Alterations in immune and antioxidant gene networks by gamma-D-glutamyl-meso-diaminopimelic acid in bovine mammary epithelial cells are attenuated by in vitro supply of methionine and arginine

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ABSTRACT

Nucleotide-binding oligomerization domain (NOD)-like receptor 1 (NOD1) is a cytosolic pattern recognition receptor with a crucial role in the innate immune response of cells triggered by the presence of compounds such as gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) present in the peptidoglycan of all gram-negative and certain gram-positive bacteria. Methionine (Met) and arginine (Arg) are functional AA with immunomodulatory properties. In the present study, we aimed to assess the effect of increased Met and Arg supply on mRNA abundance of genes associated with innate immune response, antioxidant function, and AA metabolism during iE-DAP challenge in bovine mammary epithelial cells (BMEC). Primary BMEC ($n = 4$ per treatment) were precultured in modified medium for 12 h with the following AA formulations: ideal profile of AA (control), increased Met supply (incMet), increased Arg supply (incArg), or increased supply of Met plus Arg (incMetArg). Subsequently, cells were challenged with or without iE-DAP (10 $\mu\text{g}/\text{mL}$) for 6 h. Data were analyzed as a $2 \times 2 \times 2$ factorial using the MIXED procedure of SAS 9.4. Greater mRNA abundance of *NOD1*, the antioxidant enzyme *SOD1*, and AA transporters (*SLC7A1* and *SLC3A2*) was observed in the incMet cells after iE-DAP stimulation. Although increased Met alone had no effect, incMetArg led to greater abundance of the inflammatory cytokine IL-6, and the antioxidant enzyme GPX1 after iE-DAP stimulation. The increased Arg alone downregulated *NOD1* after iE-DAP stimulation, coupled with a downregulation in the AA transporters mRNA abundance

(*SLC7A1*, *SLC7A5*, *SLC3A2*, and *SLC38A9*), and upregulation in *GSS* and *KEAP1* mRNA abundance. Overall, the data indicated that increased supply of both Met and Arg in the culture medium were more effective in modulating the innate immune response and antioxidant capacity of BMEC during in vitro iE-DAP stimulation.

Key words: amino acids, inflammation, innate immunity, NOD-like receptor 1

INTRODUCTION

Mastitis is a major issue in dairy herds impairing both economic benefit and health (Ruegg, 2017). When recognized by pattern recognition receptors (PRR), invading pathogens trigger the innate immune response in mammary gland (Kumar et al., 2011). Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) are transmembrane PRR and their crucial role in the infection of the udder has been demonstrated in dairy cows (Petzl et al., 2008). Nucleotide-binding oligomerization domain (NOD)-like receptors are cytosolic receptors and critical elements of innate immunity compensating for other pattern recognition systems (Franchi et al., 2009; Kim et al., 2016). By recognizing its specific ligand gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) produced by all gram-negative and certain gram-positive bacteria, NOD-like receptor 1 (NOD1) plays a crucial role in the innate immunity of dairy cows (Porcherie et al., 2012; Tan et al., 2012). Previous studies showed that NOD-like receptors are associated with many inflammatory diseases (Fritz et al., 2006) including the innate immune response during mastitis (Porcherie et al., 2012).

Methionine (Met), an essential sulfur-containing AA, is a key nutrient for dairy cows and showed a positive effect on the inflammatory and antioxidant response

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in peripartur dairy cows (Osorio et al., 2014; Zhou et al., 2016; Batistel et al., 2018). Through the transsulfuration pathway, methyl donors can enhance synthesis of sulfur-containing antioxidants, such as taurine and glutathione (**GSH**; Lu, 2013). Glutathione peroxidases (**GPx**) activity relies on GSH availability and plays a fundamental role in cellular protection against reactive oxygen metabolites (**ROM**; Liang et al., 2020). In addition, the semi-essential AA arginine (**Arg**) is also important for immune capacity and has anti-inflammatory properties (de Andrade Bernal Fagiani et al., 2019). Previous studies underscored the role of Arg as a functional AA with benefits on the modulation of inflammatory responses in primary bovine mammary epithelial cells (**BMEC**; Wu et al., 2016) and lactating dairy cows (Zhao et al., 2018) and also through an increase in GSH levels (Dasgupta et al., 2006).

