Methionine supplementation during a hydrogen peroxide challenge alters components of insulin signaling and antioxidant proteins in subcutaneous adipose explants from dairy cows

N. Ma,^{1,2} Y. Liang,¹ D. N. Coleman,¹ Y. Li,^{1,3} H. Ding,^{1,3} F. Liu,^{1,4} F. F. Cardoso,¹ C. Parys,⁵ F. C. Cardoso,¹ X. Shen,² and J. J. Loor^{1*}

¹Department of Animal Sciences, Division of Nutritional Sciences, University of Illinois, Urbana 61801

²College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, 210095, Jiangsu, China

³Department of Veterinary Medicine, College of Animal Science and Technology, Anhui Agricultural University, Hefei, 230036, Anhui, China

⁴Department of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou, 450086, Henan, China

⁵Evonik Operations GmbH | Nutrition & Care, Hanau 63457, Germany

ABSTRACT

Enhanced postruminal supply of methionine (Met) during the periparturient period alters protein abundance of insulin, AA, and antioxidant signaling pathways in subcutaneous adipose tissue (SAT). Whether SAT is directly responsive to supply of Met and can induce molecular alterations is unknown. Our objective was to examine whether enhanced Met supply during an oxidative stress challenge *in vitro* alters insulin, AA, inflammation, and antioxidant signaling-related protein networks. Four late-lactation Holstein cows (average 27.0 kg of milk per day) were used for SAT collection. Tissue was incubated in duplicate for 4 h in a humidified incubator with 5% CO₂ at 37°C according to the following experimental design: control medium with an “ideal” profile of essential AA (CTR; Lys:Met 2.9:1), CTR plus 100 μM H₂O₂ (HP), or CTR with greater Met supply plus 100 μM H₂O₂ (HPMET; Lys:Met 2.5:1). Molecular targets associated with insulin signaling, lipolysis, antioxidant nuclear factor, erythroid 2 like 2 (NFE2L2), inflammation, and AA metabolism were determined through reverse-transcription quantitative PCR and western blotting. Data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc.). Among proteins associated with insulin signaling, compared with CTR, HP led to lower abundance of phosphorylated AKT serine/threonine kinase (p-AKT) and solute carrier family 2 member 4 (SLC2A4; insulin-induced glucose transporter). Although incubation with HPMET restored abundance of SLC2A4 to levels in the CTR and upregulated abundance of fatty acid synthase (FASN) and phosphorylated 5'-prime-AMP-

activated protein kinase (p-AMPK), it did not alter p-AKT, which remained similar to HP. Among proteins associated with AA signaling, compared with CTR, challenge with HP led to lower abundance of phosphorylated mechanistic target of rapamycin (p-mTOR), and HPMET did not restore abundance to CTR levels. Among inflammation-related targets studied, incubation with HPMET led to greater protein abundance of nuclear factor kappa B subunit p65 (NFκB-RELA). The response in NFκB observed with HPMET was associated with a marked upregulation of the antioxidant transcription regulator NFE2L2 and the antioxidant enzyme glutathione peroxidase 1 (GPX1). No effects of treatment were detected for mRNA abundance of pro-inflammatory cytokines or antioxidant enzymes, underscoring the importance of post-transcriptional regulation. Overall, data indicated that short-term challenge with H₂O₂ was particularly effective in reducing insulin and AA signaling. Although a greater supply of Met had little effect on those pathways, it seemed to restore the protein abundance of the insulin-induced glucose transporter. Overall, the concomitant upregulation of key inflammation and antioxidant signaling proteins when a greater level of Met was supplemented to oxidant-challenged SAT highlighted the potential role of this AA in regulating the inflammatory response and oxidant status. Further studies should be conducted to assess the role of postruminal supply of Met and other AA in the regulation of immune, antioxidant, and metabolic systems in periparturient cow adipose tissue.

