Metabolism:

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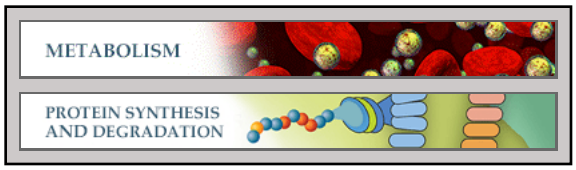

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Obesity Induces Hypothalamic Endoplasmic Reticulum Stress and Impairs Proopiomelanocortin (POMC) Post-translational Processing*

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Background: The α-MSH peptide is essential in regulating food intake and energy expenditure.
Results: ER stress induced by obesity reduces α-MSH, accumulates POMC, and decreases the enzyme PC2.
Conclusion: There is a direct link between obesity and ER stress, resulting in altered POMC processing.
Significance: These studies bring a new perspective to how ER stress can regulate energy balance by altering POMC processing.

It was shown previously that abnormal prohormone processing or inactive proconverting enzymes that are responsible for this processing cause profound obesity. Our laboratory demonstrated earlier that in the diet-induced obesity (DIO) state, the appetite-suppressing neuropeptide α-melanocyte-stimulating hormone (α-MSH) is reduced, yet the mRNA of its precursor protein proopiomelanocortin (POMC) remained unaltered. It was also shown that the DIO condition promotes the development of endoplasmic reticulum (ER) stress and leptin resistance. In the current study, using an *in vivo* model combined with *in vitro* experiments, we demonstrate that obesity-induced ER stress obstructs the post-translational processing of POMC by decreasing proconverting enzyme 2, which catalyzes the conversion of adrenocorticotropin to α-MSH, thereby decreasing α-MSH peptide production. This novel mechanism of ER stress affecting POMC processing in DIO highlights the importance of ER stress in regulating central energy balance in obesity.

Severe obesity is associated with cardiovascular disease and type-2 diabetes among other co-morbidities. Despite the great progress made in this field, our limited understanding of this condition has been disappointing. In the brain, the hypothalamic arcuate (ARC)[3] nucleus controls energy balance and body weight through the production of anorexigenic and orexigenic neuropeptides. The ARC-derived peptide, α-melanocyte stimulating hormone (α-MSH), is released by the central melanocortin system and plays a major anorexigenic role in the regulation of feeding and energy expenditure (1). α-MSH is generated from its prohormone, proopiomelanocortin (POMC), through a series of tightly regulated post-translational processing modifications. This process is conducted by two members of the family of prohormone convertases, prohormone convertase 1 and 2 (PC1 and PC2), and other processing enzymes while transported through the regulated secretory pathway (2, 3) and axon terminals. It has been shown in several landmark studies that abnormal processing for certain prohormones causes profound obesity. An early study showed that a patient with a compound heterozygous mutation in the PC1 gene resulted in the production of nonfunctional PC1 with severe childhood obesity (4). An analogous obese condition was found in a patient with a defect in POMC processing (5). This condition was reproduced in a mouse model of PC1 deficiency generated by random mutagenesis (6). Another example is the well known Cpe^{fat}/Cpe^{fat} mouse with a point mutation in the carboxyl peptidase (*cpe*) gene. This *fat*/*fat* mouse is obese, diabetic, and infertile (7, 8). Altogether, these previous studies clearly indicate that abnormal prohormone processing in particular prohormones causes obesity. Consistent with those observations, our previous studies (9) and studies presented here strongly indicate that during diet-induced obesity (DIO), there is a defect in POMC processing, including a decrease in the synthesis of the α-MSH peptide, yet DIO does not affect POMC mRNA. Along with the decrease in α-MSH, we observe a significant reduction in PC2, which catalyzes the conversion of adrenocorticotropin (ACTH) to α-MSH.

In addition to impaired prohormone processing, the DIO state is also associated with endoplasmic reticulum (ER) stress, which appears to regulate energy balance. When the ER becomes stressed because of an excessive accumulation of newly synthesized unfolded proteins, the unfolded protein response (UPR) is activated. These UPR pathways act in concert to increase ER content, expand the ER protein folding capacity, degrade misfolded proteins, and reduce the load of new pro-

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[3] The abbreviations used are: ARC, arcuate; icv, intracerebroventricular(ly); α-MSH, α-melanocyte-stimulating hormone; POMC, proopiomelanocortin; PC, prohormone convertase; DIO, diet-induced obesity; ER, endoplasmic reticulum; UPR, unfolded protein response; HFD, high fat diet; p-eIF2α and p-PERK, phosphorylated eIF2α and PERK, respectively; TUDCA, tauroursodeoxycholic acid; PBA, phenylbutyrate; HPRT, hypoxanthine phosphoribosyltransferase; NPY, neuropeptide Y; AgRP, Agouti-related peptide; PVN, paraventricular nucleus.

teins entering the ER, all of which is geared toward adaptation to resolve the protein folding defect. Early studies showed that obesity-induced ER stress impairs insulin biosynthesis (10) in pancreatic B cells by affecting proinsulin folding, processing, and insulin release. In addition, hypothalamic ER stress has been proposed as one of the possible mechanisms inducing leptin resistance and altered energy balance during obesity (11–14). In the current study, our *in vivo* and *in vitro* data strongly suggest that obesity-induced ER stress obstructs the post-translational processing of POMC and reduces α-MSH peptide production. In this study, we demonstrate a direct link between ER stress and POMC processing in the obese state, and we demonstrate that ER stress mediates high fat diet (HFD)-induced changes in α-MSH levels. Although changes in POMC gene expression are extremely important in regulating energy balance, our results underscore the importance of other cellular mechanisms, such as POMC post-translational processing, in regulating energy balance, because we demonstrate that α-MSH decreases in DIO and with ER stress, whereas POMC mRNA levels do not change in these two conditions.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant murine leptin was obtained from Dr. E. Parlow (NIDDK, National Institutes of Health, and the National Hormone and Pituitary Program, Torrance, CA). Tauroursodeoxycholic acid (TUDCA) and AG490 were from EMD Millipore. Anti-p-PERK, anti-p-eIF2α, PTP1B, pStat3, total Stat3, and SOCS3 antibodies were from Cell Signaling. CPE and β-actin were from Santa Cruz Biotechnology, Inc. Rabbit anti-ObRb was from Linco Research, Inc. Anti-PC1 and PC2 were a gift from Dr. Nabil G. Seidah (Institut de Recherches Cliniques de Montréal). Rabbit anti-ACTH anti-α-MSH antibody was developed in our laboratory (15, 16). Biotinylated goat anti-rabbit antibody was from Jackson ImmunoResearch Laboratories.

Animals—Male Sprague-Dawley rats (22 days old) from Charles River Laboratories were fed a regular diet (Purina Lab Chow, catalog no. 5001) or an HFD (Rodent Chow, catalog no. D12492, Research Diets) for 12 weeks. Regular diet provided 3.3 kcal/g energy (59.8% carbohydrate, 28.0% protein, and 12.1% fat). HFD provided 5.24 kcal/g energy (20.0% carbohydrate, 20.0% protein, and 60.0% fat). We began with 12 rats/group. However, some individuals on the HFD gain weight and fat at the same rate as their counterparts kept on the standard diet. These individuals are called DIO-resistant (17). DIO-resistant animals were excluded from our studies, and thus sample sizes for experiments ranged from eight to 10 individuals. We recently characterized hormonal and physiological characteristics of this rat DIO model (18). Food and water were available *ad libitum* unless otherwise indicated. Body weights were measured weekly. The Institutional Animal Care and Use Committee of Rhode Island Hospital/Brown University approved all of the experimental protocols and euthanasia procedures.

In Vivo Studies—Following each experiment, rats were euthanized by decapitation. Blood was collected for further analysis. The PVN (paraventricular nucleus) and ARC were collected by microdissection and subjected to the following: acid peptide extraction with 2 N acetic acid supplemented with a protease inhibitor mixture to measure peptides by HPLC and specific RIA; protein extraction with radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40) supplemented with protease inhibitor mixture for Western blot analysis; or RNA isolation with TRIzol reagent (Invitrogen) according to the manufacturer's instructions for real-time PCR analysis. Protein concentration was determined by the Bradford assay. RNA concentration was determined by spectrophotometry. RNA integrity was determined using nanochips.

To evaluate the response to fasting, lean and DIO rats were divided into two groups. One group was fed *ad libitum*, and the other was fasted for 48 h. We evaluated the response to central leptin administration in both groups of rats by stereotaxically implanting an icv cannula (Plastic One) 10 days before the experiment as described previously (19). The correct placement of the cannulas was verified by measurement of water intake in response to icv injection of angiotensin II (40 ng/rat). Fasted rats were given icv 4 μl of vehicle (artificial cerebrospinal fluid) alone or vehicle containing leptin (3.5 μg/rat). All injections were performed between 09:00 and 10:30. Animals were sacrificed 3 h later, and samples were processed as indicated above.

To inhibit leptin signaling, animals were given 10 μl icv of vehicle (13% DMSO in artificial cerebrospinal fluid) alone or containing AG-490 (Sigma-Aldrich; 54 nmol/rat) at 18:00. Animals were euthanized the following morning. To alleviate ER stress, animals were given TUDCA (Calbiochem-EMD Chemicals). TUDCA was diluted in saline, and the pH was adjusted to 7.4 using NaOH to avoid toxicity. TUDCA was then injected intraperitoneally (200 mg/kg) for 2 days. Animals were euthanized 6 h postinjection on the second day, and ARC samples were collected for RIA or Western blot analyses. Body weights of lean and DIO rats treated with and without TUDCA were taken immediately prior to the first TUDCA infusion and a second time immediately prior to sacrifice. The change in body weight due to TUDCA was measured as the percentage weight gain (g) from the first to the second measurement. Male rats (300 g) were used for tunicamycin or thapsigargin experiments. Rats received DMSO (vehicle), tunicamycin (10 μg), or thapsigargin (10 ng) in a total of 4 μl. Energy expenditure was measured using the Oxymax indirect calorimeter system (Columbus Instruments, Columbus, OH) in animals that were fasted overnight. For the thapsigargin experiment, oxygen consumption was measured up to 3 h postinjection. For the TUDCA experiment, oxygen consumption was measured overnight following injections.

