USA), with 5.4 kcal/g composed of 35% fat (lard), 35% simple carbohydrate (sucrose), 20% protein, 0.1% fiber, 0.39% sodium and 0.56% potassium – obese mice. The animals had free access to tap water and were fed *ad libitum* throughout the study. During this period, daily quantification of body weight, water and caloric ingestion was performed. After 12 weeks, non-invasive blood pressure was recorded and glucose tolerance and insulin resistance tests were performed (see below).

Twelve weeks after diet initiation, the animals were housed in metabolic cages (Techniplast, Buguggiate-VA, Italy) under controlled environmental conditions for quantification of fluid intake and for collection of 24 h urine. During this period, the normal or HFHSC diets were also given to control or obese mice, respectively. After one day of habituation (day 0), the control and obese animals were subdivided: one group of animals were maintained receiving tap water (normal salt intake, NS) whereas the other group of animals received 1.0% (w/v) NaCl in the drinking water (high salt intake, HS), during three days (day 1, 2 and 3). Urine volume was gravimetrically determined. The daily sodium intake in normal and high sodium diets averaged 1 and 3 mmol, respectively.

On the day of sacrifice, 72 h after normal or high-saline oral diet, the animals were anaesthetised with sodium pentobarbital (50 mg kg/body weight; intraperitoneally). Blood was collected directly from the heart in tubes containing lithium/heparin for later determination of plasma biochemical parameters. The intestine and the kidneys were rapidly removed after sacrifice through an abdominal midline incision. The intestine was flushed with cold saline solution (0.9% NaCl) and the following segments were collected: duodenum, jejunum, ileum and colon. The kidneys were weighed, rinsed free from blood with saline solution, decapsulated and cut in half. Fragments of duodenum, jejunum, ileum, colon and renal cortex were immersed in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany) and stored at $-80^{\circ}C$.

Blood pressure measurements

Blood pressure (systolic and diastolic) and heart rate were measured weekly in conscious restrained animals, using a photoelectric tail-cuff pulse detector (LE 5000, Letica, Barcelona, Spain). From 08:00 to 10:00, mice were placed into a semi-cylindrical container with controlled temperature (36–37°C). Before measurements, animals were conditioned to the restrainers by repeating inflation–deflation cycles for 15 min. At 12 weeks, a minimum of five reliable systolic blood pressure measurements were obtained for each animal and the average calculated.

Glucose tolerance and insulin resistance tests

Glucose tolerance and insulin resistance tests were carried out 12 weeks after diet initiation. A glucose tolerance test was performed after a 14 h fast through a single intraperitoneal injection of dextrose (1.0 g/kg). Blood glucose concentration was measured before and 15, 30, 45, 60, 90, 120 and 150 min after injection using a blood glucose meter (Freestyle Mini™ system, Abbott Diabetes, Mississauga, Canada). Insulin resistance was tested after a six hours fast through a single intraperitoneal injection of insulin, 1.5 U/kg (Actrapid® Novo Nordisk, Bargsvaerd, Denmark). Blood glucose measurements were made before and 15, 30, 45, 60 and 90 min after injection.

Ionogram and biochemistry

Plasma cholesterol and triglycerides as well as plasma and urine levels of sodium, creatinine, urea and proteins were evaluated with an automatic analyser, Cobas Mira Plus analyser (ABX Diagnostics, Montpellier, France), according to ABX Diagnostic reagents conditions. Creatinine clearance and fractional excretion of sodium were calculated as previously described.[22]

Serum insulin was quantified by enzyme-linked immunosorbent assay, according to the manufacturer's instructions (Linco Research, St Charles, MO, USA).

