Continuous low-dose infusion of tumor necrosis factor alpha in adipose tissue elevates adipose tissue interleukin 10 abundance and fails to alter metabolism in lactating dairy cows

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ABSTRACT

Repeated bolus doses of tumor necrosis factor- α (TNF α) alters systemic metabolism in lactating cows, but whether chronic release of inflammatory cytokines from adipose tissue has similar effects is unclear. Late-lactation Holstein cows ($n = 9$ – 10 /treatment) were used to evaluate the effects of continuous adipose tissue TNF α administration on glucose and fatty acid (FA) metabolism. Cows were blocked by feed intake and milk yield and randomly assigned within block to control or TNF α treatments. Treatments (4 mL of saline or 14 μ g/kg of TNF α in 4 mL of saline) were infused continuously over 7 d via 2 osmotic pumps implanted in a subcutaneous adipose depot. Plasma, milk samples, milk yield, and feed intake data were collected daily, and plasma glucose turnover rate was measured on d 7. At the end of d 7, pumps were removed and liver and contralateral tail-head adipose biopsies were collected. Results were modeled with the fixed effect of treatment and the random effect of block. Treatment with TNF α increased plasma concentrations of the acute phase protein haptoglobin, but did not alter plasma TNF α , IL-4, IL-6, or IFN- γ concentrations, feed intake, or rectal temperature. Milk yield and composition were unchanged, and treatments did not alter the proportion of short- versus long-chain FA in milk on d 7. Treatments did not alter plasma free FA concentration, liver triglyceride content, or plasma glucose turnover rate. Surprisingly, TNF α infusion tended to decrease liver TNF α and IL-1 receptor 1 mRNA abundance and significantly increased adipose tissue IL-10 protein concentration. Continuous infusion of TNF α did not induce the metabolic responses previously observed following bolus doses delivered at the same rate per

day. Metabolic homeostasis may have been protected by an adaptive anti-inflammatory response to control systemic inflammation.

Key words: cytokine, inflammation, adipose tissue, liver

INTRODUCTION

Tumor necrosis factor- α (TNF α) is a proinflammatory cytokine that was first described as cachectin, which was implicated in wasting disease following sepsis (Cerami et al., 1985). As an inflammatory mediator, TNF α plays an important role in activating components of the innate immune system in response to pathogen challenges or sterile inflammatory signals (Fiers, 1991); however, nearly all cell types express receptors for TNF α , and its reported systemic effects are pleiotropic (Olefsky and Glass, 2010). It is becoming increasingly apparent that TNF α and other cytokines play a central role in metabolic physiology, not only in acute situations, such as sepsis, but also in chronic inflammatory scenarios (Popa et al., 2007).

It is now widely accepted that TNF α is produced in adipose tissue, derived from resident macrophages and possibly adipocytes (Hotamisligil et al., 1993; Olefsky and Glass, 2010). Circulating TNF α concentrations are chronically elevated in obese individuals in several species, including sheep (Daniel et al., 2003), and the resulting inflammatory state is thought to contribute to insulin insensitivity and hepatic lipid accumulation in these individuals (Shoelson et al., 2007).

Dairy cattle commonly show signs of inflammation early in lactation (Bionaz et al., 2007). This phenomenon is likely directly related to the parturition process, but the inflammatory state is amplified in cows with excess adipose stores at the time of parturition (Ametaj et al., 2005). Excess adiposity is also a key risk factor for the development of fatty liver, one of the most prevalent metabolic disorders in dairy cattle (Bobe et al., 2004). Tumor necrosis factor- α promotes mobiliza-

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tion of energy stores by decreasing insulin sensitivity (Kushibiki et al., 2001) and feed intake (Kushibiki et al., 2003) and by directly stimulating lipolysis (Kushibiki et al., 2002), which are all changes associated with bovine fatty liver disease. Furthermore, TNF α can directly stimulate hepatic triglyceride (TG) synthesis and accumulation (Endo et al., 2007).