In Vitro Studies—N43/5 cells were used because they express POMC and PC2, and these cells have been used as an *in vitro* system that mimics hypothalamic POMC neuronal physiology (20). We also used a POMC-positive corticotropic AtT20 cell line transfected with PC2 to measure both PC2 and α-MSH release. N43–5 and AtT20 cells were each cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. At 60–70% confluence, cells were washed twice with DMEM without serum, and then DMEM containing 1% fetal bovine serum was added. Some cells were pretreated with

sodium 4-phenylbutyrate (PBA) (20 mM) or salubrinal (7.5 μM) for 14 h, after which 0.1 μg/ml tunicamycin was added for 0, 1, 2, 3, or 4 h, or thapsigargin (100 nM) was added for 6 h. At the end of the treatment period, cells were lysed with HEPES/KOH buffer.

Luciferase Assays—N43/5 cells were allowed to adhere overnight in 96-well plates. Cells were transfected using Lipofectamine 2000 with the human PC2-luciferase promoter construct from the human PC2 promoter (789 bp to −1 bp relative to the translation initiation codon) inserted in the pGL2-Basic vector (21). Cells were incubated with both PC2 promoter (0.15 μg) and a *Renilla* control (pRL-SV40, 0.15 μg; Promega) for 6 h in antibiotic-free, serum-free medium, and then the medium was changed to complete DMEM for overnight incubation. Cells were then treated with tunicamycin (0.1 μg/ml) or vehicle control. Luminescence of luciferase and *Renilla* were determined using the Dual-glo luciferase assay system (Promega), and luminescence was read with the TopCount NXT microplate luminescence counter (PerkinElmer Life Sciences).

Quantification of POMC mRNA by Real-time Quantitative PCR—Real-time quantitative PCR primers were designed using Primer3 and GenBank™ sequence data for rat POMC (accession number BC058443) and hypoxanthine phosphoribosyltransferase (HPRT) (accession number NM012583). Primers were synthesized and purified by Integrated DNA Technologies (Coralville, IA). The primer sequences used were as follows: upstream POMC, 5′- AGGACCTCACCACGGAAAG-3′; downstream POMC, 5′-ACTTCCGGGGATTTTCAGTC-3′; upstream HPRT, 5′- GCAGACTTTGCTTTCCTTGG-3′; downstream HPRT, 5′-GTCTGGCCTGTATCCAACACT-3′. Sample cDNAs were generated from total RNA using random hexamer primers and SuperScript III reverse transcriptase (Invitrogen). Each 25-μl real-time quantitative PCR used 100 ng of cDNA as template and contained 200 nM POMC or HPRT primers and ABI SYBR Green PCR core reagents (Applied Biosystems Inc.). All reactions were performed in triplicate on an ABI 7500 fast real-time PCR system. Standard curves for POMC and HPRT 1 transcript levels were generated with ABI 7500 Fast System SDS Software version 1.3.1 using diluted cDNA (100, 50, 10, 1.0, and 0.1 ng) from fed animal controls. Averaged POMC levels normalized to HPRT1 in fast and fast plus leptin-treated animals were compared with similar values obtained from fed animal controls to determine relative expression levels in both tissue types (22).

HPLC Fractionation and RIA Analysis—HPLC fractionation and RIA analysis of ACTH and α-MSH have been described previously by our laboratory (16). Briefly, samples were fractionated using a Varian ProStar Gradient HPLC system equipped with a C18 reverse phase column (Microsorb MV 300-5; Varian). Following peptide elution, samples were evaporated and reconstituted in RIA buffer (0.5 ml of phosphate buffer (pH 7.4), 500 mg/liter sodium azide, 2.5 g/liter BSA). Synthetic peptides were injected on the HPLC to determine retention times. Predicted retention times allowed for the analysis of specific regions along the gradient for RIA. The α-MSH RIA was performed using our in-house primary anti-α-MSH antiserum (1:20,000) and 5000 cpm of ^{125}I-desacetyl α-MSH tracer. The sensitivity of the assays was ~11.5 pg/tube, and the intra- and interassay variability were ~5–7% and 10–11%, respectively. The α-MSH assay used in this condition can detect 100% of acetyl and desacetyl α-MSH forms. The cross-reactivity of the α-MSH assay with ACTH is 22%; however, because there is a small amount of this peptide in the samples (see results), ACTH is not found in the HPLC profile using this assay. The ACTH RIA was performed using our in-house anti-ACTH antiserum (1:30,000) and 5000 cpm of ^{125}I-ACTH tracer. The sensitivity of the assays was ~10.0 pg/tube, and the intra- and interassay variability were ~5–7% and 10–11%, respectively. The ACTH assay used in this assay does not cross-react with any form of α-MSH. It is important to note that both assays cross-react with the POMC precursor, as was demonstrated using a purified POMC (kindly donated by Dr. Anne White, University of Manchester). However, because the prohormone presented some degree of degradation, we could not determine the percentage of cross-reactivity. Fasting glucose and insulin were measured using a rat insulin RIA kit from Millipore (catalog no. RI-13K) and the Quanti Chrom glucose assay kit from Bioassay Systems (catalog no. DIGL-100).

Western Blot Analysis—For Western blot analysis, 75 μg of total ARC or PVN lysate was separated on either 6% (p-PERK), 12% (p-eIF2α) or 10% (all other proteins) SDS-PAGE. Precision Plus Protein standards were used as molecular weight markers (Bio-Rad). After electrophoresis, proteins were electroblotted onto PVDF membranes for immunodetection and blocked with PBS in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4). Membranes were probed overnight at 4 °C with the anti-ObRb (1:100) or anti-SOCS3 (1:500), p-eIF2α (1:1000), p-PERK (1:500), PTP1B (1:1000), PC1 (1:10,000), and PC2 (1:10,000) antibodies. For POMC identification by Western blot, we used a combination of anti-ACTH and anti-α-MSH antibodies custom-made in our laboratory. These antibodies have been characterized previously (23), and together they detect the POMC precursor (31 kDa). Membranes were incubated with an HRP-linked secondary antibody (1:5000), and immunoreactive bands were visualized by enhanced chemiluminescence.

Statistical Analysis—Results are presented as the mean ± S.E. Statistical significance was determined by analysis of variance followed by post hoc Tukey's test using GraphPad Prism (version 4.0b). Differences were considered to be significant at $p < 0.05$.

RESULTS

The α-MSH Peptide Is Lower in DIO Rodents, whereas POMC mRNA Remains Unchanged—To conduct these studies, we used the rat model because of the following. 1) Because rats share with humans many characteristics of obesity physiology (24), they are considered to be an excellent model to study obesity physiology (17, 25, 26). 2) We have extensively characterized this model for energy balance studies as well as gene and peptide analyses, including prohormone processing (16, 18, 19, 27–29). 3) POMC processing was established in the rat (30–33), and we showed for the first time the changes in POMC processing in the rat ARC under nutritional changes (16). 4) Rats provide a larger source of material for peptide analysis compared with mice. 5) Most importantly, the POMC sequence in the rat is different from that the mouse, making it

