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Mechanisms underlying hypertriglyceridemia in rats with monosodium L-glutamate-induced obesity: Evidence of XBP-1/PDI/MTP axis activation

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is intimately associated with insulin resistance and hypertriglyceridemia, whereas many of the mechanisms underlying this association are still poorly understood. In the present study, we investigated the relationship between microsomal triglyceride transfer protein (MTP) and markers of endoplasmic reticulum (ER) stress in the liver of rats subjected to neonatal monosodium L-glutamate (MSG)-induced obesity. At age 120 days old, the MSG-obese animals exhibited hyperglycemia, hypertriglyceridemia, insulin resistance, and liver steatosis, while the control (CTR) group did not. Analysis using fast protein liquid chromatography of the serum lipoproteins revealed that the triacylglycerol content of the very low-density lipoprotein (VLDL) particles was twice as high in the MSG animals compared with the CTR animals. The expression of ER stress markers, GRP76 and GRP94, was increased in the MSG rats, promoting a higher expression of X-box binding protein 1 (XBP-1), protein disulfide isomerase (PDI), and MTP. As the XBP-1/PDI/MTP axis has been suggested to represent a significant lipogenic mechanism in the liver response to ER stress, our data indicate that hypertriglyceridemia and liver steatosis occurring in the MSG rats are associated with increased MTP expression.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), considered to represent the liver manifestation of the metabolic syndrome, occurs when the fatty acid liver uptake rate and/or *de novo* synthesis is greater than its oxidation and excretion as very low-density lipoprotein (VLDL) [1]. Hypertriglyceridemia is one of the main complications of type 2 *diabetes mellitus* (2DM) and the main link between liver insulin resistance and the development of NAFLD [2]. Insulin resistance increases the liver lipid production, which together with hyperglycemia induces transactivation of the transcription factors that activate the liver *de novo* synthesis of lipids [1].

Recent studies have suggested that the microsomal triglyceride transfer protein (MTP) and transcription factor *forkhead box O1* (FoxO1) participate in the aforementioned process [1,3]. MTP is an endoplasmic reticulum (ER) resident protein that participates in the insertion of triacylglycerols (TAGs) into nascent apolipoprotein B (ApoB), thus forming VLDL particles [4]. FoxO1 is a transcription factor that intermediates the inhibitory action of insulin on gene expression by way of Akt/PKB-dependent phosphorylation. In individuals with insulin resistance (IR), FoxO1 phosphorylation is reduced, resulting in the increased synthesis of MTP mRNA in hepatocytes and the increased export of VLDL into the circulation [3]. Despite this seemingly well-established mechanism, the expression of MTP varies among the various NAFLD experimental models. The liver expression of MTP was observed to be reduced in FLS (inbred fatty liver Shionogi) mice [5], increased in *ob*/*ob* mice [6], and unchanged in streptozotocin-induced diabetic mice [6]. In humans, reduced liver MTP expression is common to the various fatty liver diseases [1,7,8]. In addition to FoxO1, growth hormone (GH) is also a relevant MTP regulator, as it stimulates the assembly and secretion of VLDL particles [9,10].

The administration of monosodium L-glutamate (MSG) to newborn rodents is associated with hypothalamic nuclei injury, particularly of the arcuate nucleus, resulting in reduced production of

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GH releasing-hormone (GHRH) by the hypothalamus [11], GH by the pituitary gland, and insulin-like growth factor 1 (IGF-1) by the liver [12]. Upon reaching adulthood, these animals exhibit glucose intolerance, insulin resistance, hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and visceral obesity [13]. In addition, the liver fat content also increases in these animals, which is associated with histological changes similar to the ones observed in human NAFLD [14].