Key words: insulin signaling, adipose tissue, oxidative stress, amino acids

INTRODUCTION

Dairy cows experience negative energy balance along with systemic inflammation and oxidative stress dur-

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*Corresponding author: jloor@illinois.edu

ing the peripartal period (Sordillo and Aitken, 2009; Sordillo and Raphael, 2013; Bradford et al., 2015). Body fat mobilization during negative energy balance helps fill the deficit between intake and requirements in peripartal cows (Loor et al., 2013), but recent evidence indicates that excessive lipolysis could deplete the tissue's antioxidant reserves (Sun et al., 2016; Contreras et al., 2017b). Together, lipolysis, inflammation, and oxidative stress appear closely associated with insulin resistance and could delay recovery of fat depots in dairy cows (McNamara and Huber, 2018). In nonruminants, oxidative stress is considered a major contributing factor of insulin resistance, negatively affecting the control of glucose uptake, protein synthesis, lipogenesis, and aggravating lipolysis (Curtis et al., 2010; Hurrell and Hsu, 2017).

In dairy cows, although a direct link between oxidative stress and insulin resistance has not been concretely established, changes in circulating biomarkers of insulin action in adipose and muscle tissue (Bell and Bauman, 1997; Hayirli, 2006) along with reactive oxygen species (ROS; Bernabucci et al., 2005) especially after calving suggest a potential link. Although the metabolic picture of insulin resistance in nonruminants is markedly different than in ruminants (especially after calving), it is unknown whether increased availability of oxidants can alter insulin signaling or some of its components in bovine adipose tissue.

Methionine, an indispensable AA in lactating cows, not only improves milk protein production (Sun et al., 2016) but enhances innate immune response at the functional and transcriptional level (Zhou et al., 2016; Batistel et al., 2018; Lopreiato et al., 2019). Glutathione (GSH) is a major antioxidant in mammalian cells synthesized from metabolism of Met via the transsulfuration pathway (Martinov et al., 2010). It helps remove ROS via GSH-dependent glutathione peroxidase (GPX)-catalyzed reactions within the cytosol (Lushchak, 2012). Recently, our group reported that feeding rumen-protected Met (RPM) at a rate of approximately 0.09% of DM intake to periparturient cows led to greater mRNA (cystathionine- β -synthase, glutamate-cysteine ligase modifier subunit, glutathione reductase, and *GPX1*) and protein abundance [*GPX1*, *GPX3*, glutathione S-transferase mu 1 (*GSTM1*), and glutathione S-transferase α 4] of targets associated with GSH metabolism in subcutaneous adipose tissue (SAT; Liang et al., 2019a,b). Because protected Met also led to greater DMI (Batistel et al., 2017), the precise effect of enhanced circulating concentrations of Met (Vailati-Riboni et al., 2019) on tissue-specific mechanisms could not be discerned with certainty.

The main objective of the current study was to generate preliminary data on the effect of increasing Met

supplementation on insulin, AA, inflammation, and antioxidant signaling-related molecular networks in explants of SAT from Holstein cows. Target proteins studied were those for which commercially available antibodies have already been used successfully in our laboratory (Elolimy et al., 2019; Liang et al., 2019a,b, 2020). Although ROS encompass molecules such as superoxide anion (O_2^-) and hydroxyl radicals ($OH^\cdot$) that can lead to oxidative stress, we chose hydrogen peroxide (H_2O_2) to challenge the SAT because data from several studies (Pedernera et al., 2010; Hurrell and Hsu, 2017) support the view that it is an important redox switch. We have used this molecule in several in vitro studies including bovine hepatocytes (Zhang et al., 2020), bovine mammary epithelial cells (Ma et al., 2018a,b,c, 2019), and dairy cow SAT adipocytes (Xu et al., 2021). Our recent work also demonstrated temporal changes in H_2O_2 in SAT biopsies during the transition period, supporting the biological relevance of this molecule in the context of redox state (Liang et al., 2019b, 2020).

MATERIALS AND METHODS

Adipose Explant Culture and Treatments

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (Urbana; protocol no. 19036). Details of cows used, cell culture protocols, and molecular methods in a similar study involving SAT explants incubated with increased concentrations of Met and Arg during a challenge with *N*-acetyl-D-sphingosine were reported previously (Liang et al., 2021). Briefly, 4 healthy multiparous lactating Holstein cows from the University of Illinois dairy herd were used. Average parity, DIM, and milk yield before slaughter were 4 ± 0.4 , 248 ± 18 d, and 27.0 ± 3.5 kg/d, respectively. Cows were euthanized by captive bolt at the University of Illinois College of Veterinary Medicine diagnostic facilities (Urbana). Samples of SAT from the tail-head of each cow were obtained immediately after slaughter and brought to the laboratory in warm Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (Sigma-Aldrich) containing 1% penicillin/streptomycin (Sigma-Aldrich) within 30 min of collection. Subsequently, tissue (500–600 mg) was trimmed into pieces using a sterile scalpel blade in a sterile Petri dish (catalog no. 101VR20, Thermo Fisher Scientific), and then 200 mg incubated in duplicate in 5 mL of medium in 6-well plates. Culture media were as follows: control medium with an "ideal" profile of EAA (CTR; Lys:Met 2.9:1; Dong et al., 2018); CTR plus $100 \mu M H_2O_2$ (HP); and CTR with enhanced Met supply (Vailati-Riboni et al., 2019; Dai et al., 2020) plus