Our group recently reported the effects of increased in vitro supply of Met and Arg on mRNA and protein abundance associated with innate immune response and redox balance during an in vitro LPS challenge in BMEC (Dai et al., 2020). Similar to LPS, iE-DAP is a pathogen-associated molecular pattern that plays a fundamental role in the mastitis pathogenesis and can induce an intense response when recognized by cytosolic NOD1 (Porcherie et al., 2012). Although our group has already reported in vivo studies investigating mastitis and nutrigenomics effects in the mammary gland (Loor et al., 2011; Han et al., 2018; Vailati-Riboni et al., 2018), there are no studies that have elucidated the effects of increased supply of both Met and Arg on innate immune and antioxidant responses induced through iE-DAP stimulation in BMEC.

The general hypothesis of the present study was that an increased supply of Met and Arg in the culture medium modulate molecular biomarkers of innate immunity in BMEC induced by exogenous challenge with iE-DAP. Thus, the specific objective was to measure the mRNA abundance of genes related to immune response, antioxidant function and AA metabolism.

MATERIALS AND METHODS

Cell Culture and Treatments

The BMEC (2×10^5 cells) were seeded into 6-well plates and incubated overnight with serum-free high-glucose Dulbecco's modified Eagle's medium as described in our previous work (Dai et al., 2020). Subsequently, BMEC were pretreated for 12 h with modified medium: medium with ideal AA profile (control; **Con**), medium with increased Met supply (**incMet**), medium with increased Arg supply (**incArg**), or medium with

increased Met plus Arg supply (**incMetArg**; Dai et al., 2020). After a 12-h incubation in modified medium, cells were treated with either 10 $\mu\text{g/mL}$ of iE-DAP (iE, Sigma-Aldrich, St. Louis, MO) or PBS and incubated for another 6 h. Thus, we generated a total of 8 treatments in a factorial arrangement: (1) Con, (2) incMet, (3) incArg, (4) incMetArg, (5) Con + iE-DAP, (6) incMet + iE-DAP, (7) incArg + iE-DAP, and (8) incMetArg + iE-DAP. All treatments were conducted with 4 replicates. Formulation of the medium used in the present study was described in our previous work (Dai et al., 2020).

mRNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Cells were collected at the end of the treatment and the miRNAeasy Kit (cat. no. 217004; Qiagen, Hilden, Germany) was used to extract mRNA from BMEC according to the manufacturer's instructions. The cDNA was synthesized, and quantitative real-time PCR (**RT-qPCR**) were performed using the same protocol as reported previously (Jacometo et al., 2015). Genes associated with immune response, oxidative stress, AA transporters, Arg metabolism, and Met metabolism were measured. Primers were designed and tested following a protocol reported previously by our group (Zhou et al., 2018). Ubiquitously expressed prefoldin like chaperone (*UXT*), *GAPDH*, and ribosomal protein S9 (*RPS9*) were selected as internal control genes (**ICG**). The geometric mean of the ICG was used to normalize the gene expression data, and the generated data were calculated following the method described by Zhou et al. (2018). All primer information and RT-qPCR performance data are available in a recent manuscript from our group (Dai et al., 2020).