It might appear contradictory for MSG animals to exhibit hypertriglyceridemia, as the liver MTP expression ought to be decreased because of the lower GH availability. However, some other regulatory pathways, mainly the ones related to IR and ER stress, might actually be effective regulators of VLDL production in that animal model. Therefore, in the present study, we sought to assess the liver MTP expression and activity in rats with MSG-induced obesity and to correlate these values with the presence of dyslipidemia, IR, and ER stress.

2. Materials and methods

2.1. Neonatal induction and development of obesity

Male Wistar rat (*Rattus norvegicus)* pups were given MSG (MSG, Sigma Aldrich, USA) at a dose of 4.0 g/kg/day per subcutaneous route on alternate days during the first 10 days of life. The control (CTR) animals were given a similar volume of equiosmolar saline solution. Following weaning, the animals were weighed twice per week to assess their weight gain and feed efficiency. During the entire observation period, the animals were kept at a temperature of 23 ± 2 °C, a 12-h light/dark period, and given water and food *ad libitum*.

The animals from both groups were weighed and measured at age 30, 60, 90, and 120 days old to assess the progression of obesity using the Lee Index (LI), which is calculated by dividing the cube root of the body weight (g) by the nasal-anal length (NAL) (cm) [15]. After overnight fasting, on day 120, the animals were anesthetized (10 mg/kg xylazine, 40 mg/kg ketamine) and subjected to laparotomy to collect blood through a puncture of the abdominal aorta. The periepididymal and retroperitoneal fat pads and liver were collected and weighed for morphometric assessment. All of the protocols were approved by the animal ethics and experimentation commission of the State University of Maranhão, ruling No. 001/2009.

2.2. Serum biochemistry assessment

The blood samples were coagulated and centrifuged to separate the serum. The serum total cholesterol (TC), triacylglycerols (TAG), free fatty acids (FFA), glucose (GLC), and liver transaminase (aspartate – AST – and alanine – ALT – transaminases) levels were analyzed using spectrophotometry with the assistance of commercial kits following the manufacturer's (Labtest, Brazil) instructions.

2.3. Assessment of insulin resistance

IR was inferred from the calculation of the TyG index (*Ln*[*fasting triglycerides* (mg/dL) × *fasting glucose* (mg/dL)/2]), as previously described [16,17].

2.4. Chromatographic analysis of serum lipoproteins

The serum lipoproteins were separated using *fast protein liquid chromatography* (*FPLC*) in a Superose column 6 HR 10/30 (Amersham Biosciences, Sweden) under the constant flow of Tris buffer at 0.5 ml/min (pH 7.0; 10 mmol Tris, 150 mmol NaCl, 1 M EDTA, and 0.03% NaN_3). A total of 60 fractions were collected automatically and designated as follows: 1–15 as VLDL, 16–30 as low-density lipoprotein (LDL), 31–45 as high-density lipoprotein (HDL), and 46–60 as plasma proteins. The TC and TAG concentrations in the fractions were measured as described above (2.2), and the total protein content was measured by absorbance at 280 nm [18].

2.5. Extraction and quantification of liver lipids

The liver samples were homogenized in 1 M NaCl (100 mg/500 μL). The homogenate was extracted with 3 mL of chloroform/methanol (2:1) solution and 0.5 mL of 1 M NaCl. The organic phase was collected and left to dry for the measurement of the total fat content (mg/g of tissue). The total fat was resuspended in 0.5 mL of Triton X/methanol (2:1) for measurement of TC and TAG, as described above [19].

2.6. Histological analysis of the liver tissue

The liver samples were imbibed in 10% formaldehyde buffered solution, mounted in paraffin blocks, cut in 5-μm sections, and stained with hematoxylin-eosin (HE). Specimens were scored according to the NAFLD/NASH activity score [20], for the degree of steatosis (0–3), lobular inflammation (0–3) and hepatocellular ballooning (0–2) by two researchers in an independent blind manner.