Previous work in lactating dairy cattle supported a direct effect of TNF α on TG accumulation and metabolic gene expression in the liver (Bradford et al., 2009). Subcutaneous administration of TNF α (2 μ g/kg of BW) to late-lactating dairy cows once daily for 7 d significantly increased liver TG concentration and altered transcript profiles in a manner consistent with a shift from FA oxidation to TG storage (Bradford et al., 2009). In early lactation cows, a similar treatment regimen did not have clear effects on intermediary metabolism, but substantially decreased feed intake and milk production (Yuan et al., 2013). Thus, the objective of the present experiment was to determine whether continuous administration of the same total dose of TNF α into adipose tissue would cause similar alterations in systemic nutrient metabolism or productivity.

MATERIALS AND METHODS

Animals, Treatments, and Data and Sample Collection

Experimental procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (protocol #2753). Nineteen late-lactation Holstein cows were used, rather than early lactation cows, to ensure that animals began the experiment without hepatic inflammation and to decrease animal variability. Cows (687 kg of BW, SD = 66 kg) entered a tiestall facility 7 d before treatment to allow for environmental adaption. Cows were offered water ad libitum, fed twice daily, and milked 3 times daily in a milking parlor. The diet fed throughout the study (Table 1) was formulated to meet all nutrient requirements (NRC, 2001). Following the adaption period, cows were blocked by pretreatment feed intake and milk production and assigned randomly within block to control ($n = 10$) or TNF α ($n = 9$) treatments. Recombinant bovine TNF α was produced (GenScript, Piscataway, NJ) as previously described (Bradford et al., 2009). Saline (4 mL) or TNF α (14 μ g/kg in 4 mL of saline) was infused continuously over 7 d via 2 osmotic pumps (2ML1, Alzet, Cupertino, CA) implanted in the adipose layer in the tail-head region. The region between the tail-head and the pin bone was surgically prepared, and 2 mL of 2% lidocaine hydrochloride was injected subcutaneously to produce local anesthesia.

Table 1. Ingredient and nutrient composition of the diet

Item, % of DM	Measurement
Diet ingredient	
Corn gluten feed	30.8
Corn silage	20.8
Ground corn	18.0
Alfalfa hay	16.5
Cottonseed	5.3
Expeller soybean meal	4.7
Calcium salts of FA	0.7
Cane molasses	0.5
Menhaden fish meal	0.3
Mineral and vitamin mix ¹	2.4
Nutrient composition	
OM	92.1
NDF	33.4
Starch	23.5
CP	17.2
Ether extract	4.3

¹Contained (per kilogram of DM): Ca, 188 g; Na, 97 g; Cl, 15 g; Mg, 28 g; Fe, 2.0 g; K, 600 mg; Zn, 586 mg; Mn, 330 mg; S, 200 mg; P, 100 mg; Cu, 181 mg; Co, 36 mg; Se, 7.7 mg; I, 1.8 mg; all-*trans* retinyl acetate, 33 mg; cholecalciferol, 1.0 mg; all-*rac*- α -tocopherol, 2.0 g.

An incision (~2 cm) was made with a sterile scalpel, and the skin was separated from the underlying tissue by blunt dissection (3 $\times$ 3 cm region) for pump implantation. The incision was closed with surgical staples.

Throughout the 7-d treatment period, blood plasma, milk samples, milk yield data, and DMI data were collected daily. Two blood samples were collected (0800 h) from the coccygeal vein into evacuated tubes containing potassium EDTA or potassium oxalate with sodium fluoride as a glycolytic inhibitor (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) and centrifuged (2,000 $\times$ g for 10 min at 25°C immediately after collection); plasma was stored at -20°C. On d 5 of treatment, jugular catheters were placed and at least 18 h of recovery was allowed before sample collection via the catheters. On treatment d 7, an initial blood sample was collected (into sodium fluoride tubes), cows were given a glucose bolus containing U-¹³C-glucose (99% atom, Sigma Chemical Co., St. Louis, MO) via the jugular catheter, and blood samples were then collected at 10-min intervals for 120 min to determine glucose turnover rate (Schulze et al., 1991). Each cow received approximately 1 g of U-¹³C-glucose in 50 mL of sterile saline; syringes were weighed immediately before and after infusion to determine the exact amount administered. Catheters were flushed with a sterile solution of 3.5% sodium citrate after the labeled glucose bolus (20 mL) and after each blood sample collection (5 mL). Cows were fed every 2 h, beginning 6 h before administration of the labeled glucose and continuing through the final sample collection to promote steady-state glucose kinetics.