Statistical Analysis

Statistical analysis was performed in a similar fashion as described previously (Dai et al., 2020) with some modifications. In brief, data were analyzed as a $2 \times 2 \times 2$ factorial arrangement of treatments using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Met, Arg, and iE-DAP were the 3 factors, each containing 2 levels: basal or increased levels of the AA and with or without iE-DAP, resulting in 8 treatments. The model included the main effects of Met, Arg, and iE-DAP, as well as all the following interactions: Met $\times$ Arg $\times$ iE-DAP, Met $\times$ iE-DAP, Arg $\times$ iE-DAP, and Met $\times$ Arg. The random effect was individual cell culture well. The Shapiro-Wilk test was applied to assess normality of distribution of variables. To fit nor-

mal distribution of residuals, mRNA abundance data were log2-scale transformed before statistical analysis and then were log2 back-transformed as presented in tables and figures. Least squares means and standard errors were determined using the LSMEANS statement of SAS and were compared using Tukey's test when significant interactions were observed. Significance was set at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS

Immune Response

A 3-way interaction was observed for *TLR4* mRNA abundance ($P = 0.006$; Figure 1) due to lower *TLR4* abundance in cultures with incMetArg + iE-DAP compared with cultures without iE-DAP stimulation ($P < 0.05$; Figure 1). A 2-way interaction between Met and iE-DAP ($P = 0.008$) and between Arg and iE-DAP ($P = 0.008$) was observed for *NOD1* mRNA abundance (Table 1). This was due to greater *NOD1* mRNA abundance in cultures with incMet + iE-DAP compared with Con + iE-DAP ($P < 0.001$), and lower *NOD1* mRNA abundance in cultures with incArg + iE-DAP compared with Con + iE-DAP ($P < 0.001$).

A 3-way interaction was observed for *IL6* mRNA abundance ($P = 0.04$; Figure 1) due to greater *IL6* abundance in cultures with incMetArg + iE-DAP compared with Con + iE-DAP ($P < 0.05$) and with cultures without iE-DAP stimulation ($P < 0.05$) (Figure 1).

The *NFKB1* mRNA abundance tended to be lower overall in cultures with incMet supply ($P = 0.06$) and was lower overall in cultures with incArg supply ($P = 0.006$; Table 1). Regardless of treatment, the iE-DAP stimulation upregulated *NOS2* mRNA abundance ($P = 0.008$; Table 1).

Oxidative Stress

A 3-way interaction was observed for *GPX1* mRNA abundance ($P = 0.04$; Figure 1) due to greater *GPX1* abundance in cultures with incMetArg + iE-DAP compared with Con + iE-DAP ($P < 0.05$; Figure 1). A 2-way interaction between Met and iE-DAP was observed for *SOD1* mRNA abundance ($P = 0.02$; Table 1) due to greater *SOD1* abundance in cultures with incMet + iE-DAP compared with Con + iE-DAP ($P = 0.078$).

A 3-way interaction was observed for *NFE2L2* mRNA abundance ($P = 0.04$; Figure 1) due to lower *NFE2L2* abundance in cultures with incMetArg + iE-DAP compared with Con + iE-DAP ($P < 0.05$) and with cultures without iE-DAP stimulation ($P < 0.05$; Figure 1).

The *GSS* ($P = 0.001$) and *KEAP1* ($P = 0.04$) mRNA abundance was greater overall in cultures with incArg supply (Table 1). Regardless of treatment, the iE-DAP stimulation upregulated *GSR* mRNA abundance ($P = 0.004$; Table 1).

Amino Acid Transporters

A 3-way interaction was observed for *SLC7A1* mRNA abundance ($P = 0.002$; Figure 1) due to greater *SLC7A1* abundance in cultures with incMet + iE-DAP compared with Con + iE-DAP and with cultures without iE-DAP stimulation ($P < 0.05$). However, compared with Con – iE-DAP, cultures with incMetArg + iE-DAP had lower *SLC7A1* abundance ($P < 0.05$), and cultures with incArg and incMetArg – iE-DAP had lower *SLC7A1* abundance compared with both Con – iE-DAP and Con + iE-DAP ($P < 0.05$; Figure 1).

