Cytosolic phosphoenolpyruvate carboxykinase is a response gene involved in porcine adipocyte adaptation to heat stress

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ABSTRACT: Heat stress (HS) leads to increased lipid storage and expression of cytosolic phosphoenolpyruvate carboxykinase (PCK1) in pig adipocytes. However, the importance of PCK1 activation and lipid storage in the adaptive response to HS is unknown. Therefore, in vitro experiments were conducted to investigate the effect of PCK1 inhibition with 3-mercaptopicolinic acid (3MPA) on lipid storage and adipocyte response during HS. In vitro culture of adipocytes under HS (41.0 °C) increased ($P < 0.05$) triacylglycerol accumulation compared with control (37.0 °C). HS increased ($P < 0.05$) reactive oxygen species level and 3MPA further upregulated

($P < 0.05$) its level. Heat shock protein 70 (HSP70) gene expression was induced ($P < 0.05$) by HS compared to control, and PCK1 inhibition with 3MPA attenuated ($P < 0.05$) its induction by HS. The endoplasmic reticulum (ER) stress markers, C/EBP homologous protein (CHOP) was also upregulated by HS and 3MPA further upregulated ($P < 0.05$) CHOP mRNA level. These results suggest that with inhibition of PCK1 during HS, in vitro cultured adipocytes were less able to induce adaptive responses such as upregulation of HSP70 and triglycerides, and this exacerbated ER stress during HS. Thus, PCK1 may function to alleviate ER stress that occurs during HS.

Key words: adipocyte, ER stress, heat stress, PCK1 or PEPCK

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INTRODUCTION

Heat stress (HS) induces systemic and cellular responses that lead to metabolic change aimed at HS adaptation. However, the mechanisms of the adaptive responses to HS are still not well understood. In our previous study in adipocytes differentiated in vitro (Qu et al., 2015), we showed that, despite hypercatabolic effects of HS, adipocytes have upregulated lipogenesis in a cell autonomous manner, with the resulting increase in cellular triglyceride level. Studies have shown that HS resulted in increased carcass fat in pig (Bridges, 1998; Collin et al., 2001), through

induction of lipoprotein lipase (LPL; Kouba et al., 2001) and perhaps increased insulin signaling (Pearce et al., 2013). We also reported that HS led to an upregulation of glyceroneogenesis marker, cytosolic phosphoenolpyruvate carboxykinase (PCK1/PEPCK-C) (Qu et al., 2016) and to a limited extent, glycerol kinase in adipose tissue in vivo. However, cytosolic PCK1 is the key glyceroneogenesis enzyme in the adipocyte that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate (Chang and Lane, 1966). At present, it is unclear whether PCK1 plays any role in the adaptive response to HS either in vivo or in vitro, although glycerol, a metabolic product of PCK1 is a known chemical chaperon (Deocaris et al., 2008; Makhija et al., 2014) that may play an important role during HS response. The use of 3-mercaptopicolinic acid (3MPA) as a gluconeogenesis inhibitor, through inhibition of PCK1, has been reported previously (Jomain-Baum et al.,

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1976). This inhibitor acts as a noncompetitive inhibitor of PCK1 with respect to oxaloacetate and Mn^{2+} . Thus, we hypothesized that inhibiting PCK1 in heat-stressed adipocytes would demonstrate its importance in adipocyte HS response.

Therefore, the objective of this study was to determine the response of adipocytes to HS when the enzyme activity of PCK1 was inhibited with 3MPA.