After the glucose turnover assay was completed, osmotic pumps were removed and checked for any resid-

ual solution; all pumps were found to be empty. Liver and contralateral tail-head adipose tissue samples were then collected for analysis of liver TG and abundance of key transcripts. Liver biopsies were collected as previously described (Mullins et al., 2012). For adipose tissue biopsies, the region between the tail-head and the pin bone (contralateral to the site of pump insertion) was surgically prepared and 2 mL of 2% lidocaine hydrochloride was injected subcutaneously to produce local anesthesia. An incision (~3 cm) was made with a sterile scalpel, and subcutaneous adipose tissue samples were collected using sterile forceps and surgical scissors. After approximately 5 g of tissue was collected, the incision was closed with surgical staples.

Milk Analyses

During the treatment period, a single milk sample was collected from each cow at each milking and divided. One sample was analyzed by Heart of America DHIA (Manhattan, KS) to determine concentrations of fat, true protein, and lactose, as previously described (Mullins et al., 2012). The second sample was used for FA analysis as described by Sukhija and Palmquist (1988). Prior to FA analysis, samples were thawed, shaken, and 200 μ L were aliquoted for lyophilization. Lyophilized samples were resuspended in 1 mL of hexane containing C13:0 as an internal standard and methylated using BF_3 -methanol. The resulting FA methyl esters were extracted in hexane and injected onto a Supelco (Bellefonte, PA) SP-2560 capillary gas chromatography column (100 m $\times$ 0.25 mm $\times$ 0.2 μ m) for FA profile analysis, with a run time of 67 min.

Plasma Analyses

Colorimetric kits were used to quantify glucose, NEFA, BHBA, and insulin concentrations in all plasma samples as previously described (Bradford et al., 2009). Bovine-specific ELISA assays were used to quantify insulin (Mullins et al., 2012) and $\text{TNF}\alpha$ (Farney et al., 2011) concentrations in samples collected on d 7. Additionally, a multiplexed bovine cytokine ELISA assay (Searchlight, Bovine Cytokine Array; Aushon Biosystems; Billerica, MA) was used to determine $\text{IFN}\gamma$, IL-6, and IL-4 concentrations in plasma samples collected on d 7; this assay system has been previously described (Carroll et al., 2009). Haptoglobin was analyzed by a colorimetric method based on peroxidase activity (Cooke and Arthington, 2013). The standard curve was prepared using plasma samples (Yuan et al., 2013) determined by ELISA (kit #2410-7; Life Diagnostics, West Chester, PA).

Plasma samples collected for the glucose turnover assay were analyzed for $\text{U-}^{13}\text{C}$ -glucose enrichment (Metabolic Solutions, Inc., Nashua, NH). Glucose was extracted and converted to aldonitrile pentaacetate derivative (Tserng and Kalhan, 1983), and negative chemical ionization GC-MS (Hewlett-Packard 5890) was used to analyze derivatized samples. The isotopic composition of the glucose was determined by monitoring unlabeled ($\text{M}+0$: $m/z = 328$) versus $\text{U-}^{13}\text{C}$ -labeled ($\text{M}+6$: $m/z = 334$) glucose derivatives. This approach, as opposed to oxidation of glucose and measurement of CO_2 enrichment, ensures that results are not biased by carbon recycling via the Cori cycle. Enrichment of plasma glucose for each animal was fitted to an exponential decay curve according to the equation

$$E_t = E_0 \times e^{-kt},$$

where t = time relative to infusion (min); E_t = enrichment of plasma glucose ($\text{U-}^{13}\text{C}$ -glucose: unlabeled glucose ratio) at time t ; E_0 = enrichment at time $t = 0$; and k = rate constant (min^{-1}). After using the best-fit equations to determine k and E_0 , the total glucose pool was calculated by the equation

$$G = M \div E_0,$$

where G = total glucose pool (g) and M = mass of tracer infused (g). Plasma glucose turnover rate (GTR, g/min) was calculated as $\text{GTR} = G \times k$. Samples collected 10 min before infusion of $\text{U-}^{13}\text{C}$ -glucose were also analyzed to verify the lack of natural occurrence of the $\text{M}+6$ isotopomer.