A 2-way interaction between Met and iE-DAP was observed for *SLC3A2* mRNA abundance ($P = 0.05$; Table 1) due to greater *SLC3A2* abundance in cultures with incMet + iE-DAP compared with Con + iE-DAP ($P < 0.05$). The incArg cultures had lower overall *SLC3A2* ($P < 0.001$), *SLC7A5* ($P < 0.001$), and *SLC38A9* ($P = 0.003$) mRNA abundance (Table 1).

Arginine and Methionine Metabolism-Related Genes

There were no interactions or main effects on Met and Arg metabolism-related genes. Regardless of treatment, iE-DAP stimulation upregulated *SMS* ($P = 0.02$), *ODC1* ($P = 0.01$), and *AMD1* ($P < 0.001$) mRNA abundance (Table 1).

DISCUSSION

Bovine mammary epithelial cells contribute to the innate immune response during infection of the mammary gland by recognizing pathogens through PRR (Ibeagha-Awemu et al., 2008; Huang et al., 2019). The NOD-like receptors are cytosolic PRR, which compensate for and synergize with other PRR, such as toll-like receptors (TLR) during infection (Cardenas et al., 2011). The protein NOD1 is one of the major NOD-like receptors and initiates the innate immune response by sensing the microbial component iE-DAP (Fritz et al., 2006). At least in nonruminants, after binding with iE-DAP, downstream signaling cascades including nuclear factor kappa B (NF- κ B) are activated and promote expression of genes related to the innate immune response (e.g., inflammatory cytokines; Franchi et al., 2009).

The lower *TLR4* abundance in cultures receiving increased Met and Arg and challenged with iE-DAP is opposite to previous studies in ruminants demonstrat-

Figure 1. Abundance of mRNA for antioxidant genes in primary bovine mammary cells incubated with various AA treatments and challenged with or without 10 $\mu\text{g/mL}$ gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) for 6 h. Treatments included the ideal AA profile at a ratio of Lys:Met 2.9:1 and Lys:Arg 2:1 (con) or different levels of Met and Arg to vary the Lys:Met and Lys:Arg as follows: Lys:Met 2.5:1 and Lys:Arg 2:1 (incMet), Lys:Met 2.9:1 and Lys:Arg 1:1 (incArg) and Lys:Met 2.5:1 and Lys:Arg 1:1 (incMetArg). Data reported here had a significant ($P < 0.05$) or tended ($P = 0.10$) to have a significant triple interaction (Met $\times$ Arg $\times$ iE-DAP). ^{A-D}Means differ between treatments ($P < 0.05$). Main effects of Met, Arg, or iE-DAP along with 2-way interactions are reported in Table 1. Error bars represent the SEM. *TLR4* = toll-like receptor 4; *IL6* = interleukin 6; *GPX1* = glutathione peroxidase 1; *NFE2L2* = nuclear factor, erythroid 2 like 2; *SLC7A1* = solute carrier family 7 member 1; *SLC7A2* = solute carrier family 7 member 2.

Table 1. Abundance of mRNA (relative units) in primary bovine mammary cells incubated with different levels of methionine or arginine and challenged with or without 10 µg/mL gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP) for 6 h¹