Quantitative real-time polymerase chain reaction

Total RNA was isolated from tissues using trizol RNA extraction method, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The purity of the

sample and the RNA concentration was assayed by spectrophotometry. To evaluate the integrity, the RNA was separated by electrophoresis on a 1% agarose gel (SeaKem® LE Agarose; Lonza, Cologne, Germany), and then visualized with NucliStain (National Diagnostics, Atlanta, GA, USA). Reverse transcription was performed in a standard thermocycler (MyCycler, Bio-Rad, Hercules, CA, USA) with the iScript cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's instructions.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[23] PCR reactions using specific primers were performed using SYBR green chemistry (Bio-Rad) in the MiniOpticon thermocycler (Bio-Rad). For each gene and tissue studied, standard curves of graded dilutions from a randomly selected control sample were generated for determination of qPCR efficiency. β-Actin was used as the reference gene. Primers used for each gene analysed were referenced in other studies [18,19] or designed with the Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences used in this study were the following: 5′ TGAGTTGGAGGAGAAGGAGATGTC 3′ and 5′ AAGGGCAAGGCTGGGTTATG 3′ for amplifying UGN; 5′ GAGTGACATCGCTTGCCTTTC 3′ and 5′ TGAGTTTGTTAGCCTCGTGACTTC 3′ for amplifying GN; 5′ TGGAAGAACCACAAGCTCCCCA 3′ and 5′ AGCACGAGGAGGGCAATCAGC 3′ for amplifying GC-C; and 5′ GCCCCTGAGGAGCACCCTGT 3′ and 5′ TGGCTACGTACATGGCTGGGGT 3′ for amplifying β-actin.

For each set of primers, a no-template control and a no-reverse amplification control were included. Reaction specificities were verified by melting curve analysis. Data acquisition and analysis was performed using the CFX manager 2.0 (Bio-Rad). Fold changes in gene expression were determined using ΔΔCq method.[23]

Statistics

Results are presented as mean values ± SEM. Data were analysed using Microsoft Excel 2010 (Redmond, WA, USA) and GraphPad Prism 5 (La Jolla, CA, USA) software. Student's *t*-test or one-way analysis of variance followed by Tukey's test were applied when appropriate. A *P* value of less than 0.05 was assumed to denote a significant difference.

Table 1 Body weight and metabolic balance in control and obese mice

	Control	Obese
Body weight (g)	25.05 ± 0.39	37.39 ± 2.16*
Plasma glucose (mg/dL)	141.17 ± 7.99	167 .00 ± 7.99*
Plasma insulin (ng/mL)	0.62 ± 0.04	1.02 ± 0.16*
Plasma cholesterol (mg/dL)	74.50 ± 2.63	83 .00 ± 2.52*
Plasma triglycerides (mg/dL)	24.46 ± 2.05	35.54 ± 1.81*
Plasma urea (mg/dL)	60.36 ± 3.17	40.86 ± 2.01*
Urine urea (mg/24 h)	117.33 ± 7.89	65.27 ± 7.29*
Plasma protein (g/L)	32.90 ± 1.27	32.76 ± 0.88
Urine protein (mg/24 h)	14.64 ± 2.94	15.57 ± 2.22

Values are mean ± SEM
*Significantly different from control mice ($P < 0.05$)

Results

Metabolic studies

After 12 weeks of exposure to HFHSC diet, obese mice presented significantly increased body weight, plasma cholesterol and triglycerides, as well as hyperglycemia and hyperinsulinemia (Table 1). This was accompanied by glucose intolerance and insulin resistance (Supplementary Figure S1; please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.012232/-/DC1). Also, plasma and urinary urea were significantly reduced in obese mice in comparison to controls (Table 1). No significant differences were observed between groups in plasma and urinary levels of proteins and sodium, in fractional excretion of sodium, creatinine clearance and kidney weight (Tables 1 and 2). In addition, obese mice presented a significant increase in systolic and diastolic blood pressure (Figure 1).

The daily urinary sodium excretion was evaluated during a three-day period of HS intake (Table 3). During the first 24 h of HS diet, the obese mice presented urinary sodium excretion lower than control mice (HS day 1). However, during the remaining two days of HS diet, the urinary sodium excretion was similar between control and obese mice (HS day 2 and HS day 3). In comparison to mice subjected to NS oral diet, control and obese mice presented in the last day of HS diet no differences in creatinine clearance, but presented a significant increase in urinary sodium levels and in fractional excretion of sodium (Table 2).