Western Blot Analysis of IL-10

Plasma and adipose tissues samples collected on treatment d 7 were analyzed for IL-10 content using a semiquantitative Western blot assay. Adipose tissue (~20 mg) was homogenized at 4°C in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing a broad-spectrum protease inhibitor cocktail (Protease Inhibitor Cocktail I; Calbiochem, Gibbstown, NJ). The homogenate was centrifuged at $1,500 \times g$ for 1 min at 25°C and the protein content of the supernatant was measured with Coomassie blue. For the Western blot, samples (1 μ L of plasma or 40 μ g of protein from adipose tissue) were diluted in Laemmli sample buffer, heated at 90°C for 5 min, cooled, vortexed, separated by SDS-PAGE on a 4 to 12% Tris-HCl gel, and dry-transferred onto nitrocellulose membranes (iBlot; Invitrogen, Carlsbad, CA). Membranes were blocked in Tris buffer (pH 7.4) with 5% dry milk powder for 2 h

at room temperature, then incubated for 1 h at room temperature with a rabbit anti-IL10 antibody (catalog #sc-2313, Santa Cruz Biotechnology) diluted 1:500 for adipose samples or 1:1,000 for plasma samples. After washing, membranes were incubated for 1 h at room temperature with a secondary antibody (goat anti-rabbit IgG-HRP, catalog #sc-2033, Santa Cruz Biotechnology) diluted 10,000-fold in Tris buffer (pH 7.4). Immunodetection was performed by chemiluminescence (West-Dura; Thermo Scientific, Waltham, MA) using a digital photodocumentation system (ChemiDoc-It Imaging System; UVP Inc., Upland, CA). ImageJ software (National Institutes of Health, Bethesda, MD) was used for densitometry analysis. Western blot analysis was completed twice and densitometry values were averaged for statistical analysis. In both plasma and adipose samples, only the dimeric form of IL-10 (37 kDa) was detected.

Liver Tissue Analyses

Triglyceride content of liver tissue was measured as previously described (Mullins et al., 2012). Quantitative real-time PCR was used to measure transcript abundance of key cytokines as well as genes involved in hepatic gluconeogenesis. Total RNA was isolated from liver tissue using a commercial kit (RNeasy Lipid Tissue Mini Kit, Qiagen, Valencia, CA). Ultraviolet spectroscopy was used to quantify RNA (Nanodrop-1000, Nanodrop Technologies Inc., Wilmington, DE). Quality of RNA was assessed (Bioanalyzer, Agilent Technologies, Santa Clara, CA) and RNA integrity number values were 6.2 ± 0.8 (mean $\pm$ SD). A high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) was used for synthesis of cDNA from 2 μ g of total RNA. Quantitative real-time PCR was performed in triplicate with 5% of the cDNA product in the presence of 200 nmol/L of gene-specific forward and reverse primers using SYBR green fluorescent detection (ABI 7500 Fast, Applied Biosystems). Messenger RNA abundance was quantified using the delta cycle threshold (Ct) method, with the geometric mean of ribosomal protein submit 9, ribosomal protein submit 15, and β -actin used to normalize values. Reference gene Ct values were not affected by treatment (all $P > 0.25$). Primer sequences and reaction efficiencies are detailed in Supplemental Table S1 (<http://www.dx.doi.org/10.3168/jds.2013-7777>).

Statistical Analysis

Repeated measures results were modeled (SAS 9.2, SAS Institute, Cary, NC) with the fixed effects of treatment, day, and treatment $\times$ day interaction and the

random effect of block. Repeated measures within cow were modeled with an autoregressive (AR[1]) covariance structure, and denominator degrees of freedom were estimated by the Kenward-Rogers method. Analysis of milk variables included pretreatment values as covariates to account for differences between animals at the beginning of the study. Single time point results were modeled (JMP 8.0, SAS Institute) with the fixed effect of treatment and the random effect of block. Several variables [liver tumor necrosis factor α (*TNFA*), *IL10*, IL-1 receptor 1 (*IL1R1*), plasma haptoglobin, IL-4, IL-6, TNF α , and IFN γ] were natural log-transformed for statistical analysis to achieve normal residual distributions, and reported means were back-transformed.