MATERIALS AND METHODS

Primary Cell Isolation and Cell Culture

The Purdue Animal Care and Use Committee (PACUC) approved all animal care and use protocols described in these experiments. Isolation and culture of primary porcine preadipocytes were based on the protocol described previously (Qu et al., 2015). Briefly, preadipocytes/stromavascular cells (SVC) were isolated from the inner layer of subcutaneous adipose tissue from neonatal male piglets (less than 7 d old). Excised adipose tissue was immediately kept in buffered saline (0.15 M NaCl, 10 mM HEPES, pH 7.4) at 37 °C. The tissue was minced with a pair of scissors and then incubated with a digestion cocktail (10 mM $NaHCO_3$, 10 mM HEPES, 5 mM D-glucose, 120 mM NaCl, 4.6 mM KCl, 1.25 mM $CaCl_2$, 1.20 mM $MgSO_4$, 1.20 mM KH_2PO_4 , 3% BSA) containing collagenase (Cat. # CLS1, collagenase type I, 1 mg/mL, Worthington Biochemical Corp., Lakewood, NJ) in a shaking water bath for 45 min at 120 oscillation/min at 37 °C. When thoroughly digested, sample was centrifuged at $2,000 \times g$ at 22 °C for 10 min to separate the floating adipocytes from the precipitated SVC pellet. The SVC was washed twice in the digestion cocktail. Afterward, the pellet was suspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium (Cat. # D8900, Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; Cat. # 35010CV, Mediatech, Manassas, VA), 1% antibiotic-antimycotic (Cat. # A5955, Sigma-Aldrich), plated in sterile 24-well cell culture plates and incubated in a humidified incubator with 5% CO_2 and 95% air. Adipocytes from three pigs were pooled. Experiments were conducted with several replicates of these pooled cells. Upon reaching confluence (approximately after 4–5 d), cells were differentiated in a differentiation medium (DMEM/F12 with 10% FBS, 1% antibiotic-antimycotic, 1 μ M insulin, 1 μ M dexamethasone (1 mM), 1 μ M rosiglitazone, 1 μ M biotin, 1 μ M triiodothyronine, 1 μ M pantothenic

acid). Medium was replaced every 3 d and rosiglitazone was removed from the differentiation media after day 3 of differentiation. Cells were treated with 0.05 mM of the PCK1 inhibitor, 3MPA, (Cat. # 320386-54-7, Santa Cruz Biotechnology, Santa Cruz, CA) dissolved in DMSO or DMSO control on day 7 of differentiation and set at either 37 °C (normal) or HS (41.5 °C) temperature conditions. The treatment medium was changed after 2 d. The experiment was terminated on day 9 of differentiation. Each experiment was repeated six different times (replicates).

Gene Expression Analysis

Total RNA was extracted from cells using the QIAzol lysis reagent (Cat. # 79306, Qiagen, Valencia, CA). Extracted RNA was then dissolved in nuclease-free water (Cat. # AM9906, Ambion, Austin, TX). The concentration of extracted RNA was measured on a Nanodrop 1000 instrument (Thermo Scientific, Waltham, MA). Integrity of RNA and genomic DNA contamination were checked by electrophoresis on 0.8% agarose with ethidium bromide staining. One- μ g RNA was reverse transcribed with moloney murine leukemia virus (MMLV) reverse transcriptase (Cat. # M1701, Promega, Madison, WI). PCR assay was done on a Bio-Rad MyiQ thermocycler (Bio-Rad, Temecula, CA). The PCR reaction mixtures contained 0.5 μ g of cDNA, 0.075 nmol of forward and reverse primers, respectively, and RT2 SYBR Green qPCR master mix (Cat. # 330513, Qiagen) in a total reaction volume of 20 μ L. The reaction mix was incubated for 5 min at 95 °C for the initial denaturation step, and then 40 cycles of the following steps: 10 s at 95 °C, 20 s at 55 °C, and 20 s at 72 °C. Real time-PCR primer sequences are presented in Table 1. Abundance of mRNA for each gene was calculated after its cycle threshold (Ct) was normalized to the Ct for 18S using the $\Delta\Delta C_t$ method.