GN, UGN and GC-C receptor mRNA expression

In duodenum (Figure 2) a significant decrease of UGN and GC-C mRNA expression was observed in obese mice when compared with controls during either NS or HS diet. Regarding GN mRNA levels, a non-significant

Table 2 Renal function and sodium handling in control and obese mice, after normo-saline (NS) or high-saline (HS) diet

	NS		HS	
	Control	Obese	Control	Obese
Plasma Na^+ (mmol/L)	138.2 ± 2.7	138.6 ± 2.7	141.8 ± 2.3	138.0 ± 1.4
Urine Na^+ (mmol/24 h)	0.18 ± 0.04	0.16 ± 0.03	0.59 ± 0.12*	0.58 ± 0.12*
FE_{Na^+} (%)	1.33 ± 0.36	0.92 ± 0.29	2.43 ± 0.28*	2.69 ± 0.43*
Ccreat (ml/min)	0.08 ± 0.02	0.12 ± 0.03	0.12 ± 0.02	0.10 ± 0.01

Ccreat, creatinine clearance; FE, fractional excretion
*Significantly different from mice subjected to NS intake ($P < 0.05$)

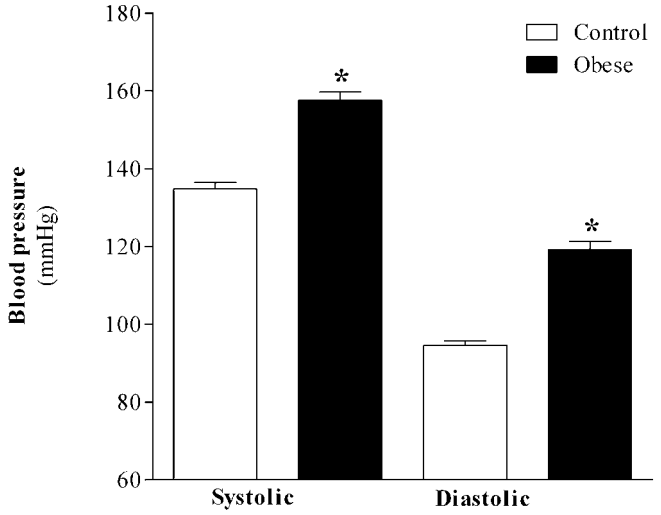

Figure 1 Systolic and diastolic blood pressure in control and obese mice. Values represent the mean ± SEM. *Significantly different from control mice ($P < 0.05$)

Table 3 Daily urinary levels of sodium (mmol/24 h) in control and obese mice, before (NS, day 0) or during high sodium intake (HS, days 1, 2 and 3)

	NS	HS		
	Day 0	Day 1	Day 2	Day 3
Control	0.18 ± 0.03	0.57 ± 0.05*	0.62 ± 0.08*	0.59 ± 0.11*
Obese	0.17 ± 0.03	0.41 ± 0.05*†	0.63 ± 0.13*	0.58 ± 0.12*

Values represent the mean ± SEM
*Significantly different from mice subject to NS intake ($P < 0.05$)
†Significantly different from control mice ($P < 0.05$)

reduction was also observed in obese mice during both NS and HS diet. Sodium load increased GC-C mRNA expression in obese mice but did not alter GN or UGN gene expression in either control or obese mice. In jejunum (Figure 3), a significant decrease of GC-C gene expression was observed in obese mice when compared with the control, during either NS or HS intake. This was accompanied by a decrease in both GN and UGN gene expression in obese mice during HS diet but not during NS intake. Sodium load did not alter GC-C or GN gene expression in control and obese mice, but increased UGN mRNA expression in control mice. In ileum (Figure 4), a significant decrease of GN, UGN and GC-C mRNA expression was observed in obese mice when compared with controls during NS conditions. Sodium load increased mRNA expression of GN, UGN and GC-C in obese mice.