RESULTS

Continuous administration of TNF α in the tail-head adipose depot caused an apparent increase in local swelling, but did not increase body temperatures (data not shown). Circulating concentrations of TNF α , IFN γ , IL-6, and IL-4 were determined to assess the effect of treatment on inflammatory mediators. None of these cytokines were altered by treatment (Table 2); however, concentrations of the acute phase protein haptoglobin were elevated by TNF α treatment (Figure 1).

Treatment failed to alter feed intake, milk production, or composition of milk (Table 3). Furthermore, the proportion of short- and medium-chain FA (<C16, derived from de novo synthesis) in milk did not differ between treatments (21.2 vs. 20.6 ± 1.2 g/100 g of FA for control vs. TNF α , respectively), providing no evidence of a shift in source of milk FA.

Liver TG concentration did not differ between treatments (Figure 2). To investigate hepatic glucose metabolism, we quantified plasma glucose turnover rate. In ruminants, the glucose turnover rate is essentially equal to the gluconeogenic rate because the net portal appearance of glucose is negligible (Reynolds et al., 1988). The glucose turnover rate did not differ between treatments (Figure 2). Likewise, mRNA abundance of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (*PCK1*) was unaltered ($P = 0.51$), although TNF α treatment tended to decrease transcript abundance of *PC*, another key gluconeogenic enzyme ($P = 0.08$, Figure 3A). Neither plasma glucose nor insulin concentrations were altered by treatment (Table 2). Plasma NEFA concentration was measured as a proxy for lipolytic rate and BHBA concentration as an index of ketogenesis; neither variable was altered by treatment (Table 2).

Additionally, liver mRNA abundance of selected pro- and anti-inflammatory cytokines were measured to assess tissue-specific responses. In the liver (Figure

Table 2. Effects of continuous infusion of tumor necrosis factor- α (TNF α) on plasma metabolites and endocrine factors¹

Item	Treatment		SEM	<i>P</i> -value
	Control	TNF α		
TNF α , pM	0.37	0.35	0.20	0.95
IFN γ , pM	0.62	0.81	0.22	0.54
IL-4, pM	0.59	0.47	0.47	0.84
IL-6, pM	2.9	6.5	2.6	0.29
Glucose, mM	3.31	3.34	0.10	0.64
NEFA, μ M	127	134	19	0.81
BHBA, μ M	496	476	37	0.84
Insulin, pM	368	363	65	0.97

¹Glucose, NEFA, and BHBA concentrations represent means over the 7-d treatment period; no treatment $\times$ time interactions were significant. Insulin and cytokine concentrations were determined on d 7 of treatment. Values are means and pooled SEM, n = 9 or 10.

3A), TNF α treatment tended to decrease *TNFA* mRNA abundance by 43% ($P = 0.07$) and *IL1R1* mRNA by 70% ($P = 0.06$), whereas *IL10* mRNA abundance was not altered by treatment ($P = 0.18$). Western blot analysis of IL-10 protein in adipose tissue showed a striking increase in this anti-inflammatory cytokine (Figure 3B). Because adipose tissue IL-10 was elevated by TNF α infusion, we also performed a semiquantitative analysis of plasma IL-10 by Western blot. No differences in plasma IL-10 were detected ($P = 0.54$, data not shown).

DISCUSSION

Previous research with once-daily subcutaneous injections of TNF α to late-lactation dairy cows found that TNF α could increase liver TG concentration and tended to decrease hepatic mRNA abundance of rate-limiting enzymes for gluconeogenesis and FA oxidation (Bradford et al., 2009). Several other studies have likewise demonstrated substantial behavioral, endocrine, and metabolic responses to similar daily bolus doses of TNF α , even though changes in circulating TNF α

Figure 1. Plasma haptoglobin concentrations in lactating dairy cows continuously infused with saline (control) or 14 μ g/kg of BW recombinant bovine tumor necrosis factor- α (TNF α) over 7 d. Values are back-transformed means $\pm$ SEM, n = 9 or 10. Treatment ($P < 0.001$) and day ($P = 0.03$) effects were significant, but no treatment $\times$ day interaction was detected ($P = 0.34$).