Immunoblotting Analysis

Cells were rinsed free of medium twice with cold $1 \times$ PBS and then suspended in $1 \times$ radio-immunoprecipitation assay (RIPA) buffer (10% Nonidet P-40, 0.5 M Tris-HCl, 2.5% deoxycholic acid, 1.5 M HCl, and 10 mM EDTA) supplemented with commercial protease and phosphatase inhibitor cocktails (Cat. # P8340 and Cat. # P2850, Sigma-Aldrich). Protein concentrations were measured with the bicinchoninic acid assay reagent (Cat. # BCA1, Sigma-Aldrich). Proteins (40 μ g per sample) were resolved on a 10% SDS polyacrylamide gel and

Table 1. List of PCR primers

Gene1	Forward	Reverse
18S	5'-ATC CCT GAG AAG TTC CAG CA-3'	5'-CCT CTT GGT GAG GTC GAT GT-3'
AQ7	5'-AGGCACTTCAGCAGACATCTA-3'	5'-TGGCGTGATCATCTTGGAGG-3'
ACOT1	5'-CCTTTCCTGGGATCGTGGAC-3'	5'-GCAAAACCCCTTCCAGCCAG-3'
CPT1	5'-GTCAGCGTAGCAAGTGGACA-3'	5'-GTGACGTTACATCCCCCTGCT-3'
DGAT2	5'-CAC CTA CTC CTT CGG GGA GA-3'	5'-CTT GGA GTA GGG CAT GAG CC-3'
FAS	5'-AGT TTG TGA TGG AGA ACA CGG CCT-3'	5'-TGT TCA CAC GTG GTG CAA GGG TTA-3'
PCK1	5'-CCC TGC CTT TGA AAA AGC CC-3'	5'-GGA GAT GAT TTC TCG GCG GT-3'
CHOP	5'-AAC AAA GTG GCC ATT CCC CA-3'	5'-ACC ATC CGG TCA ATC AGA GC-3'
XBP1	5'-CGGTGGCCGCTACGTGCACC-3'	5'-GGATATCAGACTCAGAGTCT-3'
SREBP1c	5'-ACC GCT CTT CCA TCA ATG AC-3'	5'-AAT GTA GTC GAT GGC CTT GC-3'
HSP70	5'-TTC GTG GAC AGA AGC CAC AG-3'	5'-TTG CTA GGA TCT CCA CCC GA-3'
HSP90	5'-GTC GAA AAG GTG GTT GTG TCG-3'	5'-TTT GCT GTC CAG CCG TAT GT-3'
PDK4	5'-TGC AAT GAG GGC TAC AGT CG-3'	5'-CGG TCA ATG ATC CTC AGG GG-3'
LPL	5'-ATT CAC CAG AGG GTC ACC TG-3'	5'-AGC CCT TTC TCA AAG GCT TC-3'
ATGL	5'-TGC CAA TGA GGA CGT GGG-3'	5'-GCA GCA AGT GAG TGG TTG GT-3'
LDH	5'-ATG TTG CTG GTG TCT CCC TG-3'	5'-TGT GAA CCG CTT TCC AGT GT-3'
SOD1	5'-GTT GGA GAC CTG GGC AAT GT-3'	5'-TCA GAC CAT GGC ATG AGG GA-3'
ATF4	5'-AGT CCT TTT CTG CGA GTG GG-3'	5'-GGT CGA AGG GGG ACA TCA AG-3'
ATF6	5'-GAC CTG TTT TGT CCG TTG TGG-3'	5'-ATG TCA CAA GCA AAT GGC CC-3'
GRP78	5'-TGAGTGGCTGGAAAGTCACC-3'	5'-TTGCCCTGTGCATCTCTTCC-3'