In colon (Figure 5), a significant decrease of GC-C and GN mRNA expression was observed in obese mice when compared with controls, during either NS or HS conditions. UGN mRNA expression only showed a significant decrease in obese mice subjected to NS diet. Sodium load did not alter mRNA expression of GC-C, GN and UGN in either control or obese mice. In renal cortex (Figure 6), GN mRNA expression did not differ between control and obese mice, during either NS or HS diet. On the other hand, UGN mRNA expression was increased in renal

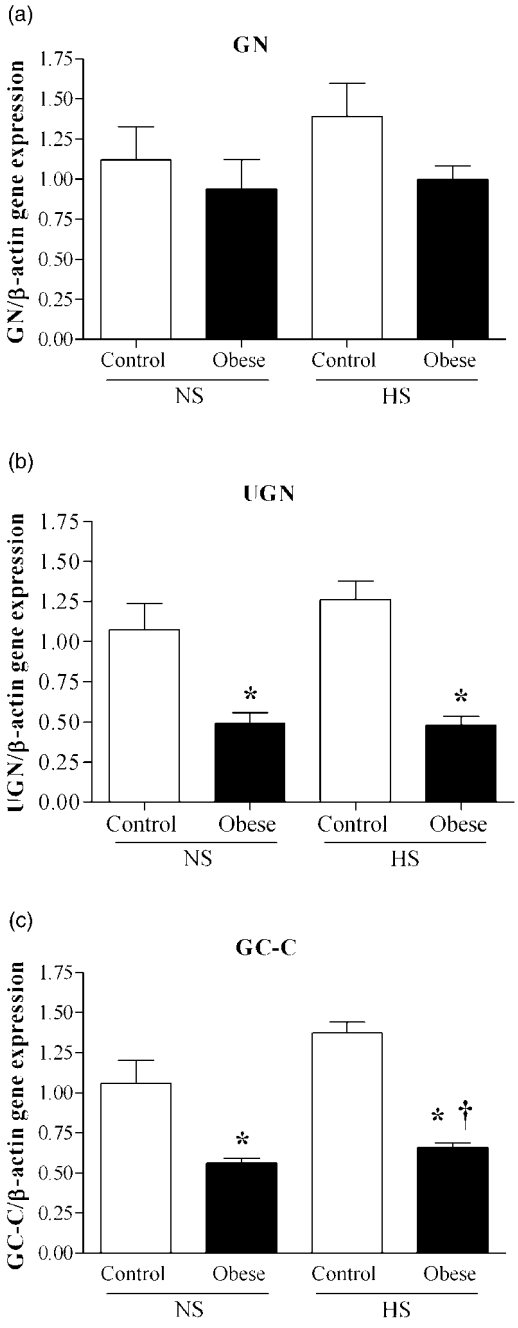

Figure 2 GN (a), UGN (b) and GC-C (c) gene expression in duodenum from control and obese mice, subject to normal (NS) or high-saline (HS) oral intake. Values represent the mean ± SEM. GN, guanylin; UGN, uroguanylin. *Significantly different from control mice ($P < 0.05$). †Significantly different from mice subjected to NS intake ($P < 0.05$)

cortex from obese mice when compared with controls during NS diet whereas during HS diet differences between control and obese mice did not achieve statistical significance. Sodium load did not alter mRNA expression of GN and UGN in either control or obese mice. GC-C mRNA expression was not detected in renal cortex.

Discussion

The exposure of C57BL/6J mice to HFHSC diet for 12 weeks resulted in the development of obesity, hyperglycemia,