100 μM H_2O_2 (**HPMET**; Lys:Met 2.5:1; Table 1). The choice of H_2O_2 dose was based on our previous in vitro experiments with bovine mammary epithelial cells (Ma et al., 2018a,b,c) and dairy cow (Xu et al., 2021) or calf (Sun et al., 2019) SAT adipocytes, demonstrating lack of negative effects (e.g., cell viability) of this molecule at concentrations between 0 and 100 μM . Similarly, other studies with nonruminant cells have also revealed no deleterious effects on cell functionality with our chosen dose (Gülden et al., 2010; Hao et al., 2017). Although tissue viability measurements have not been reported in bovine SAT explant studies published to date (e.g., Ingle et al., 1972; McNamara et al., 1995; Lanna and Bauman, 1999; Khan et al., 2013; non-exhaustive tabulation in Supplemental Table S1, <https://doi.org/10.17632/d5xdrqdkjb.1>; Loor, 2021), we used activity of superoxide dismutase (**SOD**; Xu et al., 2021) as a potential indicator of oxidative stress and cell death (Petrache et al., 2008). Incubations were carried out in a humidified incubator with 5% CO_2 at 37°C. After 4 h incubation, SAT explants were transferred from 6-well plates to screw-capped microcentrifuge tubes, snap-frozen in liquid nitrogen, and preserved at -80°C until further analysis. The choice of incubation time length is well within published research with bovine adipose tissue (Supplemental Table S1). In fact, the responsiveness of lactating dairy cow SAT explants in chronic cultures (48 h) has been reported and validated previously (Lanna and Bauman, 1999).

RNA Isolation, cDNA Synthesis, and Quantitative PCR

Detailed procedures for measuring target mRNA abundance have been reported in several of our previous publications (Han et al., 2018b; Dai et al., 2020). Briefly, total RNA was isolated from SAT using an miRNeasy kit (catalog no. 217004, Qiagen) following the manufacturer's instruction. Concentration of RNA was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Quality of RNA samples was further ascertained with an Agilent 2100 Bioanalyzer (Agilent Technologies). Average RNA integrity number of samples used was 9.47 ± 0.21 . Total RNA (100 ng/ μL RNase-free water) was reverse-transcribed to cDNA. After 1:4 dilution, cDNA was used for quantitative (q)PCR. The qPCR system included SYBR Green Fast Mix ROX (catalog no. 101414-280, Quanta Biosciences), forward and reverse primers, and nuclease-free water. The qPCR was operated in an ABI Prism 7900 HT SDS instrument (Applied Biosystems). Ribosomal protein S9, *GAPDH*, and *ACTB* were chosen as internal control genes to normalize mRNA data

by their geometric mean. For each gene, normalized qPCR data are presented as fold-change relative to CTR. To estimate standard errors for CTR data and prevent biases in statistical analysis, normalized data were transformed to obtain a perfect mean of 1.0, leaving the proportional difference between the biological replicate. The same proportional change was calculated for the treatment groups to obtain a fold-change relative to CTR. All reactions were run in triplicate. Gene symbols, primer information, and qPCR performance are included in Supplemental Tables S2 and S3 (<https://doi.org/10.17632/d5xdrqdkjb.1>; Loor, 2021).