then transferred to nitrocellulose membranes (Cat. #1620112, Bio-Rad). Thereafter, membranes were blotted with primary antibodies: anti- β -actin (Cat. # 4970, Cell Signaling Technology, Danvers, MA), HSP70 (Cat. # Cayman Chemicals, Ann Arbor, MI), anti-HSP90 (Cat. # 4877, Cell Signaling Technology), anti-CCAT/enhancer-binding homologous protein (**CHOP**) (Cat. # 2895, Cell Signaling Technology), phospho-AMP-activated protein kinase (**AMPK**) (Cat. # 2535, Cell Signaling Technology), AMPK (Cat. # 2532, Cell Signaling Technology), phospho-eukaryotic initiation factor 2 (**EIF2 α**) (Cat. # 3398, Cell Signaling Technology), EIF2 α (Cat. # 9722, Cell Signaling Technology). Secondary antibodies used were horseradish peroxidase (**HRP**)-conjugated goat anti-mouse or goat anti-rabbit IgG (Cat. # 7074, Cell Signaling Technology). Membranes were developed with Immobilon HRP substrate (Cat. # WBKLS0500, Millipore, Billerica, MA) and exposed to autoradiographic film (Cat. # sc-201697, Santa Cruz Biotechnology, Dallas, TX). Band intensities were quantified with a Kodak 1 D 3.6 imaging software (Kodak, Rochester, NY). Signal intensity of a specific protein was normalized to that of β -actin for each sample.

Triglyceride and Free Glycerol Determination

Adipocytes were washed three times with warm PBS. Total intracellular lipids, including triacylglycerol (**TAG**), was extracted with isopropanol for 30 min at room temperature with gentle agitation.

Thereafter, TAG content was measured using a TAG determination kit (Cat. # TR0100, Sigma-Aldrich) according to the manufacturer's instructions. Lipolysis was determined by measuring media glycerol concentration in unstimulated cells or cells stimulated with 10 μ M norepinephrine (Cat. # A7257, Sigma-Aldrich) for 2 h. Triglyceride and free glycerol content were normalized to cellular protein content.

Measurement of Cellular ATP

Concentration of ATP was determined in total cellular lysate recovered with 1 $\times$ RIPA buffer. Levels of ATP were measured with an ATP determination kit (Cat. # A22066, Invitrogen, Carlsbad, California). Intracellular ATP amount was normalized to total cellular protein.

Measurement of Cellular Reactive Oxygen Species

Cells were washed with PBS and then loaded with reactive oxygen species (**ROS**)-sensitive dye, CM-H2DCFDA (Cat. # C6827, Thermo Fisher Scientific, St. Louis, MO). Thereafter, cellular ROS level was determined according to the manufacturer's protocol.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Adipocyte viability was determined with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetra-

zolium Bromide (MTT) assay. After treatment of adipocytes in 96-well plates, medium was replaced with fresh medium containing MTT (Cat. # TOX1, Sigma-Aldrich) and viability was determined based on the manufacturer's protocol.

Statistical Analyses

Data were analyzed as a two-way factorial ANOVA with temperature (37 or 41.5 °C) and PCK1 inhibition treatment (DMSO control or 3MPA) as the main factors. These main factors were considered fixed effects, whereas replicate was considered a random effect. Tukey's multiple comparison analysis was used to separate the means when interaction effect was significant according to the procedures of SAS (SAS Inst. Inc., Cary, NC). Differences were considered significant with $P < 0.05$, and P values between 0.05 and 0.10 were considered as showing a strong tendency of significance. Bars in the figures represent means $\pm$ SEM.

RESULTS

Effect of HS and PCK1 Inhibition on Lipogenesis

To determine the effect of PCK1 inhibition on lipid storage during in vitro HS, cellular TAG level was measured in cells treated with 3MPA. HS-induced TAG accumulation in the adipocytes compared with control, and 3MPA treatment numerically reduced (by approximately 20%) the TAG level under HS ($P < 0.05$) (Figure 1A). However, there was no further reduction of TAG level by 3MPA under control temperature. To determine the effect of HS on the viability of adipocyte

in vitro, we performed the MTT assay. Although HS had no effect on viability compared to control, inhibition of PCK1 enzymatic activity with 3MPA treatment resulted in significantly lower ($P < 0.05$) (by approximately 18 %) cell viability than DMSO-treated cells in both 37 and 41.5 °C temperature conditions (Figure 1B).