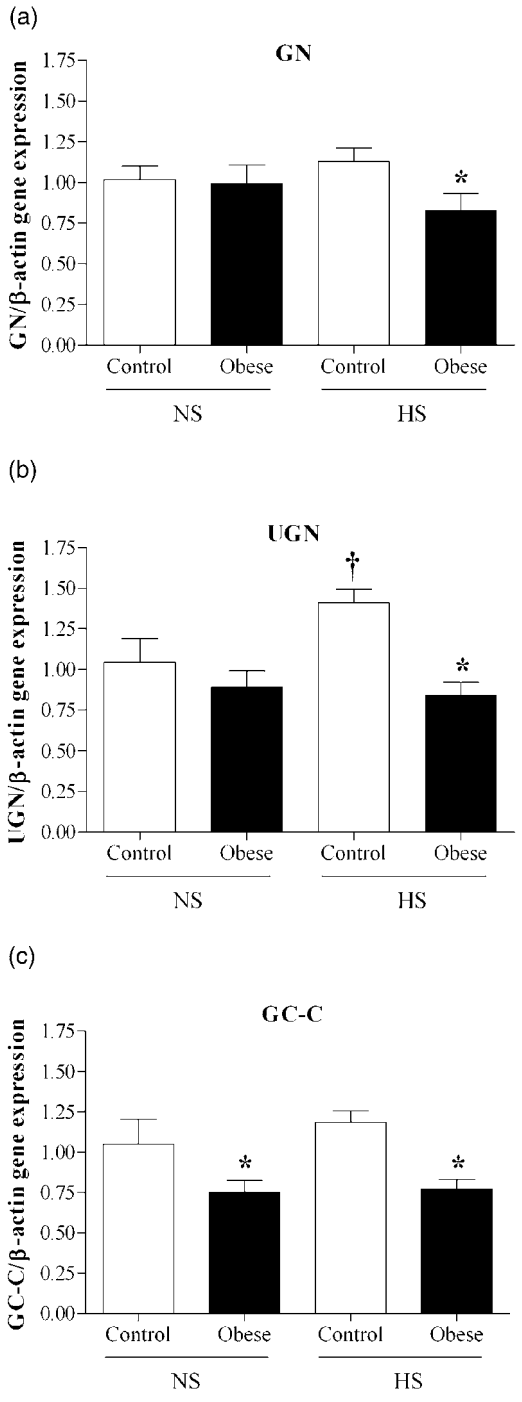

Figure 3 GN (a), UGN (b) and GC-C (c) gene expression in jejunum from control and obese mice, subject to normal (NS) or high-saline (HS) oral intake. Values represent the mean ± SEM. GN, guanylin; UGN, uroguanylin. *Significantly different from control mice ($P < 0.05$). †Significantly different from mice subjected to NS intake ($P < 0.05$)