Western Blotting

Details of the western blotting procedure were reported previously (Batistel et al., 2017; Liang et al., 2021). Briefly, approximately 100 of mg adipose tissue was homogenized in 1 mL of RIPA Lysis and Extraction Buffer (catalog no. 89900, Thermo Fisher) containing Halt protease and phosphatase inhibitor cocktail (100 $\times$, catalog no. 78442; Thermo Fisher Scientific) for 60 s and then placed on ice for 20 min. The lysate was centrifuged at $14,000 \times g$ for 15 min at 4°C. Protein concentration of the supernatant was determined using a Pierce Bicinchoninic Acid Protein Assay Kit (catalog no. 23227, Thermo Fisher Scientific). Protein samples were denatured by heating at 95°C for 5 min before loading 20 μL of protein into each lane of 4% to 20% Mini-Protean TGX precast gels (catalog no. 4561093, Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (catalog no. 1620261, Bio-Rad) in a

Table 1. Amino acid composition of control medium with an “ideal” profile of EAA (CTR; Lys:Met 2.9:1), CTR plus 100 μM H_2O_2 (HP), and CTR with added Met plus 100 μM H_2O_2 (HPMET; Lys:Met 2.5:1)

Item ($\mu\text{g}/\text{mL}$)	Treatment ¹		
	CTR	HP	HPMET
L-Lysine	175	175	175
Lys:Met	2.9:1	2.9:1	2.5:1
L-Methionine	60	60	70
L-Arginine	84	84	84
L-Histidine	74	74	74
L-Isoleucine	121	121	121
L-Leucine	206	206	206
L-Phenylalanine	93	93	93
L-Threonine	97	97	97
L-Tryptophan	16	16	16
L-Valine	142	142	142

¹CTR ratio of EAA was as follows: Lys:Met = 2.9, Lys:Thr = 1.8, Lys:His = 2.38, Lys:Val = 1.23, and Thr:Phe = 1.05, according to published recommendations (NRC, 2001; Haque et al., 2012) and our previous studies (Li et al., 2016; Vailati-Riboni et al., 2019). The level of added Met in the HPMET treatment was according to our previous work (Dong et al., 2018).

Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog no. 170-3940, Bio-Rad). After blocking with 5% nonfat milk in 1× Tris-buffered saline (1× TBST) at room temperature for 2 h, the membrane was incubated with primary antibodies (Supplemental Table S4; <https://doi.org/10.17632/d5xdrdkjb.1>; Loor, 2021) overnight at 4°C. In an attempt to assess molecular responses associated with cell death, we tested primary antibodies for HSP70 (catalog no. 4872, Cell Signaling Technology Inc.) and haptoglobin (sc-69782, Santa Cruz Biotechnology Inc.) at dilutions of 1:500 and 1:250, but were unsuccessful in detecting a signal. The membrane was washed with 1× TBST, and incubated with HRP goat anti-rabbit IgG as the secondary antibody (catalog no. 7074S, Cell Signaling Technology, diluted to 1:1,000) for 1 h. Visualization of target proteins was performed using the Clarity Western ECL Substrate (catalog no. 170-5060, Bio-Rad). Each protein was normalized against the expression of GAPDH, and data are reported as relative expression. The enhanced chemiluminescence signals were acquired using an imaging system (ChemiDoc MP, Bio-Rad), and the intensities of the bands were analyzed using Image-Pro Plus 6.0 software (Bio-Rad). All information for primary antibodies is listed in Supplemental Table S3.

SOD in SAT

Superoxide dismutase activity (catalog no. 706002, Cayman Chemical) was analyzed in SAT using a commercial kit following the manufacturer's protocols. We have previously used this kit with SAT biopsies from periparturient cows (Liang et al., 2019b).

Statistical Analysis

Normalized data (western blotting, reverse-transcription PCR) are reported as fold change relative to CTR. To estimate standard errors in CTR and prevent biases in statistical analysis, normalized data were transformed to obtain a perfect mean of 1.0, leaving the proportional difference between the biological replicate. The same proportional difference was calculated for the HP and HPMET data to obtain a fold change relative to control. The final data set was analyzed using the MIXED procedure of SAS 9.4 (SAS Institute Inc.) according to the following model with repeated measures:

$$Y_j = \mu + M_j + e_j,$$

where Y_j = dependent, continuous variable, μ = overall mean, M_j = fixed effect of treatment (j = CTR, HP, or HPMET), and e_j = residual error. Cow was the random effect. When treatment was significant, least squares means separation between groups was performed using the PDIFF statement with Tukey adjustment. Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances was checked with plots of residuals versus predicted values. Significance was set at $P \leq 0.05$.