Effect of HS and PCK1 Inhibition on Lipogenesis Markers

To determine effects of HS and PCK1 inhibition on lipogenesis markers in adipocytes differentiated in vitro, we measured gene expression of key lipogenic enzymes. Expression of PCK1 was significantly elevated ($P < 0.05$) by HS treatment than control (Figure 2A). Moreover, 3MPA significantly suppressed ($P < 0.05$) PCK1 gene expression in control and HS cells. Expression of LPL was induced ($P < 0.05$) by HS (Figure 2B), but 3MPA treatment only tended ($P < 0.08$) to downregulate its expression in both control and HS conditions. There was a tendency ($P < 0.09$) for expression of fatty acid synthase (FAS) to be induced under HS (Figure 2C). Inhibition of PCK1 with 3MPA downregulated ($P < 0.05$) FAS expression in both control and HS condition. Expression of SREBP-1c was induced by HS ($P < 0.05$), but 3MPA did not affect its expression (Figure 2D). Expression of DGAT2 was upregulated by HS treatment ($P < 0.05$), but there was no effect of 3MPA (Figure 2E).

Effect of HS and PCK1 Inhibition on Lipolysis

To determine whether HS and PCK1 had effects on lipolysis, we measured free glycerol

Figure 1. Effects of heat stress and PCK1 inhibition with 3MPA on TAG accumulation and cell viability in adipocytes. (A) Triglyceride concentration was measured in pig adipocytes under normal conditions (37 °C) and heat stress condition (41.5 °C) in cells treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). (B) Cell viability was measured with MTT assay at the end of day 9 in cells treated or not with 3MPA from days 7 to 9. Bars represent means $\pm$ SE of at least six different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

Figure 2. Expression of genes related to adipogenesis in differentiating adipocytes under normal (37 °C) or heat stress (41.5 °C) conditions treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). (A) PCK1, (B) LPL, (C) FAS, (D) SREBP1c, and (E) DGAT2. Bars represent means $\pm$ SE of at least six different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

levels in the cell culture media in unstimulated (basal lipolysis) or norepinephrine-stimulated cells. There was no difference in basal lipolysis in cells in control or HS temperature condition (Figure 3A). However, 3MPA significantly suppressed ($P < 0.05$) basal lipolysis in cells under HS. In norepinephrine-stimulated cells (Figure 3B), there was a tendency ($P < 0.07$) for reduced free media glycerol concentration in HS compared to control. Treatment with 3MPA significantly ($P < 0.05$) suppressed lipolysis in both control and cells under HS (Figure 3B). To determine whether HS regulates expression of an important lipolytic

gene, we measured the expression of adipose tri-glyceride lipase (ATGL) by PCR. Expression of ATGL was significantly induced ($P < 0.05$) by HS compared with control temperature (Figure 3C), and 3MPA downregulated ($P < 0.05$) its expression in cells under both control and HS conditions. To further determine whether HS and PCK1 inhibition affected the expression of genes involved in glycerol transport, we measured the expression of aquaporin7 (AQP7), a protein that is involved in glycerol trafficking that is abundantly expressed in adipocytes (Jin et al., 2014). Although HS induced ($P < 0.05$) AQP7 expression than in

Figure 3. Basal (unstimulated) (A) or norepinephrine-stimulated lipolysis (B) in adipocytes differentiated under normal (37 °C) or heat stress (41.5 °C) conditions and treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). Gene expression of ATGL (C) and AQP7 (D). Bars represent means $\pm$ SE of at least six different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

control cells, 3MPA treatment had no effect on its expression (Figure 3D).