Table 3. Effects of continuous infusion of tumor necrosis factor- α (TNF α) on intake, milk production, and milk composition¹

Item	Infusion treatment		SEM	P-value
	Control	TNF α		
DMI, kg/d	20.9	20.5	0.98	0.63
Milk yield, kg/d	30.0	30.5	1.23	0.72
Milk fat, kg/d	1.14	1.07	0.066	0.43
Milk fat, %	3.80	3.52	0.16	0.28
Milk protein, kg/d	0.98	0.97	0.041	0.83
Milk protein, %	3.25	3.23	0.023	0.57
Milk lactose, kg/d	1.47	1.50	0.059	0.68
Milk lactose, %	4.90	4.91	0.023	0.79

¹Values are means and pooled SEM, n = 9 or 10.

concentrations are marginal at these administration rates (Kushibiki et al., 2003; Yuan et al., 2013). We expected that continuous TNF α administration at the same dose (2 μ g/kg of BW per day) would better mimic endogenous TNF α release and would subsequently alter insulin sensitivity, increase plasma NEFA concentration through induction of lipolysis, and promote hepatic TG accumulation. In contrast, administration of TNF α through osmotic pumps did not significantly alter liver TG concentration following the 7-d treatment period (Figure 2).

Prior studies evaluating the effects of continuous TNF α administration have generally mimicked acute, rather than chronic, inflammation. Continuous administration of 100 μ g of TNF α over 7 d in mice, for

example, increased serum TNF α concentrations by approximately 500-fold (Matsuno et al., 2002). Concentrations of nearly 10 ng/mL achieved in that study are relevant for studying acute endotoxemia (Kenison et al., 1991), but TNF α concentrations of this level are clearly supraphysiological in dairy cows during the transition to lactation, when mean concentrations range from 1 to 10 pg/mL and rarely exceed 100 pg/mL in individuals (Schoenberg et al., 2011; Farney et al., 2013; Yuan et al., 2013). Other continuous infusion studies have demonstrated immune cell infiltration of muscle (Peterson et al., 2006) and suppression of plasma TG (Sweep et al., 1992a) and thyroxine (Sweep et al., 1992b) concentrations in rodents receiving 20 to 100 μ g/kg daily for 7 d. Our dose of 2 μ g/kg per day

Figure 2. Liver triglyceride (TG) concentration and plasma glucose turnover rate in lactating dairy cows continuously infused with saline (control) or 14 μ g/kg of BW recombinant bovine tumor necrosis factor- α (TNF α) over 7 d. Values are means $\pm$ SEM, n = 9 or 10. Treatment did not alter liver TG concentration (left axis, $P = 0.99$) or glucose turnover rate (right axis, $P = 0.24$).

Figure 3. Liver transcript abundance and adipose tissue IL-10 concentrations in lactating dairy cows continuously infused with saline (control) or 14 $\mu\text{g/kg}$ of BW recombinant bovine tumor necrosis factor- α (TNF α) over 7 d. (A) Transcripts involved in nutrient metabolism and inflammatory signaling in liver were quantified by quantitative real-time PCR. Values for *TNFA*, *IL1R1*, and *IL10* were back-transformed after analysis of log-transformed data. (B) Adipose tissue IL-10 protein concentration was assessed by Western blot; tissue collected was contralateral to the site of TNF α administration to avoid local effects. A representative blot image and the results of densitometry analysis are shown. All values are means $\pm$ SEM, $n = 9$ or 10 ; $^{\dagger}P < 0.10$; $^{***}P < 0.001$.

was therefore dramatically lower than those reported to have these acute responses, and the dose was chosen based on its efficacy in previous work (Kushibiki et al., 2003; Bradford et al., 2009; Yuan et al., 2013). The fact that plasma haptoglobin was elevated by our treatment regimen (Figure 1) validates the systemic relevance of this model system, even though we did not detect a change in plasma TNF α concentration (Table 2).

Despite the small number of significant treatment effects observed in the current study, 2 key insights can be gleaned from this work. First, these observations suggest that identical low doses of TNF α cause very different responses when administered in daily subcutaneous boluses versus continuous infusion in a subcutaneous adipose depot. In essentially all other respects, the design of the current study was identical to one that showed increased liver TG content and substantial alterations in metabolic transcripts in the liver following 7 d of TNF α administration (Bradford et al., 2009). Although we are unaware of reports directly comparing repeated bolus doses of TNF α to continuous administration, responses to bolus versus sustained administration of the same dose of LPS have been evaluated (Taudorf et al., 2007). This study in humans showed that bolus administration of LPS induced a more rapid TNF α response and led to significantly higher plasma IL-6 concentrations and neutrophil counts over the course of the 8-h observation period (Taudorf et al., 2007). That more dramatic increases in inflammatory agents induce greater responses than sustained, low-level administration is not surprising. Inflammatory cascades can initiate an amplification process known as the cytokine storm (Tisoncik et al., 2012). Once the threshold for this response is reached, endogenous production of TNF α , other proinflammatory cytokines, and chemokines is capable of sustaining the inflammatory profile well beyond the point when the administered agent is cleared. With continuous administration protocols, this threshold may not be reached.