RESULTS

Abundance of Insulin and AA Metabolism-Related Proteins

Treatment had a significant effect (Table 2) on the protein abundance of phosphorylated (p)-AKT ($P = 0.05$), p-MTOR ($P < 0.01$), phosphorylated 5'-prime-AMP-activated protein kinase (p-AMPK; $P = 0.05$), and fatty acid synthase (FASN; $P = 0.05$). Compared with CTR, abundance of p-AKT ($P = 0.05$) and p-MTOR ($P < 0.01$) was downregulated by H_2O_2 treatment. Compared with HP, enhanced Met supply resulted in greater protein abundance of p-AMPK and FASN. Additionally, treatment tended to influence the protein abundance of S-adenosylmethionine sensor upstream of TORC1 (SAMTOR; $P = 0.08$) and hormone-sensitive lipase (LIPE; $P = 0.10$). Treatment had a significant effect on the insulin-induced glucose transport carrier, solute carrier family 2 member 4 (SLC2A4; $P = 0.05$; Table 2). Compared with CTR, HP led to downregulation of SLC2A4, and supply of Met restored protein abundance to the levels detected with CTR.

Abundance of Immune Response Proteins and Genes

Protein abundance of nuclear factor kappa B subunit p65 (RELA; $P = 0.02$; Table 3) was significantly affected by treatment. Although HP had no effect, protein abundance of RELA was greater in response to HPMET. At the mRNA level, abundance of the cytokines *IL6*, *TNF*, *IL10*, and *IL1B* was not affected by treatment (all $P > 0.10$; Table 4).

Abundance of Antioxidant Signaling and Metabolism-Related Proteins

Treatment affected the protein abundance of GPX1, the antioxidant transcription regulator nuclear factor, erythroid 2 like 2 (NFE2L2), and the cytosolic oxidant

Table 2. Response in the abundance of proteins associated with insulin and AA signaling, transsulfuration, and nutrient transport in subcutaneous adipose tissue (SAT; $n = 4/\text{treatment}$) cultured in medium with an “ideal” profile of EAA (CTR; Lys:Met 2.9:1), SAT incubated with CTR plus 100 μM H_2O_2 (HP), or SAT incubated with CTR plus enhanced Met supply plus 100 μM H_2O_2 (HPMET; Lys:Met 2.5:1); values are means expressed relative to CTR, which was set to 1.0

Item ¹	Treatment ²			SEM	P-value
	CTR	HP	HPMET		
Insulin signaling					
p-AKT	1.00 ^a	0.26 ^b	0.27 ^b	0.21	0.05
PPARG	1.00	0.56	0.87	0.16	0.25
p-ACACA	1.00	1.24	0.71	0.21	0.24
FASN	1.00 ^b	0.93 ^b	1.78 ^a	0.21	0.05
p-AMPK	1.00 ^b	0.81 ^b	2.76 ^a	0.47	0.05
LIPE	1.00	0.54	1.02	0.15	0.10
AA signaling					
p-MTOR	1.00 ^a	0.40 ^b	0.20 ^b	0.08	<0.01
SAMTOR	1.00	0.48	0.41	0.17	0.08
Transsulfuration					
CBS	1.00	0.50	0.76	0.32	0.59
Glucose and AA transport					
SLC1A3	1.00	0.70	0.90	0.30	0.87
SLC2A4	1.00 ^{ab}	0.48 ^b	1.23 ^a	0.19	0.05

^{a,b}Means between treatments with different lowercase superscripts differ ($P \leq 0.05$).

¹CBS = cystathionine β -synthase; FASN = fatty acid synthase; LIPE = hormone-sensitive lipase; p-ACACA = phosphorylated acetyl-CoA carboxylase α ; p-AKT = phosphorylated AKT serine/threonine kinase; p-AMPK = phosphorylated 5'-prime-AMP-activated protein kinase; p-MTOR = phosphorylated mechanistic target of rapamycin; PPARG = peroxisome proliferator activated receptor gamma; SAMTOR = S-adenosylmethionine sensor upstream of TORC1; SLC1A3 = solute carrier family 1 member 3; SLC2A4 = solute carrier family 2 member 4 (GLUT4).

²Each protein was first normalized against the expression of GAPDH before calculating differences relative to CTR (which was set to 1.0).

product detoxification enzyme GSTM1 (Table 3). Compared with the HP cultures, enhanced supply of Met led to greater NFE2L2 ($P = 0.03$) and GPX1 ($P = 0.05$) but lower GSTM1 ($P = 0.02$) protein abundance.