Effect of HS and PCK1 Inhibition on Metabolic Markers and Cellular ATP Level

Expression of beta-oxidation markers, acyl-CoA thioesterase 1 (ACOT1) was not affected by HS or 3MPA (Figures 4A). There was a significant ($P < 0.05$) temperature $\times$ treatment interaction effect on the expression of carnitine palmitoyl-transferase 1 (CPT1) such that control cells treated with 3MPA had the highest expression, whereas its expression was lowest in HS cells treated with this inhibitor. To investigate whether HS had effects on glucose metabolism, we measured gene expression of pyruvate dehydrogenase kinase 4 (PDK4) and lactate dehydrogenase (LDH). Both PDK4 and LDH were induced ($P < 0.05$) by HS and 3MPA had no effects on their expression (Figure 4C and D). HS significantly increased ($P < 0.05$) ATP level (Figures 4E) and 3MPA treatment upregulated ($P < 0.05$) ATP level in cells under both control and HS temperature conditions. We also measured the cellular levels of phosphorylated and total AMPK as markers of cellular energy status. HS treatment

tended ($P < 0.1$) to increase phospho-AMPK/AMPK. However, there was a significant ($P < 0.05$) interaction effect on phospho-AMPK/AMPK ratio such that control cells treated with 3MPA treatment had the same level of phospho-AMPK/AMPK ratio as the two HS treatments, whereas control cells without the inhibitor had the lowest ratio (Figures 4F).

Effect of HS and PCK1 Inhibition on Endoplasmic Reticulum Stress Markers

To determine whether HS-regulated endoplasmic reticulum (ER) stress in in vitro differentiated adipocytes, we measured expression of ER and HS markers. HS tended ($P < 0.08$) to increase ROS level as indicated by the fluorescence of the ROS-sensitive CM-H2DCFDA dye (Figure 5A) and 3MPA further upregulated ($P < 0.05$) its level under both control and HS conditions. Additionally, HS increased ($P < 0.05$) heat shock protein 70 (HSP70) gene expression compared to control (Figure 5B). Treatment with 3MPA downregulated ($P < 0.05$) HSP70 expression only in HS cells (Figure 5B). Inhibition of PCK1 with 3MPA upregulated HSP70 protein

Figure 4. Expression of metabolic markers genes ACOT1 (A), CPT1 (B), PDK4 (C), and LDH (D) in adipocytes differentiated under normal (37 °C) or heat stress (41.5 °C) conditions and treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). Cellular concentration of ATP (nM/μg protein) (E) and p-AMPK/AMPK ratio (F) determined by western blotting. Bars represent means ± SE of at least six different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

in cells in control temperature ($P < 0.05$), but decreased it under HS ($P < 0.05$) (Figure 5C). HS also tended ($P < 0.09$) to induce HSP90 gene expression but had no significant effects on HSP90 protein expression (Figure 5D and E) and 3MPA increased ($P < 0.05$) its mRNA abundance in both control and HS cells (Figure 5D). HS induced ($P < 0.05$) the expression of other stress markers, ATF6 and superoxide dismutase 1 (SOD1), activating transcription factor 4 (ATF4), and p-eIF2 α compared to control (Figure 6A and B), and 3MPA further increased ($P < 0.05$) their expression in both control and HS cells. Expression of ATF4 was induced ($P < 0.05$) by HS, but not affected by 3MPA (Figure 6C). The expression of ER stress markers, CHOP and

x-box binding protein 1 (XBP1) (Figure 7A and C) were induced ($P < 0.05$) in HS and further upregulated ($P < 0.05$) by 3MPA in both control and HS conditions. Protein abundance of CHOP (Figure 7B) was not affected by either HS or 3MPA. Expression of 78 kDa glucose-regulated protein (GRP78) was induced only by HS (Figure 7D), but there was no effect of 3MPA.