Item ²	Met			Arg			iE-DAP			P-value		
	-Met	+Met	SEM	-Arg	+Arg	SEM	-iE-DAP	+iE-DAP	SEM	Met × Arg	Met × iE-DAP	Arg × iE-DAP
Immune response												
<i>TLR2</i>	0.82	0.76	0.03	0.79	0.79	0.03	0.76	0.81	0.03	0.14	0.25	0.99
<i>IL1B</i>	0.011	0.012	0.001	0.012	0.011	0.001	0.012	0.011	0.001	0.21	0.51	0.85
<i>NOD1</i>	1.24	1.28	0.02	1.36	1.16	0.03	1.39	1.13	0.03	0.30	<0.001	<0.001
<i>NFKB1</i>	0.87	0.82	0.008	0.88	0.81	0.01	0.85	0.84	0.01	0.06	0.77	0.12
<i>CXCL2</i>	0.17	0.16	0.005	0.16	0.17	0.005	0.17	0.16	0.005	0.28	0.29	0.02
<i>CXCL8</i>	0.15	0.16	0.004	0.16	0.15	0.004	0.15	0.15	0.004	0.25	0.82	0.02
<i>NOS2</i>	0.25	0.29	0.02	0.25	0.28	0.02	0.23	0.31	0.02	0.13	0.008	0.89
Oxidative stress												
<i>GSS</i>	0.98	0.92	0.02	0.90	1.01	0.02	0.88	1.03	0.02	0.05	<0.001	0.27
<i>GSR</i>	1.02	0.99	0.02	1.02	0.99	0.02	0.96	1.05	0.02	0.17	0.004	0.09
<i>SOD1</i>	1.14	1.14	0.02	1.20	1.08	0.02	1.21	1.08	0.02	0.85	<0.001	0.99
<i>SOD2</i>	0.93	0.93	0.01	0.94	0.91	0.01	0.94	0.92	0.02	0.99	0.39	0.96
<i>KEAP1</i>	1.03	1.04	0.02	1.01	1.07	0.02	0.98	1.10	0.02	0.74	<0.001	0.19
<i>CUL3</i>	1.07	1.07	0.01	1.08	1.06	0.01	1.05	1.08	0.01	0.90	0.17	0.90
AA												
transporters												
<i>SLC38A9</i>	1.17	1.15	0.02	1.21	1.11	0.02	1.17	1.20	0.02	0.57	0.78	0.15
<i>SLC38A2</i>	1.08	1.08	0.03	1.10	1.06	0.03	1.07	1.11	0.03	0.90	0.59	0.52
<i>SLC7A5</i>	1.03	1.05	0.03	1.19	0.91	0.03	1.02	1.06	0.03	0.62	0.25	0.81
<i>SLC3A2</i>	1.05	1.08	0.02	1.15	0.99	0.02	1.06	1.07	0.02	0.23	0.61	0.39
<i>SLC36A1</i>	1.08	1.04	0.01	1.07	1.04	0.01	1.08	1.09	0.01	0.06	0.12	0.65
Arginine												
metabolism												
<i>SRM</i>	1.05	1.03	0.01	1.05	1.03	0.01	1.02	1.05	0.01	0.32	0.06	0.41
<i>SMS</i>	1.06	1.06	0.02	1.08	1.03	0.02	1.03	1.09	0.02	0.99	0.02	0.06
<i>SOD1</i>	0.45	0.46	0.01	0.45	0.46	0.01	0.43	0.47	0.01	0.36	0.01	0.27
<i>ARG1</i>	0.99	1.11	0.08	1.03	1.07	0.08	0.96	1.16	0.08	0.31	0.09	0.69
Methionine												
metabolism												
<i>MAT2A</i>	1.10	1.03	0.03	1.08	1.05	0.03	1.04	1.09	0.03	0.12	0.26	0.34
<i>AMD1</i>	1.10	1.07	0.02	1.06	1.12	0.02	1.04	1.14	0.02	0.24	<0.001	0.01

¹Treatments were the ideal AA profile to achieve ratios of Lys:Met 2.9:1 and Lys:Arg 2:1 (con) or treatments containing different levels of Met and Arg to vary the Lys:Met and Lys:Arg as follows: Lys:Met 2.5:1 and Lys:Arg 2:1 (incMet), Lys:Met 2.9:1 and Lys:Arg 1:1 (incArg), and Lys:Met 2.5:1 and Lys:Arg 1:1 (incMetArg). No statistically significant ($P > 0.10$) triple interactions (Met × Arg × iE-DAP) were detected for these data.