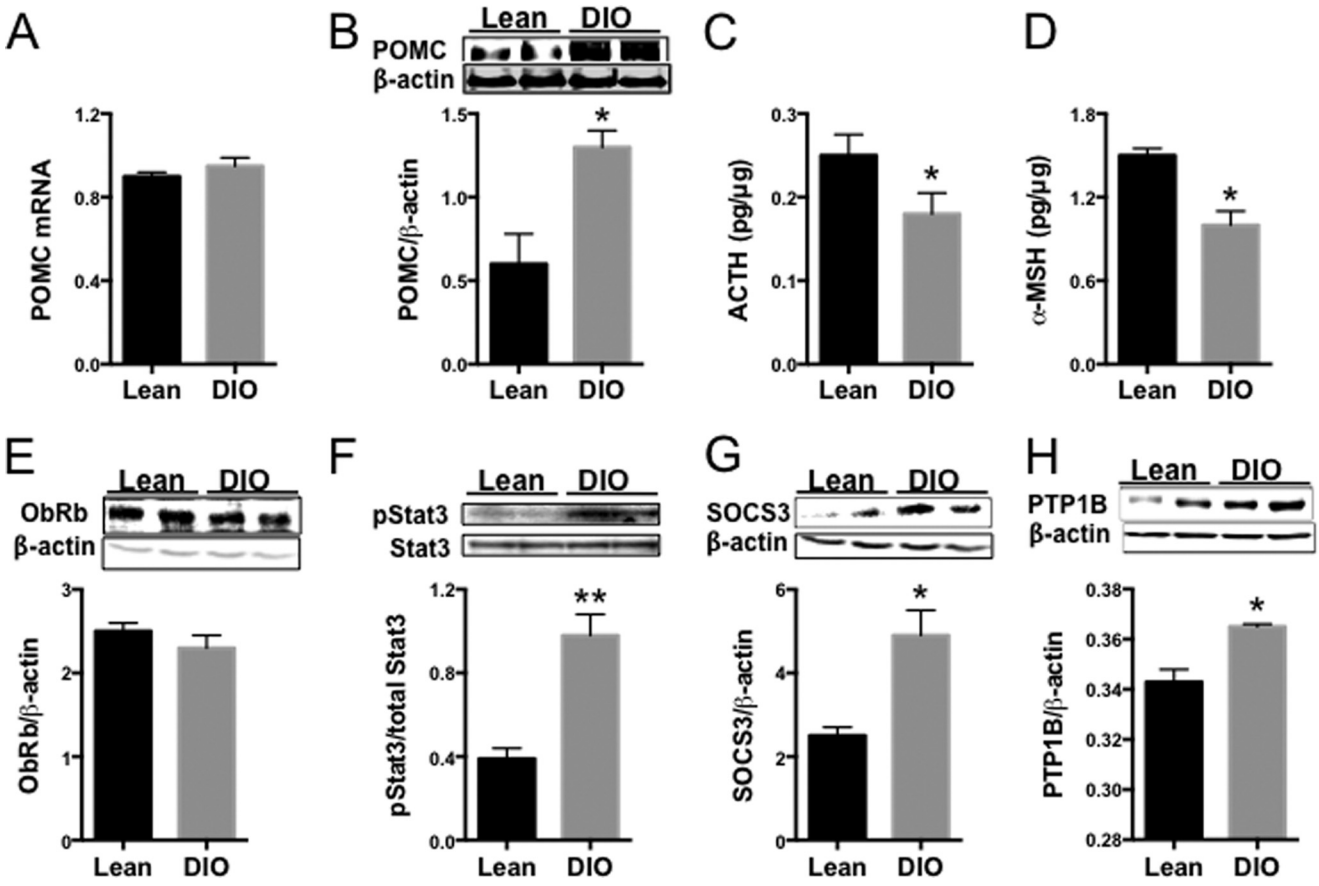

FIGURE 1. **α-MSH peptide is lower in DIO rodents, whereas POMC mRNA remains unchanged.** Feeding rats a high fat diet for 12 weeks generated DIO (*gray bars*), whereas lean controls (*black bars*) were kept on regular chow. *A*, POMC mRNA in lean and DIO ARC was measured by RT-PCR. *B*, Western blot analysis depicted the accumulation of POMC protein in the DIO ARC. *C*, RIA of ACTH in lean and DIO rats. *D*, RIA of α-MSH in lean and DIO rats. *E*, ObRb levels in lean and DIO rat ARC as measured by Western blot. *F*, Western blot of pSTAT3 over total STAT3 in the ARC of lean and DIO rats. *G*, SOCS3 protein levels in lean and DIO ARC were measured by Western blot. *H*, PTP1B protein levels in lean and DIO ARC measured by Western blot. $n = 12$ lean and 8 DIO. Data are mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$ *versus* lean control.

difficult to extrapolate a mouse model to the rat unless POMC processing in the mouse is characterized.

Our recently published results reveal that lean and DIO mice have similar POMC mRNA levels, yet α-MSH levels were lower in DIO mice (9), which strongly suggests that α-MSH biosynthesis through processing is altered in the obese state. Consistent with this observation, high fat feeding in rats presented the same profile of lower α-MSH peptide with unaltered POMC mRNA compared with lean counterparts (Fig. 1). Interestingly, we also observed in the rat that POMC protein accumulated during HFD feeding (Fig. 1*B*), suggesting that less properly folded POMC is able to reach the Golgi complex and secretory granules for final processing and storage but instead remained in the ER. Therefore, two processing products of POMC, ACTH and α-MSH, were diminished (Fig. 1, *C* and *D*).

Leptin-positive Regulation on POMC mRNA Is Blunted in the DIO State—Studies have shown that DIO animals are partially sensitive to leptin-induced STAT3 phosphorylation in the ARC (34), and STAT3 contributes to hypothalamic POMC expression (35, 36). Because we do not see any change in POMC mRNA in the obese state, we explored some of the factors affecting leptin signaling in our DIO rats. Fig. 1*E* shows that ObRb leptin receptor protein levels were similar between lean and DIO ARC. Phosphorylated Stat3 is elevated in DIO (Fig. 1*F*). However, the negative leptin regulators suppressor of cytokine signaling-3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B) (37) also increased in DIO compared with lean controls (Fig. 1, *G* and *H*). Elevated SOCS3 and PTP1B may explain why pSTAT3 is elevated in DIO, but POMC mRNA levels are not elevated in the DIO ARC (Fig. 1*A* and 2*A*). These results are consistent with previous observations that DIO rodents develop resistance to leptin-induced STAT3 phosphorylation in the ARC (38).

POMC Processing Is Altered in DIO Animals—Although leptin has a clear role in regulating POMC expression in the lean animal (16), different mechanisms appear to play a larger role in regulating the decrease in α-MSH peptide seen in the DIO. We propose these mechanisms to be defective POMC folding and processing. For example, we demonstrate that POMC processing is altered during different nutritional states including DIO. For example, we found that mRNA and peptide levels of POMC as well as levels of the POMC-derived peptides ACTH and α-MSH decrease during fasting in lean rats (16), where the ACTH/α-MSH ratio was 0.11 for fed *versus* 0.69 for fasted states (16). This ratio provides a measure of the conversion of ACTH to α-MSH in the ARC, and our results demonstrate that fasting resulted in a 6.27-fold increase in ACTH/α-MSH ratio. Administration of leptin partially reversed these effects, dem-

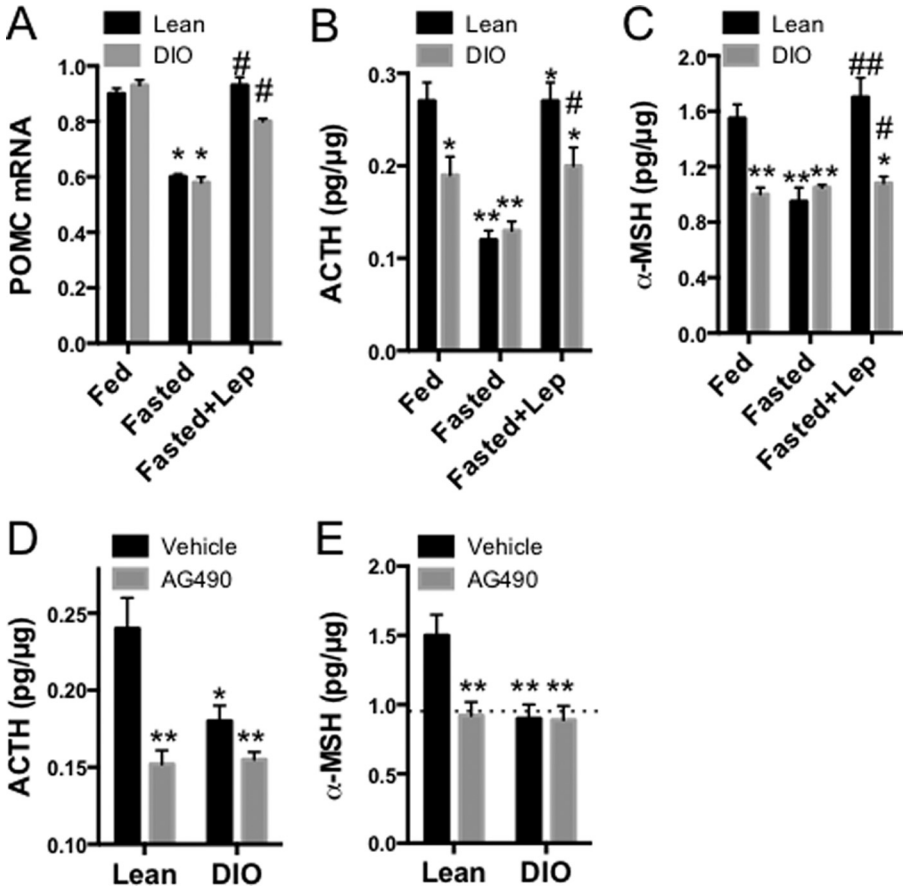

FIGURE 2. **Leptin action on POMC, ACTH, and α-MSH in DIO rats.** For leptin experiments, fasted animals were without food for 48 h, and leptin was infused icv (3.5 μg/rat). *Black bars*, lean; *gray bars*, DIO. For *C* and *D*, AG490 (54 nmol/rat; *gray bars*) or vehicle control (*black bars*) was infused icv. *A*, RT-PCR of POMC mRNA levels. *B*, ACTH RIA analyses in lean and DIO ARC. *C*, α-MSH RIA analyses in lean and DIO ARC. *D*, ACTH response to AG490 in lean and DIO ARC. *E*, α-MSH response to AG490 in lean and DIO ARC. $n = 12$ lean and 8 DIO (*A–D*). Data are mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$ *versus* lean fed vehicle (*A–C*) or lean vehicle (*D* and *E*); #, $p < 0.05$; ##, $p < 0.01$ *versus* fasted within the same group (lean or DIO).

onstrating that during fasting in lean animals, the biosynthesis of POMC and its derived peptides is nutrient- and leptin-dependent through STAT3 signaling (16, 36). However, this was not the case in the DIO condition. Our current results corroborate previous evidence that fasting decreases and leptin increases POMC, ACTH, and α-MSH (Fig. 2, *A–C*) levels in control lean rats. In contrast, although POMC mRNA and ACTH peptide content in DIO rats responded to fasting and leptin to a slightly lesser degree but in a similar manner as that in lean rats (Fig. 2, *A* and *B*), α-MSH levels in DIO rats were insensitive to either fasting or leptin administration (Fig. 2*C*). These results strongly suggest that the conversion from ACTH to α-MSH is impaired in the DIO condition.