DISCUSSION

Adipocytes exposed to HS in vitro respond by increasing cellular triglyceride storage (Qu et al., 2015). We have demonstrated before (Qu et al., 2015) and in this experiment that this response is accompanied by increased expression of lipogenic genes

Figure 5. Concentration of ROS measured by CM-H2DCFDA fluorescence (A), HSP70 gene expression (B), HSP70 protein abundance (C), HSP90 gene expression (D), and HSP90 protein abundance (E) in adipocytes differentiated under normal (37 °C) or heat stress (41.5 °C) conditions and treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). Bars represent means $\pm$ SE of at least six different replicates. Superscript letters (a, b and c) indicate significant mean difference at $P < 0.05$.

such as LPL, PCK1, PPAR γ , SREBP1c, FAS, and DGAT2. Previous *in vivo* studies have shown that HS resulted in increased carcass fat in pig (Bridges, 1998; Collin et al., 2001), through induction of LPL (Kouba et al., 2001) and insulin-signaling pathway (Pearce et al., 2013). Our previous experiment also confirmed induction of numerous lipogenic genes

such as PCK1 and LPL in the subcutaneous fat depot of pigs exposed to HS for 7 d (Qu et al., 2016). Thus, although the precise mechanisms are still not completely known, HS involves increased lipogenesis in pigs, and this response is always accompanied by increased PCK1 expression, suggesting that this gene might play a critical role in the

Figure 6. Expression of endoplasmic reticulum stress marker genes ATF6 (A), SOD1 (B), ATF4 (C), and protein abundance of EIF2α (D) in adipocytes differentiated under normal (37 °C) or heat stress (41.5 °C) conditions and treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). Bars represent means $\pm$ SE of at least six different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

increased lipid storage in adipocytes during HS. We have further demonstrated increased lipid storage in adipocytes under HS in this experiment and inhibition of PCK1 activity with 3MPA led to about 20% lower TAG storage under HS. This suggests that PCK1 might play an important role in the lipogenic response of adipocytes under HS challenge. Increased expression of PCK1 in adipocytes under HS demonstrates an increased need for glycerol during hyperthermia in adipocytes. Indeed, HS was accompanied by increased expression of AQP7, a gene that is involved in glycerol uptake, confirming this possibility. This agrees with studies that showed induction of AQP7 under HS (Sugimoto et al., 2013; Wang et al., 2015). However, AQP7 expression was not affected by PCK1 inhibition.

Expression of genes involved in beta-oxidation (ACOT1 and CPT1) was not affected by HS in this study. However, HS led to increased expression of PDK4 and LDH. This induction of these genes suggests that HS may indeed enhance channeling

of substrates into glycolysis and glyceroneogenesis. Pyruvate entry into mitochondrial tricarboxylic acid cycle as acetyl-coA is inhibited by PDK4 (Harris et al., 2002). PDK4 is known as a gate-keeper directing the carbon flux into glycolysis via inhibition of the pyruvate dehydrogenase complex. PDK4 is key to the shift from glycolysis to oxidative phosphorylation (Liu et al., 2017). LDH catalyzes the final step in anaerobic glycolysis through the conversion of pyruvate to lactate (Vander Heiden et al., 2009). This step is critical for allowing anaerobic respiration and glycolysis to continue. The induction of these genes may provide more substrates for PCK1 for glyceroneogenesis in support of increased adipogenesis under HS.

A potential mechanism for the increased lipid storage in adipocyte under HS might include reduced norepinephrine-stimulated lipolysis. Although basal lipolysis was not different between control cells and those under HS, there was a tendency for lower stimulation of free glycerol release

Figure 7. Expression of endoplasmic reticulum stress markers CHOP mRNA (A), CHOP protein (B), XBP1 mRNA (C), and GRP78 mRNA (D) in adipocytes differentiated under normal (37 °C) or heat stress (41.5 °C) conditions and treated from days 7 to 9 of differentiation with or without 0.05 mM of the PCK1 inhibitor (3MPA). Bars represent means $\pm$ SE of at least six different replicates. Superscript letters (a and b) indicate significant mean difference at $P < 0.05$.

in cells under HS. The reduction of lipolysis in cells under HS may not involve downregulation of ATGL expression because the expression of this gene is actually upregulated by HS. Although PCK1 inhibition further reduced lipolytic response to norepinephrine in both control and heat-stressed cells, and this lipolytic desensitization was accompanied by reduction in the expression of ATGL. This suggests that ATGL might still be involved in some aspects of lipolysis in adipocytes.