²*AMD1* = adenosylmethionine decarboxylase 1; *ARG1* = arginase 1; *CUL3* = cullin-3; *CXCL2* = C-X-C motif chemokine ligand 2; *GSR* = glutathione reductase; *GSS* = glutathione synthetase; *IL1B* = interleukin 1β; *CXCL8* = C-X-C motif chemokine ligand 8; *KEAP1* = kelch-like ECH-associated protein 1; *MAT2A* = methionine adenosyltransferase 2A; *NFKB1* = nuclear factor κB; *NOS2* = nitric oxide synthase 2; *ODC1* = ornithine decarboxylase 1; *SLC36A1* = solute carrier family 36 member 1; *SLC38A2* = solute carrier family 38 member 2; *SLC38A9* = solute carrier family 38 member 9; *SLC3A2* = solute carrier family 3 member 2; *SLC7A5* = solute carrier family 7 member 5; *SMS* = spermine synthase; *SOD2* = superoxide dismutase 2; *SRM* = spermidine synthase; *TLR2* = toll-like receptor 2.

ing that mammary cells increase *TLR4* transcription after LPS stimulation (Bulgari et al., 2017; Wang et al., 2017). However, our recent work evaluating the increased Met and Arg supply in BMEC during the LPS challenge showed a similar response. A *TLR4* downregulation was observed in BMEC after LPS challenge, coupled with a trend for lower *TLR4* abundance in incMet + LPS cultures (Dai et al., 2020). Thus, the assays with pathogen-associated molecular patterns (e.g., LPS and iE-DAP) in BMEC with increased Met and Arg led to a downregulation in *TLR4* abundance, a response that might be associated with a protective effect of both AA to prevent an exacerbated inflammatory response.

In nonruminant cells, recognition of iE-DAP through the NOD1 receptor leads to activation of the NF- κ B pathway and stimulates transcription of inflammatory cytokines and others transcription factors (Chamailard et al., 2003). Intriguingly, upregulation of *NOD1* in cultures with incMet during the iE-DAP challenge did not stimulate greater *NFKB1* abundance. Such response was opposite to the lower overall *NFKB1* and *NOD1* abundance during iE-DAP challenge in response to incArg suggesting this AA relieved the inflammatory response as reported in previous work with BMEC stimulated with LPS (Wu et al., 2016).

Interleukin 6 is a soluble mediator that contributes to host defense by modulating the immune response (Tanaka et al., 2014). A recent study demonstrated that iE-DAP stimulation of BMEC led to upregulation of both *NOD1* and *NFKB1* with a consequent increase in IL-6 abundance (Gao et al., 2020). In the present study, we speculate that greater IL-6 abundance in incMetArg cultures without changes in *NFKB1* might have been associated with stimulation of other transcription factors as already demonstrated in previous studies in nonruminants, such as nuclear factor IL-6, interferon regulatory factor 1, and activator protein 1 (Akira and Kishimoto, 1992; Tanaka et al., 2014). Our previous work supplementing multiparous Holstein cows with rumen-protected Met detected greater IL-6 plasma concentrations in cows fed rumen-protected Met during the periparturient period (Zhou et al., 2016). We also demonstrated that blood polymorphonuclear cells exposed to LPS stimulation tended to have greater IL-6 abundance as exogenous Met supplementation increased (Vailati-Riboni et al., 2019). In the present study, the greater IL-6 abundance in incMetArg cultures after iE-DAP stimulation suggested that increased availability of both AA played an important role in regulating the immune response. This cytokine has a dynamic functionality according to the interaction with specific receptors, acting as a proinflammatory inducer (trans signaling) or as an anti-inflammatory modulator (clas-

sic signaling; Scheller et al., 2011). Understanding the meaning of the increased IL-6 abundance in cultures with IncMetArg after iE-DAP stimulation becomes challenging without evaluating protein abundance and receptor interactions. However, the absence of a significant difference in other proinflammatory-related genes (e.g., *IL1B*, *NOS2*) indicates that a possible IL-6 anti-inflammatory role might have contributed to modulating the immune response in cells challenged with iE-DAP and receiving exogenous Arg and Met in the culture medium.