SOD Activity

Although activity of SOD was numerically lower (~75%) with HP (0.53 ± 0.06 U/mg of protein) and

Table 3. Response in the abundance of inflammation- and antioxidant-related proteins in subcutaneous adipose tissue (SAT; $n = 4/\text{treatment}$) cultured in medium with an “ideal” profile of EAA (CTR; Lys:Met 2.9:1), SAT incubated with CTR plus 100 μM H_2O_2 (HP), or SAT incubated with CTR plus enhanced Met supply plus 100 μM H_2O_2 (HPMET; Lys:Met 2.5:1); values are means expressed relative to CTR, which was set to 1.0

Item ¹	Treatment ²			SEM	P-value
	CTR	HP	HPMET		
Inflammation					
RELA	1.00 ^b	0.64 ^b	1.83 ^a	0.25	0.02
Antioxidant signaling					
NFE2L2	1.00 ^b	0.99 ^b	3.82 ^a	0.65	0.03
KEAP1	1.00	1.61	2.01	0.35	0.27
Glutathione metabolism					
GPX1	1.00 ^b	0.60 ^b	1.95 ^a	0.28	0.05
GSTM1	0.98 ^a	0.79 ^a	0.43 ^b	0.11	0.02

^{a,b}Means between treatment with different lowercase superscripts differ ($P \leq 0.05$).

¹GPX1 = glutathione peroxidase 1; GSTM1 = glutathione S-transferase mu 1; KEAP1 = kelch-like ECH-associated protein 1; NFE2L2 = nuclear factor, erythroid 2 like 2; RELA = nuclear factor kappa B subunit p65.

²Each protein was first normalized against the expression of GAPDH before calculating differences relative to CTR (which was set to 1.0).

HPMET (0.42 ± 0.06 U/mg of protein) relative to CTR (0.61 ± 0.06 U/mg of protein), no statistical effect was detected ($P = 0.19$).

DISCUSSION

Work from our laboratory using transcriptomics and bioinformatics on adipose tissue from periparturient or dry cows (Moisá et al., 2017; Minuti et al., 2020) has uncovered several biologically relevant pathways, of which AA signaling via MTOR seems particularly important, given its role in coordinating aspects of tissue protein synthesis as a function of AA availability. In nonruminants, mechanistic target of rapamycin C1 (MTORC1) silencing contributes to the manifestation of some stress-related phenotypes, suggesting that signaling through this kinase could regulate aspects of adipose tissue function (Cai et al., 2016). For instance, adipocyte MTORC1 deficiency in mice markedly decreased lipid storage in adipose tissue and induced hepatic steatosis along with insulin resistance (Chimin et al., 2017). Activation of MTOR can upregulate mitochondrial biogenesis and oxidative metabolism (Cunningham et al., 2007), and ROS production contributes to the inhibition of MTOR signaling (Zhao et al., 2017).

Although $100 \mu\text{M}$ H_2O_2 did not affect the proliferation of primary bovine mammary epithelial cells (Ma et al., 2018a) or other mammalian cell lines within a 4-h incubation (Davies, 1999; Gülden et al., 2010), the

observed decrease in protein abundance of AKT and MTOR in adipose explants suggested that intracellular oxidants might impair some aspects of normal bovine adipose function. This idea is partly supported by data from SAT adipocytes isolated from biopsies of clinically healthy dairy cows at 17 DIM (Xu et al., 2021). In that study, relative to the control, a linear increase was observed in malondialdehyde (MDA) and ROS concentrations and a decrease in SOD and glutathione peroxidase (GPX) activity in adipocytes incubated with $100 \mu\text{M}$ H_2O_2 . However, although those enzymes clearly were negatively affected, cell viability actually peaked at $100 \mu\text{M}$ H_2O_2 , a response that we surmised was due to upregulation of the abundance of SOD and GST proteins (Xu et al., 2021). Our own in vivo data on SOD revealed little change in activity around parturition in SAT despite the gradual increases in ROS (Liang et al., 2019b). Although the present study was performed with SAT from late-lactation cows, these data led us to speculate that SAT can mount a robust antioxidant response driven in no small part via molecular changes for several proteins.