To further support our findings, we blocked STAT3 signaling with the JAK2 inhibitor AG490 (Fig. 2, *D* and *E*), because leptin activation of STAT3 is mediated by the JAK2 receptor. icv infused AG490 attenuated ACTH in the ARC of control and DIO animals. Although AG490 reduced α-MSH levels in lean controls, it had no effect on α-MSH levels in DIO animals. That inhibiting leptin signaling failed to decrease α-MSH suggests that leptin may regulate α-MSH levels indirectly in the DIO condition. Collectively, the results substantiate our previous findings that the conversion of ACTH to α-MSH is impaired in DIO.

These results led us to explore whether the POMC processing cascade is impaired in DIO. PC1, PC2, and CPE regulate POMC post-translational processing (Fig. 3*A*). Fig. 3 shows that PC1 mRNA and protein remain unchanged in the ARC of lean and DIO rats. For CPE, mRNA was unchanged, but its protein level showed a tendency to decrease. In contrast, PC2 protein levels were significantly lower (~53.1%) in the DIO ARC compared with controls, yet there was no difference in PC2 mRNA levels between lean and DIO rats. These data suggest that PC2, which is an enzyme critical for the conversion of ACTH to α-MSH, is the key regulator of α-MSH levels in DIO. Therefore, in the next series of studies, we focused mainly on PC2.

ER Stress Is Established in the ARC of DIO Animals—The above results prompted us to ask the following question. If POMC mRNA does not change in the DIO condition, what mechanism(s) could affect POMC biosynthesis/processing such that α-MSH production is decreased? One important possibility is the development of ER stress. As described in the Introduction, recent studies have linked ER stress, obesity, and dysregulation of appetite-regulating hormones (39–41). For example, DIO in mice causes hypothalamic ER stress, which in turn contributes to leptin resistance (39). We therefore investigated whether DIO rats present ER stress in two hypothalamic brain regions important for appetite control, the ARC and

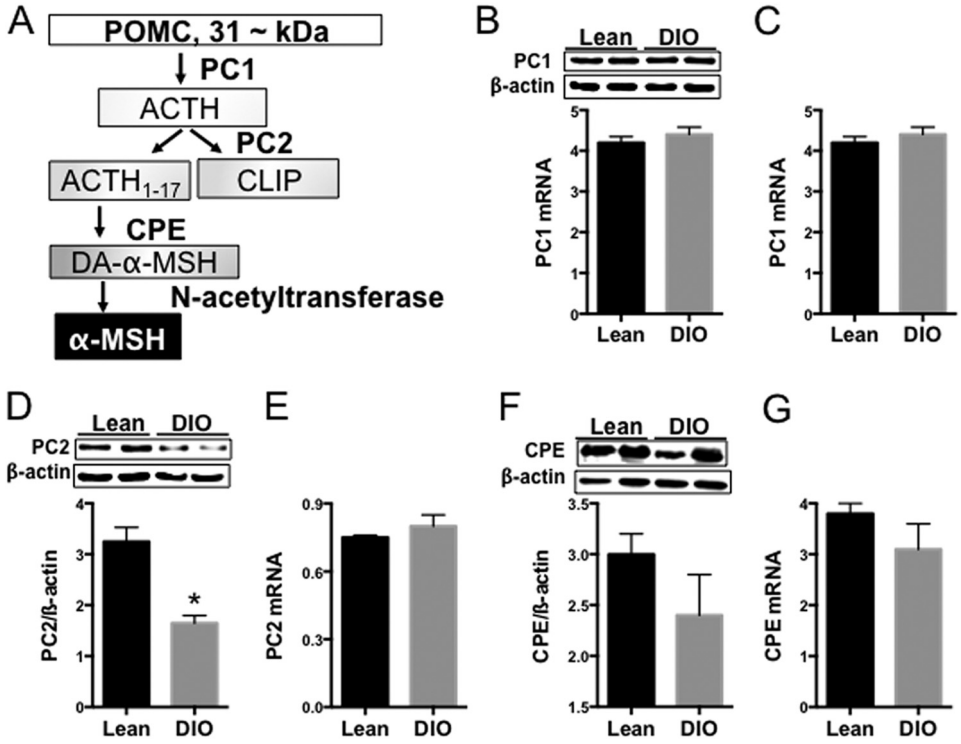

FIGURE 3. **POMC processing is altered in DIO.** *A*, the POMC processing cascade is modeled here and has been described previously. *Black bars*, lean; *gray bars*, DIO. *B*, Western blot of PC1 protein levels in lean and DIO ARC. *C*, RT-PCR of PC1 mRNA levels in lean and DIO ARC. *D*, Western blot of PC2 protein levels in lean and DIO ARC. *E*, RT-PCR of PC2 mRNA levels in lean and DIO ARC. *F*, Western blot of CPE protein levels in lean and DIO ARC. *G*, RT-PCR of CPE mRNA levels in lean and DIO ARC. Depicted analyses were performed on $n = 8$ lean and 8 DIO (*A–D*) (*B*, *D*, and *F* were repeated $n = 8$ with the same results. Data are mean ± S.E. (*error bars*). *, $p < 0.05$ *versus* lean control.

PVN. We focused on one of the branches of ER stress that is activated in the mouse hypothalamus during high fat feeding and leptin resistance (39, 42). The results show that ER stress markers p-PERK and p-eIF2α were significantly elevated in the ARC of DIO animals compared with controls (Fig. 4, *A* and *B*). In contrast, p-PERK and p-eIF2α levels were similar in the PVN of DIO and lean control rats (data not shown). These results demonstrate that ER stress developed in the ARC but not the PVN of rats subjected to 12 weeks of HFD. We further investigated whether ER stress in the ARC of lean animals could be induced pharmacologically in a manner similar to that observed in the DIO state. To this end, lean rats were icv infused with tunicamycin or thapsigargin, two well known ER stress inducers (39, 42). Both tunicamycin and thapsigargin significantly increased p-PERK and p-eIF2α levels in the ARC (Fig. 4, *C* and *D*). Once it was established that DIO causes ER stress to develop in the ARC of our rats, we then determined whether the negative leptin regulators SOCS3 and PTP1B were affected by ER stress. Tunicamycin and thapsigargin each increased SOCS3 mRNA (Fig. 4*E*) as well as SOCS3 and PTP1B proteins in the ARC (Fig. 4*F*). Taken together, these results indicate that ER stress occurs in the DIO ARC and that ER stress plays a role in increasing negative leptin regulators.

ER Stress Impairs POMC Processing—We then determined that inducing ER stress impairs POMC processing. For example, we found that, when ER stress was induced in the ARC using tunicamycin, there was a 150% increase in the ACTH/α-MSH ratio, indicating that the catalytic activity of PC2 was markedly reduced (Fig. 5, *A* and *B*, and Table 1). This was consistent with the lower PC2 protein levels seen in the ARC of rats kept on HFD feeding for 12 weeks (Fig. 3*D*). In addition, PC2 protein, but not mRNA, was significantly lower in the ARC of lean rats treated with either tunicamycin or thapsigargin to induce ER stress (Fig. 5, *C* and *D*) compared with controls. Our results demonstrate that when ER stress is induced, either by obesity or a pharmacological agent, both α-MSH and PC2 are significantly attenuated. Furthermore, pharmacologically induced ER stress tended to decrease oxygen consumption and significantly increased plasma glucose (Fig. 5, *E* and *F*), indicating a shift to a positive energy balance.

We next determined whether ameliorating the UPR activated by ER stress in DIO animals could reverse the DIO-induced reduction of the POMC-derived peptides ACTH and α-MSH. Consequently, we treated both lean and DIO rats for 2 days with TUDCA, which is a chemical chaperone that has been used to successfully alleviate ER stress in rats (44). We found that TUDCA reduced p-eIF2α in the DIO ARC (Fig. 6*A*) and that TUDCA rescued the decrease in both ACTH and α-MSH in DIO rats (Fig. 6, *B* and *C*), indicating that ER stress is responsible for the reduction in these peptides in the DIO condition. We also found that DIO rats gained more weight than lean controls over this 2-day time period, but this was not the case for DIO rats treated with TUDCA. TUDCA-treated DIO rats gained less weight than vehicle-treated DIO rats. In fact, weight gain in TUDCA-treated DIO rats was similar to lean rats (Fig. 6*D*). Food intake significantly decreased and oxygen consumption tended to increase in DIO rats treated with TUDCA (Fig. 6, *E* and *F*) Fasting glucose levels tend to be higher in our DIO rats,