2.7. Determination of protein expression by western blotting

The liver samples were homogenized using sonication in lysis buffer containing protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, and 10 mM PMSF). In total, 30 μg of total protein was diluted in the sample buffer, subjected to SDS–PAGE, and transferred to nitrocellulose membranes. The detection of specific proteins was performed using incubation with anti-PDI (Enzo Life Sciences, USA), anti-MTP (Bristol-Myers-Squibb, USA), anti-KDEL (Enzo Life Sciences, USA), and anti-XBP1 (Enzo Life Sciences, USA) primary antibodies, then with peroxidase-conjugated secondary antibodies, and developed using the chemiluminescence method (peroxidase-H_2O_2-luminol).

2.8. Statistical analysis

The CTR and MSG groups were compared using Student's *t*-test. The significance level was established as $p < 0.05$. The results were expressed as the mean ± standard error of the mean and analyzed using the software Prism 5 (GraphPad, USA).

3. Results and discussion

3.1. Neonatal administration of MSG induces obesity associated with insulin resistance

The neonatal administration of MSG induced obesity and dyslipidemia in the treated rats. The data listed in Table 1 demonstrate that at age 120 days old, the LI values were greater in the MSG group compared with the CTR group, although the weight of this group was 23% lower. Although the food intake did not differ between the groups, the MSG group exhibited intense deposition of both visceral (periepididymal pad) and non-visceral (retroperitoneal pad) fat. The MSG animals exhibit vagal hyperactivity and sympatho-adrenal hypoactivity, resulting in hyperinsulinemia and an increase of the white adipose tissue [21,22]. The reduction of GH in MSG animals [11,12], in this case exhibited by the striking

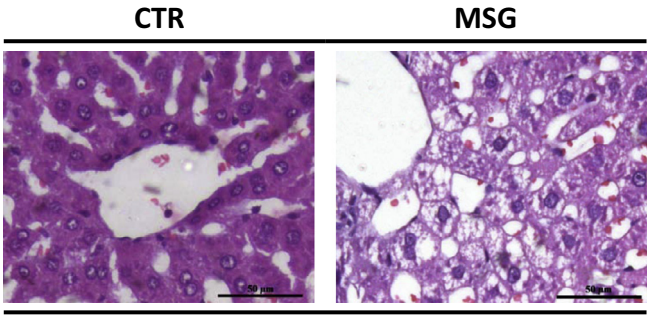
Table 1
Morphophysiological and biochemical parameters of control and MSG-obese rats.

	CTR	MSG	p
Morphophysiological parameters			
Food intake (g/100 g bw/day)	7.3 ± 0.8	7.3 ± 0.4	ns
Body weight (g)	366.1 ± 10.6	283.0 ± 12.8	0.0001
Nasal-anal length (cm)	23.7 ± 0.2	20.08 ± 0.3	<0.0001
Lee index ($g^{1/3}$/cm)	301.5 ± 1.9	330.6 ± 4.1	<0.0001
Feed efficiency [intake (g)/ Δbw (g)]	1.16 ± 0.04	0.76 ± 0.04	<0.0001
Retroperitoneal fat (g/ 100 g bw)	0.74 ± 0.11	2.53 ± 0.09	<0.0001
Periepididymal fat (g/ 100 g bw)	0.78 ± 0.008	2.99 ± 0.23	<0.0001
Liver (g/100 g bw)	2.34 ± 0.02	2.18 ± 0.02	0.0002
Serum biochemical parameters			
Free fatty acids (mmol/L)	0.77 ± 0.02	1.23 ± 0.14	0.0029
Total cholesterol (mg/dL)	43.38 ± 3.64	69.44 ± 6.82	0.0039
Triacylglycerols (mg/dL)	24.32 ± 2.56	83.61 ± 12.99	0.0004
Glucose (mg/dL)	67.21 ± 5.39	118.70 ± 15.09	0.0054
TyG index	3.66 ± 0.08	4.55 ± 0.07	<0.0001
AST (IU/L)	51.29 ± 4.13	80.44 ± 10.22	0.0157
ALT (IU/L)	27.17 ± 2.92	42.90 ± 5.47	0.0207
Liver biochemical parameters			
Total fat (mg/g liver)	194.40 ± 15.23	247.70 ± 13.82	0.0196
Total cholesterol (mg/g liver)	4.08 ± 0.12	3.87 ± 0.19	ns
Triacylglycerols (mg/g liver)	8.61 ± 0.36	12.97 ± 1.26	0.0043