Glutathione peroxidase 1 (**GPX1**) and superoxide dismutase 1 (**SOD1**) are key free-radical scavengers in the first line of antioxidant defense, and protect cells against ROM generated during cellular metabolism (Loor et al., 2013; Mohammadi et al., 2016; Ighodaro and Akinloye, 2018). The *GSS* gene encodes the enzyme glutathione synthetase (**GSS**), which plays a crucial role in the GSH synthesis through the gamma-glutamyl cycle. Glutathione is the most potent cellular antioxidant used by GPX1 against cytosolic hydrogen peroxide (Aquilano et al., 2014). Nuclear factor, erythroid 2 like 2 (**NFE2L2**) is a transcription factor that once activated promotes the expression of genes with antioxidant response elements (e.g., *GPX1*, *GSH*, and *SOD1*) on their promoters (Pias et al., 2003; Ruiz et al., 2013).

The increased Met supply in BMEC was previously reported to be beneficial for the antioxidant response through increasing GPX1 and SOD1 enzymatic activity during hyperthermia-induced BMEC injury (Han et al., 2015). Our previous study with multiparous Holstein cows demonstrated that enhanced postparturient Met supply altered mammary gland antioxidant gene networks via phosphorylation of NFE2L2 (i.e., a response that led to greater *GPX1* abundance in cows supplemented with Met during periparturient period; Han et al., 2018). In the present study, the greater *GPX1* abundance with incMetArg after iE-DAP stimulation indicated that an increase in the supply of both AA can enhance the antioxidant capacity of BMEC during the iE-DAP challenge. In addition, the greater overall *GSS* abundance with Arg supply underscored a more potent effect in the antioxidant response.

From a physiological point of view, the lower *NFE2L2* abundance observed in cultures with incMetArg and incArg after iE-DAP stimulation is not in accordance with *GPX1*, *GSS* and *SOD1* responses. The *NFE2L2* downregulation might be partly explained by the overall upregulation of *KEAP1* observed in cultures with incArg. The *KEAP1* gene encodes the kelch-like ECH-associated protein 1 (**KEAP1**), which is often characterized as an inhibitor of NFE2L2 activity through its sequestration in the cytosol (Ho et al., 2005; Han et al.,

2018). The antagonistic response between *NFE2L2* and *KEAP1* was demonstrated in our previous work with mammary tissue of cows fed rumen-protected Met; that is, *NFE2L2* upregulation and *KEAP1* downregulation with increased Met supply (Han et al., 2018). Thus, we speculate that increased Met and Arg might modulate transcription of antioxidant-related genes by affecting the interactions of NFE2L2/KEAP1. Further studies are required to elucidate the underlying mechanisms in detail.

Amino acid transporters play a crucial role in providing these molecules to help maintain cellular redox balance and key intracellular signaling mechanisms (Broer and Palacin, 2011). The *SLC7A1* gene encodes a transporter for Arg and other cationic AA (Bronte and Zanovello, 2005). The former is often in short supply during stress conditions (Bansal and Ochoa, 2003). Thus, the greater *SLC7A1* abundance in response to incMet + iE-DAP suggested an increase in uptake of Arg to ensure a sufficient supply during the challenge. Recently our group demonstrated that increased Met supply in unstimulated BMEC upregulated *SLC7A1* abundance, whereas greater Arg supply downregulated 5 of the 7 known mechanistic target of rapamycin complex 1-target AA transporters genes (*SLC1A5*, *SLC3A2*, *SLC7A1*, and *SLC7A5*; Hu et al., 2020). The lower *SLC7A1* mRNA abundance in response to greater Arg supply in unstimulated cells was also detected in previous studies with nonruminants and ruminants (Closs, 2002; Hatzoglou et al., 2004; Dai et al., 2020). According to (Hu et al., 2020), the opposite response in *SLC7A1* abundance due to greater Met or Arg supply suggested that BMEC has a different sensitivity to the additional Met or Arg supply. The marked downregulation in several AA transporters (*SLC7A1*, *SLC7A5*, *SLC3A2*, and *SLC38A9*) in cultures with incArg suggested that a negative feedback mechanism helps regulate AA uptake in situations where the AA levels are already adequate (Hu et al., 2020).