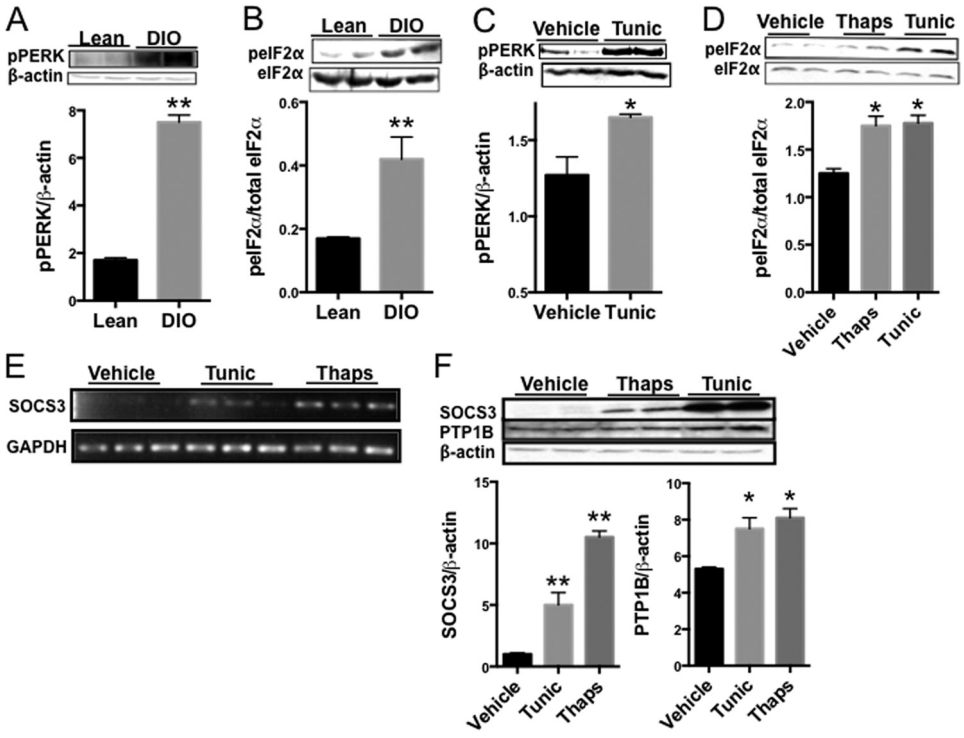

FIGURE 4. **DIO and pharmacologically induced ER stress increases p-PERK and SOCS3 and PTP1B expression.** *Black bars*, lean; *gray bars*, DIO. Protein levels were analyzed by Western blot. *A*, p-PERK protein levels in lean and DIO ARC. *B*, p-eIF2α protein levels in lean and DIO ARC. For *C–F*, ER stress inducers thapsigargin (*thaps*; 10 ng/rat; *dark gray bars*) or tunicamycin (*tunic*; 10 μg/rat; *light gray bars*) were icv infused in lean rats and compared with vehicle controls (*black bars*). *C*, p-PERK protein levels in the ARC of lean rats treated with tunic or vehicle control. *D*, protein levels of p-eIF2α in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. *E*, analysis of SOCS3 expression by end point PCR in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. *F*, SOCS3 and PTP1B protein levels in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. $n = 12$ lean and 8 DIO (*A–D* and *F*) $n = 3$ (*E*). Data are mean $\pm$ S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$ *versus* lean control (*A* and *B*) or vehicle control (*C–F*).

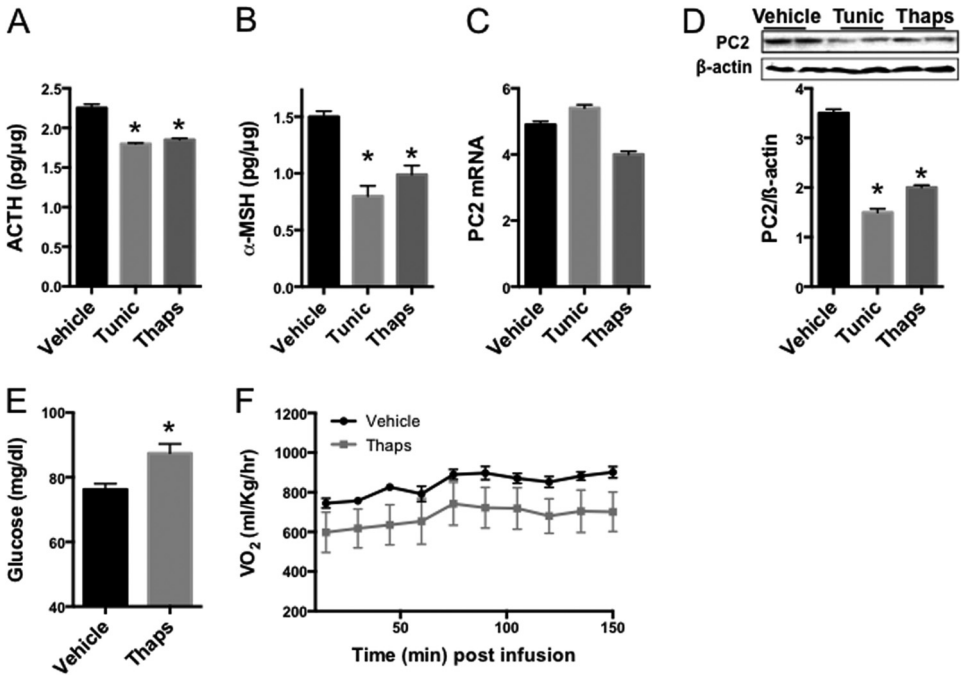

FIGURE 5. **Pharmacologically induced ER stress alters ACTH, α-MSH, and PC2.** *A–D*, ER stress inducers thapsigargin (*thaps*; 10 ng/rat; *dark gray bars*) or tunicamycin (*tunic*; 10 μg/rat; *light gray bars*) were icv infused in lean rats and compared with vehicle controls (*black bars*). *A–D*, $n = 8$/group; *E* and *F*, $n = 6$/group. *A*, RIA of ACTH peptide content in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. *B*, RIA of α-MSH peptide content in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. *C*, RT-PCR of PC2 mRNA levels in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. *D*, Western blot of PC2 protein levels in the ARC of lean rats treated with tunicamycin, thapsigargin, or vehicle control. *E*, fasting glucose levels in rats treated with thapsigargin or vehicle control. *F*, oxygen consumption following thapsigargin or vehicle control infusions. Data are mean $\pm$ S.E. (*error bars*). *, $p < 0.05$ *versus* vehicle control.

and treating these DIO rats with TUDCA tended to mitigate that increase in fasting glucose (Fig. 6*G*).

In addition, this 2-day TUDCA treatment did not appear to alter leptin signaling. For example, pSTAT3 levels were not altered by TUDCA in either lean or DIO rats (Fig. 7*A*). Similarly, TUDCA did not change the levels of the negative leptin regulator SOCS3 in either lean or DIO rats (Fig. 7*B*). SOCS3 was elevated in DIO rats and remained elevated in DIO rats treated with TUDCA. In addition, protein levels of the leptin receptor OBRB were statistically similar to controls (Fig. 7*C*). These data indicate that mitigating ER stress for 2 days augments ACTH and α-MSH levels, which does not require changes in leptin signaling.

Because the clearance enzyme prolylcarboxypeptidase can regulate α-MSH levels, we also measured prolylcarboxypeptidase in lean and DIO rats treated with and without TUDCA but found no differences in any comparison (diet, TUDCA, or interaction; Fig. 7*D*). Furthermore, a reduction in PC2 could affect not only the biosynthesis of α-MSH but also other energy-regulating peptides, such as neuropeptide Y (NPY) and Agouti-related peptide (AgRP). We measured NPY peptide in lean and DIO rats treated with and without TUDCA and observed no difference in any comparison (Fig. 7*E*). Most studies measure AgRP using mRNA analyses. However, because we found changes in the processing enzyme PC2, we wanted to measure peptide levels. We attempted to measure AgRP peptide in lean and DIO rats treated with and without TUDCA using the rat AgRP ELISA kit (MBS730302) from MyBioSource, but we were unable to detect AgRP.

To address whether the effect of ER stress on PC2 can be reversed by chemical chaperones specifically in POMC cells, we pretreated N43/5 cells (a mouse hypothalamic POMC cell line that expresses PC2) with the chemical chaperone PBA, followed by induction of ER stress with tunicamycin. PBA has been used successfully to moderate ER stress in a neuronal cell line (45). We found that treatment with 0.1 μg/ml tunicamycin did not change the levels of PC1 in either the presence or absence of PBA (data not shown). These results are consistent with the *in vivo* results in which PC1 levels in the ARC are similar between lean and DIO rats (Fig. 3*B*). In contrast, tunicamycin significantly reduced PC2 levels at 2, 3, and 4 h of treatment (Fig. 8*A*). Furthermore, the ER stress marker p-eIF2α increased with tunicamycin treatment (Fig. 8*A*). These PC2 and p-eIF2α observations in N43/5 cells subjected to ER stress

TABLE 1

Molar ratio of ACTH/α-MSH and POMC content in the ARC of lean rats treated with the ER stress inducer tunicamycin or vehicle control

Rats treated with tunicamycin or vehicle were euthanized, and the ARC was collected for HPLC and RIA analyses of POMC, ACTH, and α-MSH. The first row depicts the molar ratio of ACTH/α-MSH and the percentage increase detected in tunicamycin-treated rats. The second row depicts POMC content (pmol) and the amount of POMC content (pmol) accumulated in tunicamycin-treated rats.

	Vehicle	Tunicamycin	Change
ACTH/α-MSH	1.4	2.1	150% increase
POMC	0.035	0.05	2-Fold accumulation