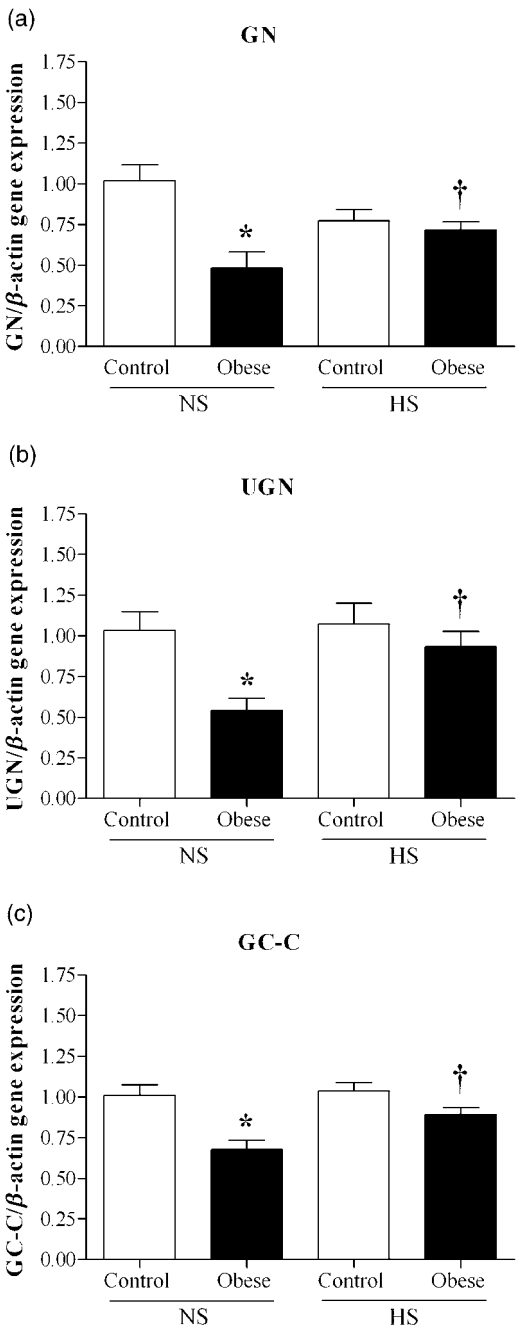

Figure 4 GN (a), UGN (b) and GC-C (c) gene expression in ileum from control and obese mice, subject to normal (NS) or high-saline (HS) oral intake. Values represent the mean ± SEM. GN, guanylin; UGN, uroguanylin. *Significantly different from control mice ($P < 0.05$). †Significantly different from mice subjected to NS intake ($P < 0.05$)

glucose intolerance, insulin resistance, hyperinsulinemia and hypertension, as already described by others.[24–27] In addition, the hypercaloric diet induced a down-regulation of intestinal GPS mRNA expression. The blunted intestinal GPS mRNA expression in obese mice is due not only to a reduced gene expression of GN and UGN peptides, but also to a reduced gene expression of its GC-C receptor. The importance of the GC-C receptor in intestinal fluid secretion was evidenced in compromised GC-C transgenic mice that presented a reduction of intestinal fluid secretion.[28,29] In the present study, obese mice presented difficulties in handling an oral sodium load, reflected by a reduced urinary sodium excretion during the first day of HS intake. Therefore, one can hypothesize that the decrease in the gene expression levels of intestinal GPS may contribute to increase intestinal sodium absorption, thus contributing for the development of sodium-sensitive hypertension in diet-induced obesity.