Data are mean ± SEM (n = 7), compared by Student's *t*-test.
ns = non-significant, bw = body weight.
TyG index = ln([fasting triacylglycerols] × [fasting glucose]/2).

NAL reduction, impairs the development of visceral tissues and is associated with the reduction of feed efficiency and body weight (Table 1) [23].

IR is the main factor linking the various comorbidities included in the metabolic syndrome together [1]. In the present study, IR was assessed through the calculation of the TyG index, which has been described as a sensitive and specific method for the assessment of insulin sensitivity [16,17]. The TyG value was 25% higher in the MSG group compared with the CTR group (Table 1). The accumulation of visceral adipose tissue is the initial cause of IR, mainly as a function of its high lipolysis rate. Under such conditions, a large amount of FFA is released into the circulation, with the consequent activation of serine kinases (JNK and IKKß) via protein kinase C [24]. The serum FFA levels were 60% higher in the MSG group compared with the CTR group (Table 1). In addition, the MSG rats exhibited massive dyslipidemia, with significant elevation of the serum lipids and fasting blood glucose. Moreover, the serum TAG levels were more than threefold higher in the MSG animals compared with the CTR animals. These results, together with the remainder of the findings described above, confirm MSG-induced obesity as a model for metabolic syndrome.



	CTR	MSG	*p*
NAFLD/NASH scale			
Steatosis	0 ± 0	1.25 ± 0.25	0.0008
Lobular Inflammation	0 ± 0	0.25 ± 0.25	ns
Hepatocellular ballooning	0 ± 0	1.25 ± 0.25	0.0008
Total Score	0 ± 0	2.75 ± 0.75	0.0042

Fig. 1. Histopathological appearance of liver in MSG-obese rats. Representative HE stained liver sections from 120 days old CTR and MSG rats are shown in upper panel (bar = 50μm). Specimens were scored according to the NAFLD/NASH activity score, as described under materials and methods. Data are expressed as mean ± SEM (n = 4–5) and compared by Student's *t*-test. ns: non-significant.

3.2. MSG-obese rats exhibit early non-alcoholic fatty liver disease associated with increased VLDL synthesis and export

Histological analysis of the CTR and MSG livers according to the NAFLD/NASH activity scale [20] revealed grade 1 fatty degeneration in the MSG animals, which is characterized by microvacuolar steatosis and ballooning, without significant