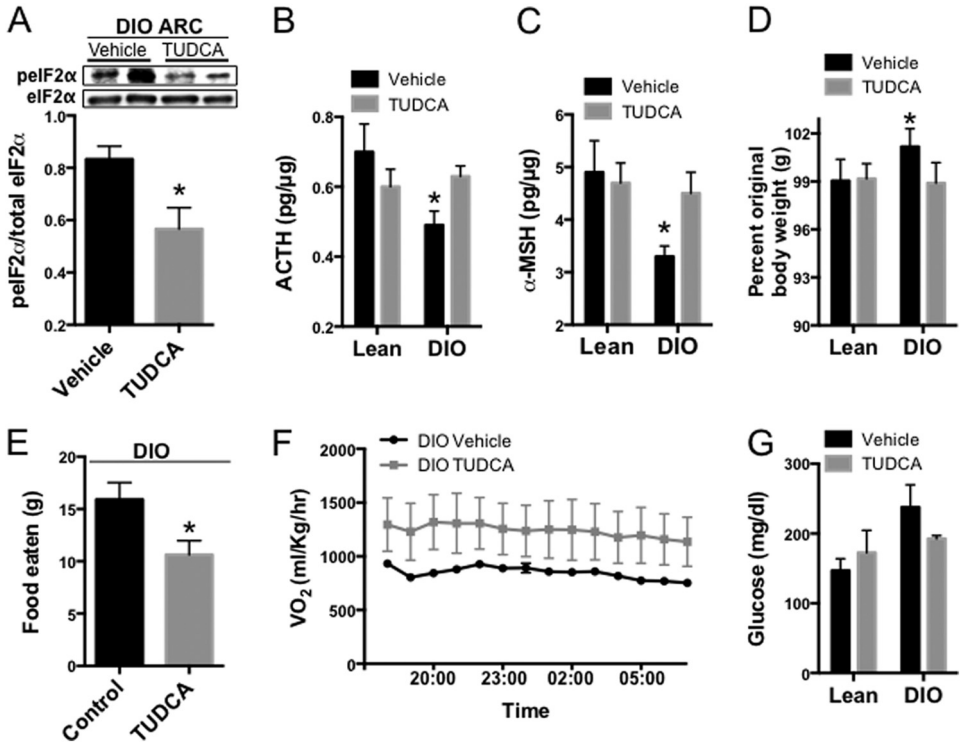

FIGURE 6. **Changes in ACTH and α-MSH in DIO are reversed with a chemical chaperone.** Lean and DIO rats were treated with (*gray bars*) and without (*black bars*) the chemical chaperone TUDCA (200 mg/kg for 2 days). *LV*, lean vehicle ($n = 4$); *LT*, lean TUDCA ($n = 4$); *DV*, DIO vehicle ($n = 5$); *DT*, DIO TUDCA ($n = 5$). *A*, TUDCA reduced p-eIF2α in the ARC of DIO rats. *B*, RIA of ACTH peptide content in the ARC of lean and DIO rats treated with and without TUDCA. *C*, RIA of α-MSH peptide content in the ARC of lean and DIO rats treated with and without TUDCA. *D*, body weight of lean and DIO rats treated with and without TUDCA. Animals were weighed immediately prior to the first TUDCA infusion and a second time immediately prior to sacrifice. Weight was measured as the percentage weight gain (g) from the first to the second measurement. *E*, TUDCA reduced food intake in DIO rats. *F*, oxygen consumption in DIO rats following TUDCA or vehicle control infusions. *G*, fasting glucose levels in rats treated with TUDCA or vehicle control. Data are mean ± S.E. (*error bars*). *, $p < 0.05$ *versus* vehicle control.

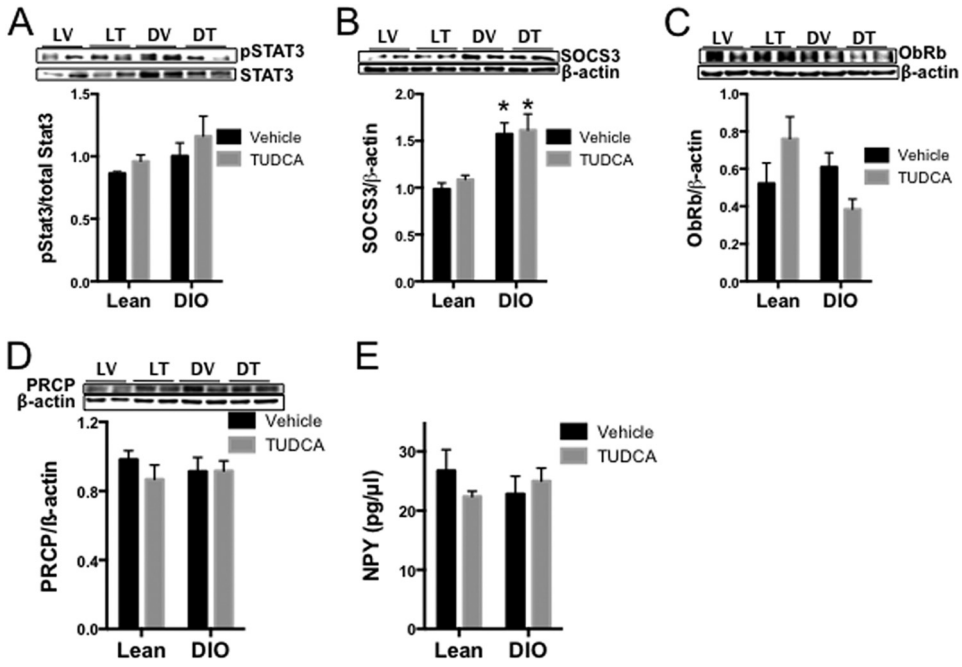

FIGURE 7. **There were no changes detected in leptin signaling, prolylcarboxypeptidase (*PRCP*), or NPY due to TUDCA.** Lean and DIO rats were treated with (*gray bars*) and without (*black bars*) the chemical chaperone TUDCA (200 mg/kg for 2 days). *LV*, lean vehicle ($n = 4$); *LT*, lean TUDCA ($n = 4$); *DV*, DIO vehicle ($n = 5$); DT, DIO TUDCA ($n = 5$). *A*, Western blot of pSTAT3 protein levels in the ARC of lean and DIO rats treated with and without TUDCA. *B*, Western blot of SOCS3 protein levels in the ARC of lean and DIO rats treated with and without TUDCA. *C*, Western blot of ObRb protein levels in the ARC of lean and DIO rats treated with and without TUDCA. *D*, Western blot of PRCP protein levels in the ARC of lean and DIO rats treated with and without TUDCA. *E*, RIA of NPY content in the ARC of lean and DIO rats treated with and without TUDCA. Data are mean ± S.E. (*error bars*). *, $p < 0.05$ *versus* vehicle control.

mimic the data observed in the DIO ARC as well as in the ARC of animals treated with tunicamycin and thapsigargin. When cells were pretreated with PBA, PC2 levels did not decrease compared with untreated control cells, and p-eIF2α was resistant to tunicamycin treatment, indicating that ER stress reduced PC2 (Fig. 8*A*). In addition, the ER stress inducer thapsigargin also decreased PC2 in N43/5 cells. Because pharmacological agents such as tunicamycin and thapsigargin affect cellular processes other than ER stress, we also used salubrinal in our studies because salubrinal specifically inhibits the dephosphorylation of p-eIF2α. We first ran a dose response, and found that enhancing the phosphorylation of eIF2α with salubrinal increased PC2 levels (Fig. 8*B*). We then found that salubrinal reversed the effect of thapsigargin on PC2 (Fig. 8*C*).

Using the N45/5 neuronal POMC-positive cell line, we next determined whether ER stress affected PC2 transcription or caused changes in the processing of PC2 itself, because the active form of the enzyme is the smaller, processed form that acts in secretory granules to cleave the ACTH peptide to generate α-MSH. Therefore, we measured PC2 promoter activity in N43/5 cells treated with and without tunicamycin. Tunicamycin did not alter PC2 promoter activity (Fig. 8*D*) or PC1 activity as expected (data not shown). These results indicate that the mechanism by which ER stress reduces PC2 occurs after transcription, which supports our *in vivo* finding that PC2 mRNA in DIO rats is similar to that in lean rats (Fig. 3*E*). Thus, we also tested whether the 7B2 peptide, a facilitator of PC2 maturation (46), was affected by ER stress. Fig. 8*E* shows that tunicamycin did not alter the level of pro7B2 (27 kDa) or its smaller active form of 21 kDa, suggesting that 7B2 peptide does not participate in the down-regulation of PC2 activity during ER stress. Consistent with the results seen in the N43/5 cells, DIO rats did not show changes in 7B2 compared with lean rats (Fig. 8*F*). Data from lean and DIO rats show that PC2 effects on POMC processing are nutrient-dependent (Fig. 3*D*) and are not likely to be driven by changes in transcription (Fig. 3*E*) or processing (Fig. 8*F*). Instead, the attenuation of PC2 during ER stress is probably caused by reduced translation efficiency or enhanced clearance of unfolded PC2 by the ER-associated degradation system or by a combination of both processes.

To corroborate our results using N45/5 cells, we also evaluated the affect of ER stress on PC2 in AtT-20 cells, which are corticotropic cells that express POMC. Furthermore, AtT20 is the only cell line to our knowledge in which both PC2 and α-MSH can be measured if PC2 is transfected. Induction of ER stress in these cells with thapsigargin (using conditions that have been shown to induce ER stress in previous studies (47)) decreased PC2, but pretreatment with PBA prevented this effect (Fig. 9*A*). Similar results were observed for α-MSH release (Fig. 9*B*). We also found that enhancing PC2 expression in thapsigargin-treated cells overcame the effect of ER stress on PC2 levels (Fig. 9*C*). Additionally, we measured the release of α-MSH into the medium and found the same pattern that thapsigargin decreased α-MSH, but enhanced PC2 overexpression in thapsigargin-treated cells significantly increased α-MSH compared with thapsigargin with less PC2 (Fig. 9*D*).