At least in nonruminants, insulin signaling controls glucose absorption, lipogenesis, lipolysis, and protein synthesis (Kenéz et al., 2019), all of which play vital roles in adipose tissue metabolism. A transient state of insulin resistance around parturition prevents peripheral tissues from using glucose, to guarantee glucose supply for the mammary gland (Contreras et al.,

Table 4. mRNA abundance of inflammation, antioxidant, and glutathione metabolism-related genes in subcutaneous adipose tissue (SAT; $n = 4/\text{treatment}$) cultured in medium with an “ideal” profile of EAA (CTR; Lys:Met 2.9:1), SAT incubated with CTR plus $100 \mu\text{M}$ H_2O_2 (HP), or SAT incubated with CTR plus enhanced Met supply plus $100 \mu\text{M}$ H_2O_2 (HPMET; Lys:Met 2.5:1); values are means expressed relative to CTR, which was set to 1.0

Item ¹	Treatment ²			SEM	P-value
	CTR	HP	HPMET		
Inflammation					
<i>IL6</i>	1.00	0.90	0.76	0.11	0.34
<i>TNF</i>	1.00	0.44	0.51	0.27	0.33
<i>IL10</i>	1.00	0.53	0.57	0.16	0.15
<i>IL1B</i>	1.00	0.52	0.48	0.27	0.37
Antioxidant enzymes					
<i>SOD1</i>	1.00	1.04	0.85	0.06	0.14
<i>SOD2</i>	1.00	0.71	0.68	0.19	0.47
<i>CAT</i>	1.00	1.40	1.16	0.10	0.07
<i>GCLC</i>	1.00 ^a	0.84 ^b	0.81 ^b	0.04	0.04
<i>GCLM</i>	1.00	0.97	0.76	0.10	0.25

^{a,b}Means between treatment with different lowercase superscripts differ ($P \leq 0.05$).

¹*CAT* = catalase; *GCLC* = glutamate-cysteine ligase catalytic subunit; *GCLM* = glutamate-cysteine ligase modifier subunit; *IL6* = interleukin 6; *IL10* = interleukin 10; *IL1B* = interleukin 1β; *SOD1* = superoxide dismutase 1; *SOD2* = superoxide dismutase 2, mitochondrial; *TNF* = tumor necrosis factor.

²Ribosomal protein S9, *GAPDH*, and *ACTB* were chosen as internal control genes to normalize mRNA data after calculating their geometric mean. For each gene, treatment differences were calculated relative to CTR (which was set to 1.0).

2017a). This physiological state is characterized by low insulin concentration in plasma and reduced insulin responsiveness in skeletal and adipose tissue (Bell and Bauman, 1997; Etherton and Bauman, 1998). Lower insulin responsiveness postpartum versus prepartum, assessed via glucose tolerance tests, underscored the state of insulin resistance (Holtenius et al., 2003). The fact that downregulation of SLC2A4 caused by oxidative stress dampened the sensitivity of adipose tissue to insulin (Hurrell and Hsu, 2017) led us to speculate that H₂O₂ challenge might have caused oxidative stress and a subsequent decrease in protein abundance of SLC2A4.

The AMP-activated protein kinase (AMPK) plays an important role in controlling cellular energy homeostasis, partly through its role in altering glucose uptake via enhancing the translocation of intracellular storage vesicles of SLC2A4 to the plasma membrane (Hardie et al., 2012). Thus, upregulation of p-AMPK in response to enhanced Met supply partly explained the increase in protein abundance of SLC2A4. Activation of AMPK reduced de novo fatty acid synthesis by downregulating *ACACA* and *FASN* mRNA in bovine mammary epithelial cells (McFadden and Corl, 2009). Thus, the upregulation of p-AMPK and *FASN* in response to Met supply suggested this AA might contribute to de novo fatty acid synthesis independent of the AMPK pathway. Whether other AA in the culture medium induced a lipogenic effect, as by feeding the TCA cycle, cannot be discerned. Future research is warranted to investigate the mechanisms whereby Met and other AA may participate in the regulation of lipid synthesis in bovine adipose tissue.