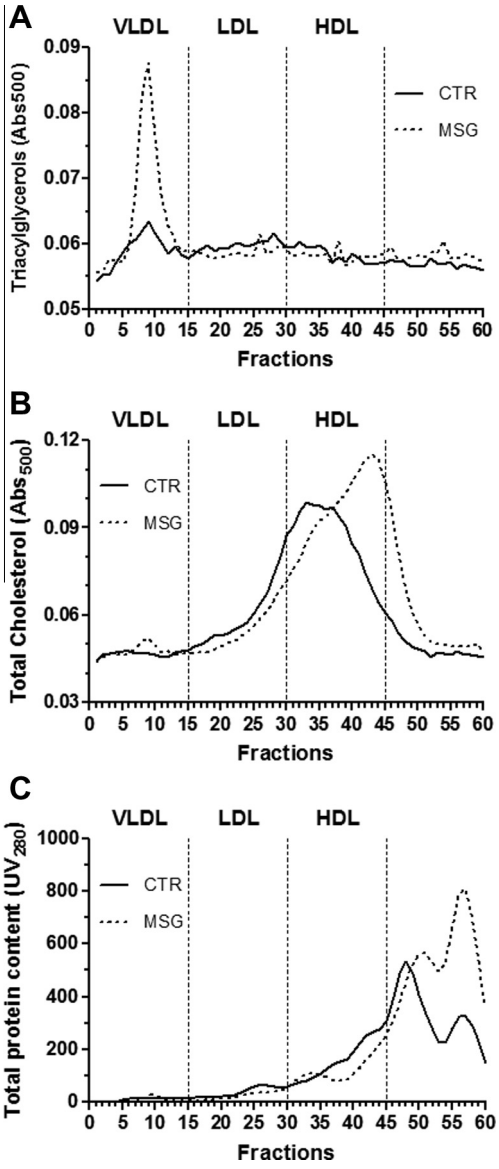


Fig. 2. Serum lipoprotein profile in MSG-obese rats. Serum samples from 120 days old CTR and MSG rats (n = 6) were separated into 60 FPLC-derived fractions, which were assayed for determination of triacylglycerol (A) and total cholesterol (B) levels in VLDL, LDL, and HDL particles at 500 nm. Total protein content (C) in each fraction was detected at UV 280 nm.

inflammatory infiltration (Fig. 1), while the CTR animals exhibited no changes whatsoever. The activity of the liver enzymes AST and ALT was almost twice as high in the MSG group compared with the CTR group (Table 1). The activity of these enzymes bears a positive correlation with the risk factors for metabolic syndrome and NAFLD [25]. The liver total fat content was approximately 25% higher in the MSG group compared with the CTR group, with a 50% increase in TAG and no difference in the TC level (Table 1). These findings, together with the serum lipid profile point to hypertriglyceridemia as the main liver lipid metabolism disorder in MSG animals.

To characterize the TAG synthesis and export more thoroughly, the serum lipoprotein profile was analyzed using FPLC. The TAG content in the VLDL particles was twice as high in the MSG group compared with the CTR group (Fig. 2A), which denotes a remarkable increase in liver VLDL synthesis. However, the TAG content did not increase in LDL and HDL particles (Fig. 2A). The TC content in the VLDL particles exhibited a 75% increase in the MSG animals (Fig. 2B). Although the ApoB levels were not measured directly, the total protein quantification at 280 nm is suggestive of increased VLDL particle protein content in the MSG group (Fig. 2C). Such an increase in the VLDL particle synthesis rate appears to arise from greater MTP expression and/or activity due to IR [2]. In addition, hypertriglyceridemia itself is a relevant inducer of ER stress in hepatocytes, whose activation increases the liver IR [26].

3.3. The liver expression of endoplasmic reticulum stress markers is increased in MSG-obese rats

The ER homeostasis is maintained by a complex signaling system known as the *unfolded protein response* (UPR), which includes three cell stress sensors: PKR-like ER kinase (PERK), activating transcription factor – 6 (ATF6), and inositol requiring ER-to-nucleus signal kinase – 1alpha (IRE1α). Under homeostatic conditions, the activity of these sensor proteins is inhibited by their binding to a chaperone 78-kDa glucose-regulated protein (GRP78). However, when ER stress occurs, the proteins unbind from GRP78, which is thus dislocated to provide support to the unfolded proteins, while the sensor ones undergo autophosphorylation, consequently activating their mechanisms of gene expression regulation [26,27]. In addition, GRP94 also participates in the detection of unfolded proteins and contributes to the proper protein folding [28]. To assess GRPs expression, we applied an anti-KDEL antibody, which react with ER-retaining KDEL motif on those chaperones. The liver expression of GRP78 and GRP94 was increased in the MSG group