DISCUSSION

In this study, we demonstrate that there is a direct link between ER stress and POMC processing in the obesity state and that ER stress mediates the HFD-induced changes in POMC processing. We show that ER stress is the mechanism

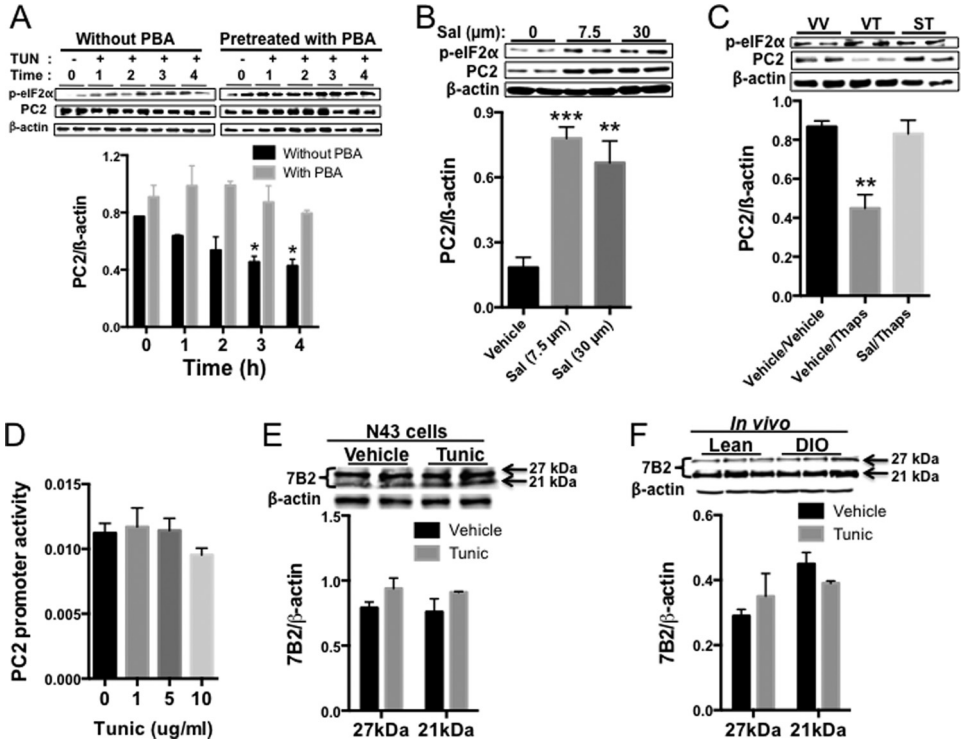

FIGURE 8. **ER stress attenuates PC2 protein (but not PC2 promoter activity or processing) specifically in POMC-positive N43/5 neuronal cells, and salubrinal-enhanced p-eIF2α increases PC2 and appears to protect against the ER stress decrease in PC2.** *A*, PC2 and p-eIF2α protein in POMC-positive N43/5 cells treated with tunicamycin (*tunic*; 0.1 μg/ml; *gray bars*) and with and without PBA (20 mM, 14 h) pretreatment. $n = 6$; results were replicated in three independent experiments. *B*, dose-response curve for PC2 protein in N43/5 cells treated with vehicle, 7.5 μg of salubrinal, and 30 μg of salubrinal. p-eIF2α is also shown. $n = 4$/group. *C*, PC2 protein in N43/5 cells pretreated with vehicle (*VV*), pretreated with vehicle then thapsigargin (*thaps*; for 6 h) (*VT*), or pretreated with 7.5 μg of salubrinal (14 h) and then thapsigargin (6 h) (*ST*). p-eIF2α is also shown. $n = 6$/group. *D*, PC2 promoter activity with and without tunicamycin (1.5 h) treatment as analyzed by luciferase assay. $n = 8$. *E*, Western blot analysis of 7B2 in control (*white bars*) and tunicamycin-treated (0.1 μg/ml, 2 h; *gray bars*) N43/5 cells. *F*, Western blot analysis of 7B2 in the ARC of lean (*white bars*) and DIO (*gray bars*) animals. *E* and *F*, $n = 6$–9. Data are mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* vehicle control at time 0 (*A*), vehicle (*B* and *D*), vehicle/vehicle (*C*), or lean controls (*F*).

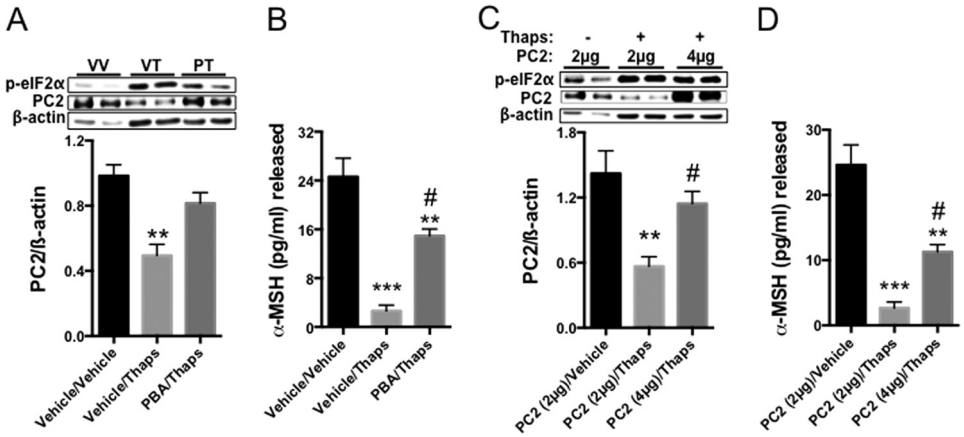

FIGURE 9. **PC2 protein and α-MSH release decreases with induction of ER stress in POMC-positive AtT20 cells.** PC2 protein (*A*) and α-MSH release into the medium (*B*) in AtT20 cells pretreated with vehicle (*VV*), pretreated with vehicle and then thapsigargin (*thaps* for 6 h) (*VT*), or pretreated with 20 mM PBA (14 h) and then thapsigargin (6 h) (*PT*). PC2 protein (*C*) and α-MSH release into the medium (*D*) in AtT20 cells transfected with 2 μg of PC2 cDNA and treated with vehicle, transfected with 2 μg of PC2 cDNA and treated with thapsigargin, or transfected with 4 μg PC2 cDNA and treated with thapsigargin (all $n = 5$/group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* vehicle/vehicle (*A* and *B*) or PC2 (2 μg)/vehicle (*C* and *D*). #, $p < 0.05$ *versus* vehicle/thapsigargin (*A* and *B*) or PC2 (2 μg)/thapsigargin (*C* and *D*). *Error bars*, S.E.

affecting POMC processing, which results in lower α-MSH production independent of POMC mRNA levels. In addition, we demonstrate that PC2, the key specific enzyme involved in the conversion of ACTH to α-MSH, is most likely responsible for the reduced processing of the α-MSH precursor.

Obesity is associated with hypothalamic leptin resistance, which contributes to maintaining obesity despite hyperleptinemia. The importance of leptin regulation of POMC neurons was demonstrated in earlier studies, where deletion of the leptin receptor (LepR) specifically in POMC neurons (POMC-Cre: $LepR^{loxP/loxP}$) resulted in increased body weight and adiposity, hyperleptinemia, and altered hypothalamic neuropeptide expression (48). Early work suggested that an improvement of ER function and leptin signaling could be achieved by adminis-

trating chemical chaperones in DIO animals (39). Our results are consistent with that paradigm, showing an increase in the phosphorylation of ER stress markers, PERK, and eIF2α, which were also activated by pharmacological induction of ER stress in lean rats (Fig. 4). We extended these findings and showed that the negative regulators of leptin signaling, SOCS3 and PTP1B, are up-regulated in lean rats treated with either tunicamycin or thapsigargin compared with controls, which is consistent with previous *in vitro* studies demonstrating that PTP1B is increased during ER stress (49, 50) and contributes to ER stress-induced leptin resistance (50). The effect of ER stress on SOCS3 has been less clear, with one study reporting that the N-terminal truncated isoform of SOCS3, resulting from alternative translation initiation, significantly increased with ER stress through the induction of p-eIF2α (51). Overall, *in vivo* results from our studies and others indicate that ER stress occurs in DIO and contributes to leptin resistance. The up-regulation of SOCS3 and PTP1B by DIO-induced ER stress may override the leptin signaling demand for more POMC production in obesity, preventing an increase in POMC mRNA in the DIO condition even with hyperleptinemia. Furthermore, it is possible that elevated SOCS3 during ER stress and DIO conditions contributes to a decrease in particular proteins, potentially including PC2, by targeting these proteins for proteosomal degradation through polyubiquitination (52).

Early ER stress studies were an important turning point in our understanding of ER stress and leptin resistance. The current work described here represents a significant and novel advance concerning the role of ER stress on prohormone processing. Together, results indicate that ER stress affects cellular events such as POMC processing and that these changes in POMC processing do not require changes in leptin signaling. In our studies, two bioactive POMC products, ACTH and α-MSH, are lower in DIO rats experiencing ER stress compared with lean controls, which is a phenomenon that cannot be attributed to changes in leptin signaling that would affect POMC mRNA because we observe no changes in POMC mRNA in DIO compared with lean rats. Although some studies have reported that POMC mRNA decreases in DIO rodents (53, 54), most studies do not demonstrate lower POMC mRNA in DIO *versus* lean rodents (9, 55–58). In contrast to mechanisms that would regulate POMC mRNA, we discovered that ER stress-induced attenuation of the anorexigenic α-MSH peptide is due to a post-translational mechanism. We first established that ACTH and α-MSH are reduced when the ARC is under ER stress due to DIO or pharmacological induction. We further confirmed that the reduction in these POMC-derived peptides seen in DIO is specifically caused by ER stress because levels of these peptides are rescued after treatment with the chemical chaperon TUDCA. Thus, enhancing the folding capacity of the ER in obese rats, a physiological obesity model similar to humans, can restore the potent appetite suppressor α-MSH to levels similar to those seen in lean individuals.