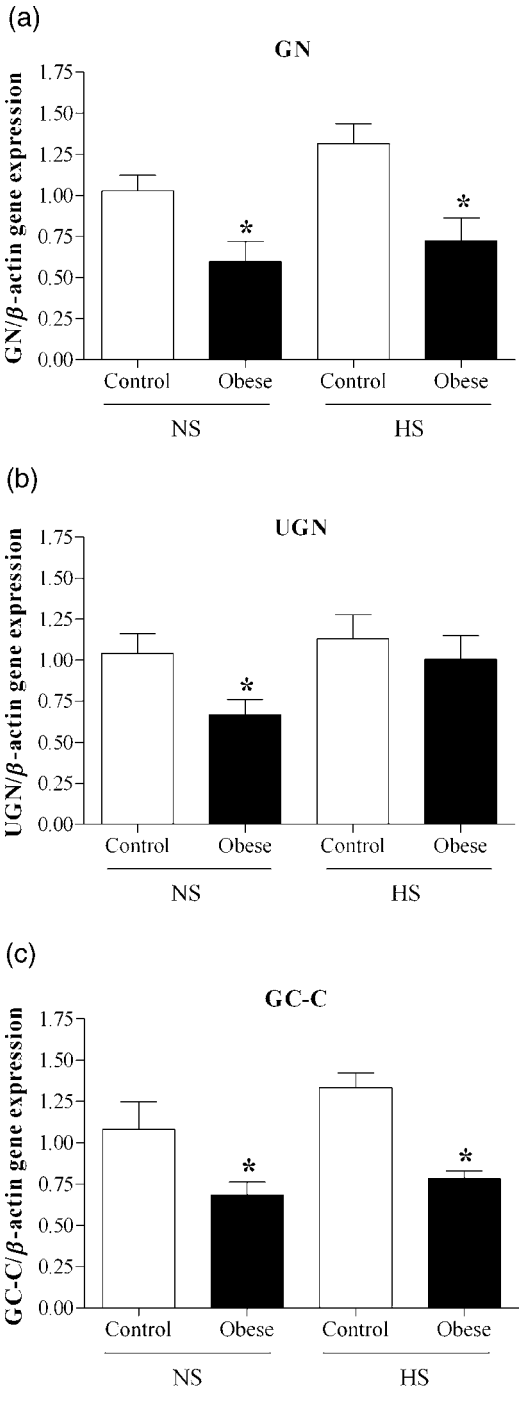

Figure 5 GN (a), UGN (b) and GC-C (c) gene expression in colon from control and obese mice, subject to normal (NS) or high-saline (HS) oral intake. Values represent the mean ± SEM. GN, guanylin; UGN, uroguanylin. *Significantly different from control mice ($P < 0.05$)

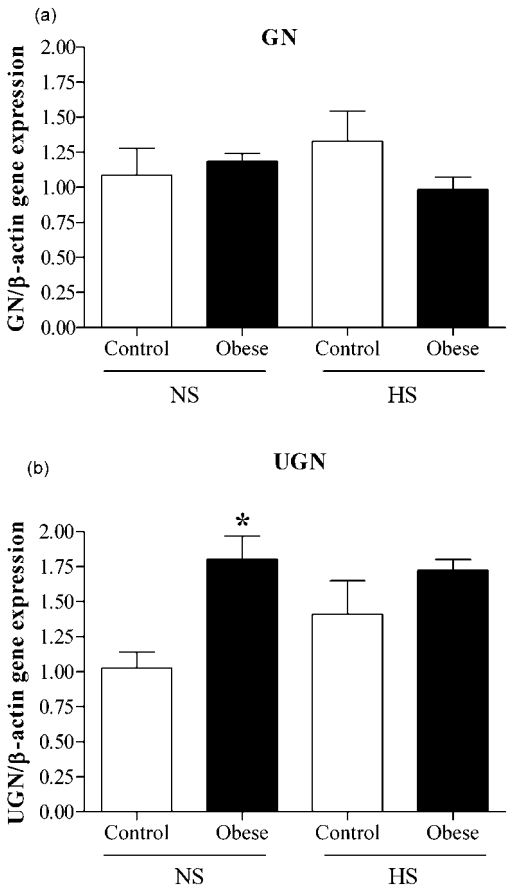

Figure 6 GN (a) and UGN (b) gene expression in renal cortex from control and obese mice, subject to normal (NS) or high-saline (HS) oral intake. Values represent the mean ± SEM. GN, guanylin; UGN, uroguanylin. *Significantly different from control mice ($P < 0.05$)

In the kidney, the increase of UGN gene expression in obese mice during normal salt intake, suggests a renal compensatory response to the reduced intestinal production of GN peptides, which supports the occurrence of complementary functions between the intestine and the kidney with GPS playing a role in renal–intestinal cross-talk. Interestingly, although the enhanced renal UGN gene expression in obese mice may contribute to sodium excretion, these animals presented an elevated blood pressure. The particular contribution of the renal GPS, when viewed together with all the natriuretic systems of the kidney, may be minor. In fact, the activation of other systems like the sympathetic nervous and renin–angiotensin–aldosterone systems appear to play a major role in sodium and water retention in obese individuals.[3] In agreement with this view, are studies showing that a reduced natriuretic response to an oral salt load is observed in subjects with heart failure despite the elevated plasma proGN and proUGN and the increased urinary bioactivity of UGN presented by these patients.[30,31] In the kidney, GC-C receptor mRNA was not detected, which is consistent with previous studies and supports the well-established hypothesis that UGN signaling in the kidney involves a different pathway and/or a receptor that is distinct from GC-C.[6,32–35]