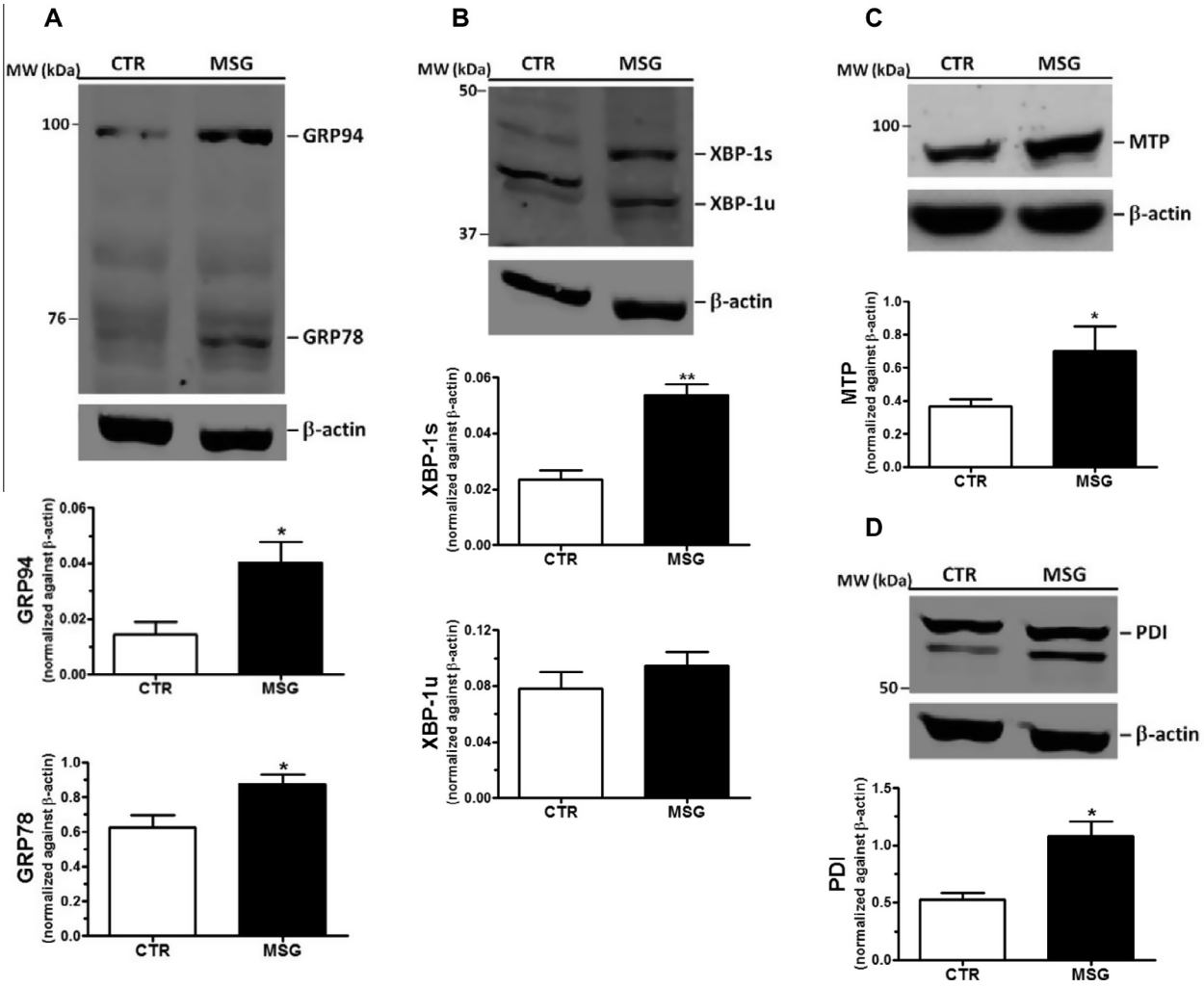


Fig. 3. Endoplasmic reticulum stress-related protein expression in the liver of MSG-obese rats. Liver samples from CTR and MSG rats were subjected to SDS–PAGE and western blotting to assess the expression of (A) 78-kDa (GRP78) and 94-kDa (GRP94) glucose-regulated proteins, (B) unspliced and spliced X-box binding protein 1 (XBP-1u and XBP-1s, respectively), (C) microsomal triglyceride transfer protein (MTP), and (D) protein disulfide isomerase (PDI). Data are expressed as mean ± SEM (n = 3–4) and compared by Student's t-test.*$p < 0.05$ and **$p < 0.01$ versus CTR.

compared with CTR (Fig. 3A), which denotes the occurrence of adaptive response to ER stress.