It is well known that leptin regulates POMC mRNA and thus α-MSH production and that leptin resistance contributes to the obese condition (59, 60). Our data reveal that inhibition of STAT3 signaling fails to suppress α-MSH production in the DIO conditions, which supports the hypothesis that leptin resistance affects α-MSH in DIO. It has also been reported that ER stress established in DIO contributes to leptin resistance (39). Because we do not detect any change in POMC mRNA in the ARC of our DIO rats, it is unlikely that ER stress-induced effects on leptin's actions on POMC gene expression contribute to the decrease in α-MSH in DIO. It is possible, however, that ER stress effects on leptin sensitivity regulate α-MSH levels indirectly. As Ozcan *et al.* (39) demonstrated previously, chemical chaperones can sensitize rodents to leptin. Thus, we tested whether the TUDCA-induced increase in ACTH and α-MSH in our DIO rats was due to enhanced leptin signaling. However, we detected no changes in leptin signaling molecules in the ARC of our TUDCA-treated rats. This is probably because we treated them with TUDCA for 2 days, whereas animals in the previous study were given TUDCA for longer periods of time, including TUDCA pretreatment regimes. Thus, it appears that treatment with TUDCA for 2 days is sufficient to reverse the DIO-induced attenuation of ACTH and α-MSH as well as to reduce weight gain, decrease food intake, and slightly increase energy expenditure in the DIO condition but not to improve the leptin resistance established in the DIO ARC. We detected a slight, albeit not significant, decrease in ObRb in the ARC of our TUDCA-treated DIO rats, suggesting that longer term treatment would probably affect leptin sensitivity, as demonstrated by Ozcan *et al.* (39), and thus potentially alter α-MSH. However, data from the current study indicate that it is possible to rescue the changes in ACTH and α-MSH during DIO by mitigating ER stress, and this does not require changes in leptin signaling. Future studies will characterize the role of leptin resistance during ER stress and DIO in regulating α-MSH and other energy-regulating peptides.

Our studies pharmacologically inducing ER stress in lean animals strongly support the hypotheses that ER stress is the mechanism driving the reduction of post-translational POMC processing in DIO. For example, there was a 150% increase in the ACTH/α-MSH ratio in tunicamycin-treated rats, indicating that the conversion of ACTH to α-MSH was markedly reduced. Induction of ER stress by icv thapsigargin in the current study resulted in downstream metabolic changes, including increased fasting glucose levels and a tendency toward decreased energy expenditure. We also show that PC2, but not PC1 or CPE, protein levels are significantly reduced under both conditions, although there was a tendency for CPE to decrease in DIO. A previous study demonstrated a significant decrease in CPE mRNA in the DIO mouse (61), but we detected only a slight decrease in CPE protein expression in our DIO rats. This discrepancy in the magnitude of change in CPE may be due to species differences. In early studies, we showed that leptin up-regulates both PC1 and PC2 via STAT3 signaling (62). However, the slight increase in STAT3 signaling during DIO failed to increase PC1 or override the reduction in PC2, suggesting that leptin does not influence α-MSH levels in DIO by affecting POMC-processing enzymes. Our *in vivo* results were complemented by studies using POMC-positive N43/5 and AtT20 cells, in which ER stress significantly decreased PC2. Further experiments demonstrated that ER stress specifically reduces PC2 because pretreatment with the chemical chaperone PBA prevented the ER stress-induced attenuation of PC2. Similarly,

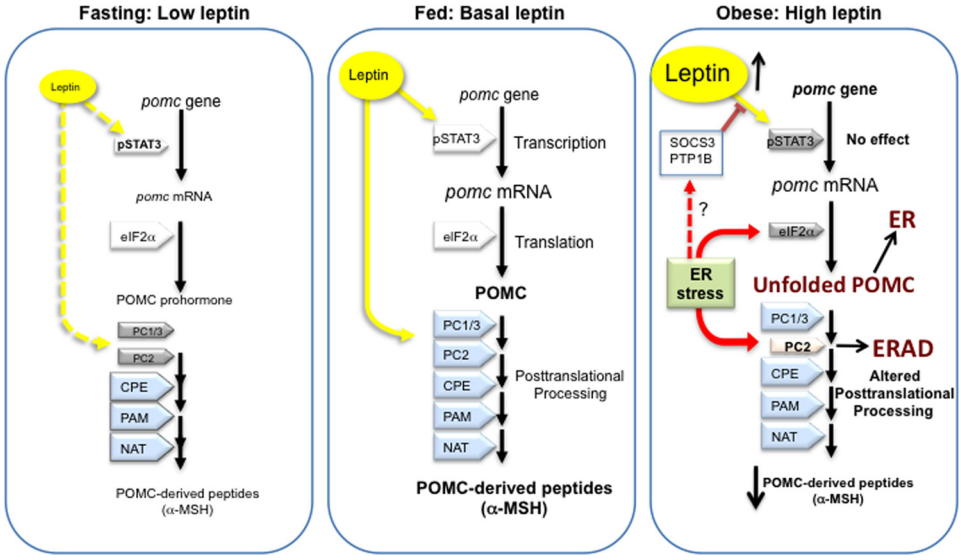

FIGURE 10. **Model depicting the changes in POMC, its processing enzyme PC2, and α-MSH under conditions of fasting (*i.e.* low leptin; *first panel*), fed (*i.e.* basal leptin; *second panel*), and DIO (*i.e.* high leptin; *third panel*).** Compared with the fed (basal leptin) condition, fasting causes diminished α-MSH by leptin-mediated reduction in POMC mRNA along with reduced POMC processing due to lower PC1 and PC2 levels. In the DIO state, ER stress triggers an unfolded response causing an accumulation of POMC in the ER partly caused by an unfolded response and a possible degradation of PC2 through the ER-associated degradation system, conditions that ultimately cause less α-MSH production.

ER stress reduced the amount of α-MSH released into the medium, and pretreatment with a chemical chaperone partially rescued the effect. Salubrinal, which selectively inhibits the dephosphorylation of p-eIF2α subunit α (63), has been shown to enhance the FFA-induced activation of the PERK pathway but not the ATF6 and IRE1 branches (64). We found that blocking the dephosphorylation of eIF2α with salubrinal increased PC2 and reversed the effect of thapsigargin on PC2 levels in our POMC-positive N43/5 cells, suggesting that this aspect of the UPR could protect the decrease in PC2 during ER stress. Furthermore, enhanced expression of PC2 in cells experiencing ER stress increased both PC2 protein levels and α-MSH levels compared with cells under ER stress with less PC2. Collectively, the results indicate that the effect of obesity-induced ER stress on α-MSH biosynthesis is specific and that ER stress-mediated attenuation of PC2 is primarily responsible for the reduced α-MSH observed in DIO rats. Fig. 10 depicts the proposed model by which ER stress affects POMC biosynthesis and processing in the obese state.

This is the first study, to our knowledge, to demonstrate that ER stress alters neuropeptide processing. A previous study demonstrated that palmitic acid impedes the processing of proinsulin as well as PC2 and PC1 in the pancreatic beta cell line MIN6 (65). Because palmitic acid has been shown to elicit ER stress and the UPR (66), it is possible that ER stress caused these physiological changes. Furukawa *et al.* (65) showed that reduction of PC2 might have been due to impaired processing of the PC2 facilitator 7B2. However, we found no difference in 7B2 levels in DIO or in N43/5 cells subjected to tunicamycin. Our results suggest that ER stress does not mediate changes in PC2 mRNA because neither DIO nor pharmacological induction of ER stress altered PC2 mRNA. However, it is difficult to measure PC2 mRNA expression specifically in POMC neurons because the ARC contains a heterogeneous mixture of POMC and NPY/AgRP cells that have opposing actions on food intake are both regulated by PC2 at the intracellular level (67–69). Thus, we also analyzed PC2 promoter activity in N43/5 POMC-positive cells and found no effect of tunicamycin, which further supports the idea that ER stress does not alter PC2 mRNA. Given our PC2 mRNA and our 7B2 results, it appears that ER stress does not control ARC PC2 transcription or processing. Thus, lower PC2 levels during ER stress are probably due to diminished translation efficiency and/or enhanced clearance of misfolded PC2 to the ER-associated degradation system. Future research will investigate these possibilities and whether obesity-induced ER stress affects processing of prohormones other than POMC, such as proAgrp and proNPY.

Interestingly, we observed an accumulation of POMC protein in DIO compared with lean rats despite the decrease in POMC-derived peptides. Professional secretory cells, such as POMC neurons, have an enhanced capacity to store large amounts of proteins/peptides and as such may be more susceptible to accumulation of these proteins/peptides during ER stress than other cell types. We propose that during DIO, there is an accumulation of unfolded proteins (including POMC) in the ER of ARC cells, which activates the UPR. The UPR increases p-PERK, which phosphorylates eIF2α to then inhibit translation. eIF2α phosphorylation is essential to attenuate translation when the protein-folding load in the ER exceeds the protein-folding capacity. However, long term ER stress in the DIO condition may lead to excess unfolded POMC that collects in the ER, potentially exacerbating the ER stress phenomenon and resulting in less properly packaged POMC precursor for transit and processing. This may explain why we observe less ACTH in DIO despite unaltered POMC mRNA and PC1. Although beyond the scope of the current study, the next generation of this work will explore in detail the changes in POMC folding during obesity-induced ER stress. Recent evidence reveals that pancreatic beta-cell function is dependent on translation inhibition by p-eIF2α during HFD (43). In this

study, attenuation of p-eIF2α, achieved by using a mouse model in which an alanine was substituted for the serine 51 phosphorylation site, led to accumulation of insulin in the ER, beta-cell failure, and type 2 diabetes. Here, the data indicate that the increase in p-eIF2α after 12 weeks of HFD is not sufficient to counterbalance the accumulation of POMC in the ER.

Understanding the central control of energy-regulating neuropeptides during DIO is important for the identification of therapeutic targets to prevent and/or mitigate obesity pathology. Our data have determined that obesity-induced ER stress impairs the production of the anorexigenic neuropeptide α-MSH by affecting its precursor POMC in a post-transcriptional manner. In particular, the processing of POMC is impaired by ER stress that develops in the DIO condition. This mechanism appears to be highly specific, primarily affecting the enzyme PC2, which, within the POMC processing cascade, is essential for converting ACTH to α-MSH. Overall, the data indicate that ER stress regulates POMC processing and that PC2 is a major player in this regulation.

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