At least in nonruminants, nuclear factor, erythroid 2 like 2, a master regulator of cellular redox homeostasis, controls the transcription of many antioxidant enzymes such as SOD, glutathione peroxidase, *CAT*, NAD(P)H dehydrogenase 1, and GSH metabolism-related enzymes (e.g., *GSR*, *GPX1*, *GPX2*, and *GPX3*) (Lushchak, 2012). Kelch-like ECH-associated protein 1 (KEAP1), a negative regulator of NFE2L2, prevents movement of the NFE2L2 protein into the nucleus of the cell, inhibiting its activity. Greater RPM supplementation upregulated NFE2L2 and downregulated KEAP1 in bovine mammary tissue during the periparturient period (Han et al., 2018a). In addition, compared with controls, RPM led to greater abundance of p-NFE2L2 in SAT at 30 d after parturition (Liang et al., 2019b). Thus, available data suggest that increased postnatal Met supply might alter the interactions between NFE2L2 and KEAP1, with a net result of enhancing the antioxidant activity of the tissue. Such an effect helps explain the observed upregulation of NFE2L2 in SAT explants.

Glutamate-cysteine ligase catalytic subunit (GCLC) is the rate-limiting enzyme in GSH synthesis (Lu, 2013). Thus, downregulation of *GCLC* caused by H₂O₂ stimulation of adipose explants suggested that H₂O₂ might reduce GSH synthesis and contribute to enhancing the oxidant status. Glutathione S-transferase mu 1 (GSTM1), one of the GSH S-transferases, catalyzes the conjugation of GSH to electrophilic compounds (Han et al., 2018b), which led us to speculate that greater protein abundance of GSTM1 in bovine SAT from cows fed RPM was indicative of a reduction in oxidant status within the tissue (Liang et al., 2019b). As such, the observed downregulation of GSTM1 in explants receiving added Met was somewhat unexpected. It could be that inherent differences in the responsiveness of SAT around parturition compared with later in lactation (Contreras et al., 2017b) account for some of these differences.

The complex formed by NF- κ B and RELA mediates the transcription of pro-inflammatory cytokines such as *IL1B*, *IL6*, and *TNF* (Vallabhapurapu and Karin, 2009). In a previous in vitro study, we demonstrated that greater supply of Met reduced pro-inflammatory responses in bovine mammary epithelial cells exposed to a short-term challenge with lipopolysaccharide (Dai et al., 2020). Although the upregulation of RELA in response to Met supply in explants challenged with H₂O₂ appears paradoxical given the positive effect of this AA on NFE2L2 and GPX1, it could be that activation of the immune response under the conditions of the study represented a homeostatic response (Contreras et al., 2017b). Our previous work and others have clearly demonstrated that immune responsiveness of SAT is an inherent feature of the tissue, which serves key functions such as allowing proper remodeling (Vailati-Riboni et al., 2016; Moisés et al., 2017; Lopreato et al., 2018).

As in our recent paper with SAT explants (Liang et al., 2021), some limitations of the present study should be acknowledged. First, by design, the present study narrowly focused on only a few components of insulin, AA, inflammation, and antioxidant signaling-related pathways during H₂O₂ stimulation under basal conditions. Thus, we could not clarify the effect of insulin. Second, in spite of its use in many published bovine cell studies (e.g., Xu et al., 2021), a supraphysiological concentration of a single pro-oxidant (H₂O₂) under basal conditions (i.e., with no insulin challenge) was used. Thus, responses detected might not adequately reflect the fact that SAT might experience a challenge from more than one pro-oxidant molecule in vivo. Lastly, SAT was obtained from late-lactation cows, which cannot reflect the responsiveness of tissue from periparturient cows.

CONCLUSIONS

Although direct measures of oxidant and antioxidant molecules were not performed, overall, the responses observed indicated that exogenous H_2O_2 was able to alter tissue oxidant status. In fact, the short-term stimulation of adipose tissue with H_2O_2 had a negative influence on some of the components of insulin signaling that were chosen for study. It did so without negative effects on tissue SOD activity, which in studies with isolated adipocytes has proven sensitive to H_2O_2 . Increasing the supply of Met attenuated the decrease in abundance of insulin signaling-related proteins and seemed to enhance NFE2L2 and GSH-related antioxidant responses. Further in vivo research is warranted to investigate the role of Met and other AA in regulating immune, antioxidant, and metabolic systems in bovine adipose tissue. Such work should encompass not only molecular tools but also mass spectrometry, isotopic tracing technologies, and assays for key enzyme activities in those pathways.

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