In an interesting study, Carrithers *et al.*[36] reported that the administration of a sodium load (7%) to Sprague–Dawley rats during four days slightly increased UGN mRNA expression in the jejunum and markedly increased GN mRNA expression in duodenum and jejunum. In our study, the oral sodium load (1%) administered to control mice increased UGN mRNA expression in the jejunum, but did not alter GN mRNA expression. The different results obtained in these two studies may be related with the different sodium loads administered to the animals

as well as with the different animals's species used. Our study also reports that regarding obese mice, sodium load increased GC-C mRNA expression in the duodenum, and GC-C, GN and UGN mRNA expression in ileum. Nevertheless, these data demonstrate that variations in sodium intake lead to intestinal segment-specific changes in GPS mRNA expression, as previously suggested.[36] In addition, the absence of response of renal GPS to a high sodium load reported in our study fits well to what was reported by Carrithers *et al.*[36] but is not in agreement to what was described by Potthast *et al.*[32] They found that mice receiving a sodium load (1% saline) in drinking water presented increased renal UGN mRNA expression.[32] The existence of controversial results regarding the effect of HS intake on the regulation of GPS justifies the need to clarify the effect of oral sodium intake on peptide synthesis and/or secretion, and the resultant changes in intestinal and renal transport of sodium. Recently, an interaction between the atrial natriuretic peptide and the GN peptides was reported in isolated perfused rat kidney; this interaction may play a role in the regulation of kidney function in other pathophysiological states, such as in the natriuresis following ingestion of salty meals.[37]

Valentino *et al.*[20] suggested that silencing of GC-C in mice disrupts satiation, resulting in hyperphagia and subsequent obesity and metabolic syndrome. Given this, one can hypothesize that the reduced GPS mRNA expression in intestine may contribute to the development of obesity in these hypercaloric fed mice. Specifically, the reduced UGN mRNA expression in these obese mice could lead to a reduced proUGN secretion into circulation, resulting in a reduced activation of GC-C in the hypothalamus. The down-regulation of intestinal GPS could then reduce satiation and increase appetite behaving as an amplificatory mechanism for obesity induction and maintenance.

The altered GPS gene expression in obese mice presented in this study may not directly translate in expression/activity changes of the corresponding proteins. Given that these peptides suffer a strong post-transcriptional regulation, the parallel evaluation of protein expression of GN peptides in renal and intestinal tissues is critical to fully understand this system.[38]

The mechanisms responsible for the observed changes in GPS mRNA expression in obese mice are not clarified in the present study. It is likely that they could be directly related to the diet, obesity or other mechanisms also altered in these mice. Given that GC-C is also the receptor for diarrheagenic bacterial enterotoxins,[39–41] one possible hypothesis is the modulation of GPS by the gut microbiota. In accordance, several studies described that diet alterations in mice lead to changes in gut microbiota;[42–44] and it was demonstrated in germ-free mice that, besides modulating energy harvest, the gut intestinal microbiota may also influence gene expression, host's metabolic homeostasis and inflammatory signaling pathways.[45–50] Nevertheless, other regulatory mechanisms controlling GPS gene expression cannot be ruled out.

In conclusion, the data obtained suggest that in a mouse model of diet-induced obesity a down-regulation of intestinal GPS mRNA expression is accompanied by a compensatory increase of renal UGN mRNA expression. The blunted intestinal GPS in this mouse model is due not only to a reduced gene expression of GN and UGN peptides, but also to a reduced gene expression of its GC-C receptor. We hypothesize that the decrease in gene expression levels of intestinal GPS may contribute to the development of hypertension and obesity during hypercaloric diet intake.

Author contributions: LS-S, MP, IS-S and BS-M participated in the experimental design; all authors participated in interpretation of the studies and analysis of the data and review of the manuscript; LS-S, MM-R, JQ-S, CF-C and IS-S conducted the experiments; LS-S, IS-S and BS-M wrote the manuscript.

ACKNOWLEDGEMENTS

We acknowledge the contribution to this work of Odília Queirós, Augusta Vieira-Coelho and Eduardo Lopes (Bio-Rad). BS-M was supported by the fellowship SFRH/BPD/21782/2005, MM-R by the fellowship SFRH/BPD/64703/2009 and JQ-S by the fellowship SFRH/BD/39066/2007 from Fundação para a Ciência e Tecnologia/FEDER.

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(Received June 23, 2012, Accepted October 2, 2012)