3.4. ER stress activates the XBP-1/PDI/MTP axis in MSG-obese rats

Recent data suggest that X-box binding protein 1 (XBP-1) is one of the main factors linking ER stress and lipid biosynthesis [29]. When ER stress occurs, IRE1α promotes alternative XBP-1 mRNA processing, involving the removal of a 26-nucleotide intron; this process results in the production of spliced XBP-1 (XBP-1s), which is a powerful gene transactivation factor, particularly relative to lipogenic genes [27]. The data depicted in Fig. 3B demonstrate that XBP-1s expression was 2.5 times higher in the MSG animals compared with the CTR animals. These findings corroborate previous reports indicating that XBP-1s expression is increased in obese rats with 2DM induced by a fat-rich diet and streptozotocin [30]. In addition, our data suggest that both the increase in the liver lipid accumulation and NAFLD observed in the MSG animals are possibly due to greater lipid biosynthesis resulting from the action of XBP-1s.

MSG rats are resistant to the action of insulin, which is favorable for greater MTP expression because of lesser FoxO1 phosphorylation [3]. The relationship between MTP expression and NAFLD is a matter of much controversy. In humans, liver steatosis bears a positive correlation with the reduction of MTP expression [7,8]. However, in rodents, MTP expression appears to be particularly regulated by the FFA supply to the hepatocytes [2]. Because in the present study, the FFA level was 60% higher in the MSG rats compared with the CTR rats (Table 1), their liver MTP expression was investigated. The results indicated that the liver MTP expression was twice as high in the MSG group compared with the CTR group (Fig. 3C). Such a remarkable increase in MTP expression appears to be related to the IR observed in MSG rats. In these animals, the liver FoxO1 gene expression was significantly increased compared with CTR (data not shown). Therefore, the loss of insulin sensitivity results in the reduction of the suppressive effect on the FoxO1 expression, which in turn promotes the expression of MTP.

MTP activity is maintained through the formation of a heterodimer with protein disulfide isomerase (PDI). PDI is a ubiquitous ER oxidoreductase chaperone that maintains the lipid-transferring activity of MTP in hepatocytes and enterocytes in addition to preventing aggregate formation [31]. The PDI catalytic activity was observed to not be essential for MTP–PDI complex formation, as site-directed mutations affecting both PDI functions did not interfere with either complex formation or MTP activity [32]. However, in a recently published study, Wang and colleagues [33] suggested that PDI is a novel target of XBP-1 regulatory action in hepatocytes. These authors demonstrated that XBP-1 overexpression promotes an increase in PDI expression and increases MTP activity without interfering with the latter's expression as such. The data depicted in Fig. 3D patently demonstrate that PDI expression was also increased in the MSG rats' livers, thus providing a relevant link between XBP-1 expression and increased MTP secretory activity, which here was indicated by the remarkable increase of the TAG content of the MSG rats' VLDL particles (Fig. 2A).

All together, the results of the present study suggest for the first time that activation of the XBP-1/PDI/MTP axis might be a relevant mechanism underlying the occurrence of hypertriglyceridemia and NAFLD in MSG-obese rats. Nevertheless, more thorough studies are needed, particularly aiming at characterizing the expression of the other UPR/ER stress sensor proteins, PERK and ATF6. In addition, the activation of that axis must also be investigated in younger animals, in which IR and ER stress are less developed.

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