Exposure to HS in vitro induces of HSP which can minimize cellular damage to maintain cellular homeostasis (Cvoro et al., 2004). Among the many HSPs family, HSP70 and HSP90 are well-known members that protects cells and tissues from different stress conditions. HSP70 is a molecular chaperone that is involved in protein folding, transportation, and translocation (Cvoro et al., 2004). Accumulation of unfolded or misfolded proteins results in ER stress or unfolded protein response (UPR). Endoplasmic

reticulum stress activates markers such as ATF4, ATF6, SOD1, XBP1, and GRP78 to restore ER homeostasis (Fulda et al., 2010). The UPR also induces generation of ROS which could disrupt cellular membrane integrity and function. In this study, we demonstrate that HSP70 is induced by HS and there was a tendency for HSP90 to be similarly induced. However, PCK1 inhibition with 3MPA reduced HSP70 gene and protein expression. The reduction of HSP70 expression by 3MPA during HS could be linked to the reduced viability of cells in HS that were treated with 3MPA because HSP70 is a recognized survival factor (Silver and Noble, 2012). Additionally, HSP70 could be redirected to the surface lipid droplets in adipocytes during HS (Jiang et al., 2007) to prevent misfolding of lipid droplet-associated metabolic enzymes and other critical proteins, and translocation of HSP70 to the surface of lipid droplets could potentially lead to stabilization of lipid droplets during HS

(Jiang et al., 2007). The reduction of lipolysis in norepinephrine-stimulated cells under HS might be a reflection of lipid droplet stabilization by HSP70 during HS. Reduction of HSP70 level by 3MPA could contribute to enhanced protein denaturation under HS, and the observed increase in ER stress and cell death.

The increase of ROS level, CHOP expression, and the decrease in cell viability in 3MPA-treated cells supports the idea that PCK1 might be critical for adipocyte survival and maintenance of cellular homeostasis, especially during HS. This may also be linked to decreased HSP70 by 3MPA in HS because HSP70 may be crucial for cellular survival under HS-induced ER stress. The ER has many important functions, including posttranslational modification, folding, and assembly of newly synthesized proteins. Proper ER function is essential for the survival of cells (Araki et al., 2003). We demonstrated that in vitro HS promotes oxidative stress and ER stress which is evidenced by intracellular ROS accumulation and increased expression of ER stress markers such as CHOP, XBP1, ATF4, ATF6, SOD1, and GRP78. Thus, the increase in cellular ROS and ER stress markers like CHOP and the decreased cell viability by 3MPA support a critical role for PCK1 or lipogenesis in alleviating HS-induced cytotoxicity.

HS induces increase of cellular ATP concentration for meeting increased cellular need for extra ATP for synthesis of new chaperone proteins such as HSP and others (Soini et al., 2005). Heat adaptation is an energy-demanding process that involves interaction of different co-chaperones, co-factors, and their substrates, and these interactions often involve ATP hydrolysis, which in turn affects substrate turnover rates (Mallouk et al., 1999; Kampinga and Craig, 2010). Although AMPK, is typically activated when intracellular ATP level is low (Mihaylova and Shaw, 2011), the tendency for an increase in AMPK activation in heat-stressed cells, despite the high ATP level in these cells, may be to support the increased need for ATP for the adaptive response to HS. Thus, AMPK might be a cellular signal for increased energy generation during HS in adipocyte.

In summary, inhibiting PCK1 enzymatic activity in vitro decreases the thermotolerance of the adipocyte. Overall, in addition to its well-characterized metabolic function in glyceroneogenesis and lipogenesis, this work indicates a possible implication of PCK1 in HS adaptation in pig adipocytes. These findings suggest that upregulation of PCK1 is a critical adaptive feature of pig adipocytes

exposed to HS in vitro. Therefore, PCK1 may be an important target for stress alleviation in the adipocyte during HS or other stressful conditions such as obesity and type II diabetes treatment.

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