The *SLC3A2* gene is a member of the solute carrier family 3 member 2, which encodes a bidirectional antiporter that regulates the exchange of L-AA between the extracellular and intracellular compartments (Nicklin et al., 2009). Our recent work underscored that enhanced postruminal supply of Met led to greater *SLC3A2* mRNA abundance in adipose tissue of Holstein cows during the periparturient period (Liang et al., 2019). As in the present study, upregulation of *SLC3A2* in cultures with incMet was also observed in MAC-T cells (Dong et al., 2018) and human muscle tissue with increased essential AA supplementation (Drummond et al., 2010). Mechanistically, the upregulation of *SLC3A2* with greater Met supply supports the role of AA for maintenance of an efficient immune response as pre-

viously demonstrated in rodents (Ikeda et al., 2017). Overall, the greater *SLC7A1* abundance with incMet in both iE-DAP stimulated and nonstimulated cultures compared with controls suggested a beneficial effect of Met on AA utilization in BMEC, a response supported by the upregulation of *SLC3A2* with incMet supply during the iE-DAP challenge.

Polyamines (e.g., putrescine, spermidine, and spermine) are bioactive amines that act on cell regulation, DNA transcription and RNA translation, protein synthesis, and modulation of the immune response (Larqué et al., 2007). Among polyamines, spermine is considered the most active in the control of various biological processes (Yuan et al., 2001). The *SMS* gene encodes spermine synthase, which catalyzes the production of spermine from spermidine (Kalač, 2014). The *ODC1* gene encodes ornithine decarboxylase 1 (**ODC1**), a rate-limiting enzyme in the polyamine biosynthesis pathway, which catalyzes the production of putrescine from ornithine (Schipper and Verhofstad, 2002). The *AMD1* gene encodes adenosylmethionine decarboxylase 1 (**AMD1**), an intermediate enzyme in polyamine biosynthesis (Larqué et al., 2007). The upregulation in mRNA abundance of the various components of the polyamine pathway after iE-DAP stimulation, regardless of treatment, might have been related to a cellular protection mechanism in an attempt to counteract the inflammatory response.

Spermine is considered the most effective polyamine in regulating ODC1 activity and its concentrations are often increased during infectious and inflammatory processes (Zhang et al., 1997; Yuan et al., 2001). An increase in spermine in human mononuclear cells during LPS challenge inhibited proinflammatory cytokine synthesis (Zhang et al., 1997). Upregulation of *NOS2*, which encodes an inducible nitric oxide synthase (**NOS**), during iE-DAP stimulation suggested an increase in nitric oxide (NO) production, an essential component of the oxidative burst during the phagocytic response (Bogdan, 2015). Previous studies in rodents reported that spermine downregulated *NOS* mRNA abundance and NO concentrations in macrophages challenged with LPS (Southan et al., 1994; Mossner et al., 2001). Although we did not determine concentrations of polyamines, we speculate that upregulation of the polyamine pathway to increase spermine levels was necessary. It represented a counter-regulatory mechanism to dampen production of NO and an exacerbated proinflammatory response during iE-DAP challenge.

CONCLUSIONS

Enhanced supply of Met and Arg in the culture medium was beneficial for modulation of the innate

immune response and antioxidant capacity in BMEC during inflammatory stimulation with the peptidoglycan iE-DAP. Such effects encompass proinflammatory and antioxidant responses coupled with AA transport mechanisms. The functional relevance of these responses in vivo remains to be determined.

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