The other intriguing finding herein was the anti-inflammatory response observed in liver and adipose tissue. In our prior study with daily bolus doses, peripheral administration of TNF α increased *TNFA* mRNA in the liver, suggesting the activation of inflammatory transcriptional programs (Bradford et al., 2009). Therefore, it was surprising to find evidence of increased IL-10 in adipose tissue, as well as possible decreases in *TNFA* and *IL1R1* mRNA in liver. Interleukin 1 receptor 1 mediates most of the effects of the proinflammatory cytokine IL-1 β (Sims et al., 1993), so a decrease in its expression is consistent with a decrease in inflammatory signal transduction, as is the decrease in *TNFA*. Interleukin-10 is one of the best-characterized anti-inflammatory cytokines (Fiorentino

et al., 1991; Banchereau et al., 2012), and an increase in production of this protein in adipose tissue distant from the site of pump implantation clearly demonstrates an anti-inflammatory shift in this tissue.

Although the proinflammatory effects of TNF α are typically the most overt, evidence of anti-inflammatory components of the cellular responses to TNF α has also been reported, involving at least 3 different mechanisms. Interleukin-10 production can be induced by inflammatory agents, such as TNF α (Giambartolomei et al., 2002). This response is usually considered a component of the resolution phase of inflammation, the well-characterized, preprogrammed wave of anti-inflammatory compound release that follows the initial cytokine storm (Serhan, 2011). Another mechanism that may contribute to desensitization (tolerance) during continued exposure to TNF α is the induction of suppressor of cytokine signaling 3. This protein interferes with signaling downstream of cytokine receptors, and TNF α increases its abundance by inhibiting degradation of both SOCS3 mRNA (Ehrling et al., 2007) and protein (Dagvadorj et al., 2010).

Transmembrane receptors for TNF α mediate its inflammatory effects on cells, but these receptors can also be cleaved to release soluble forms that neutralize TNF α activity by preventing it from binding to intact receptors (Van Zee et al., 1992). Interestingly, TNF α itself promotes this cleavage process (Dri et al., 2000), and increased concentrations of soluble TNF α receptors have been directly implicated in the tolerance induced by low-dose pretreatment with either LPS (van Mierlo et al., 2008) or TNF α (Sass et al., 2002). Therefore, continuous low-dose TNF α treatment may induce receptor cleavage and increase soluble TNF α receptor concentrations, decreasing subsequent responses to the cytokine.

We propose that a combination of resolving phase signals, disruption of intracellular cytokine signaling, and TNF α receptor shedding may have led to a state in which inflammation was suppressed in TNF α -treated cows to a greater extent than in the control animals. The idea that low-dose pretreatment with inflammatory agents can promote tolerance to larger subsequent challenges is well-documented, as discussed herein. More broadly, this phenomenon brings to mind the concept of hormesis, which proposes that compounds can generate very different responses at different doses, to the extent that a harmful agent at a high dose may be beneficial or protective at a low dose (Kolb and Eizirik, 2012). In fact, a fairly long list of immune system signals have been documented as inducing hormetic responses in a variety of model systems (Calabrese, 2005). The current work suggests that such responses may also depend on the pulsatility of administration.

Our findings leave open the question about the relevance of adipose tissue-derived cytokines in dairy cattle. Because of the well-established links between adipose-associated inflammation and metabolic syndrome in humans and rodents (Olefsky and Glass, 2010), it has been proposed that adipose-derived inflammatory signals may provide the mechanistic link between high adipose tissue stores at parturition and increased risk of metabolic disorders in dairy cows. Recent evidence, however, has not supported this hypothesis. Plasma TNF α concentrations are not greatly elevated during the periparturient period (Schoenberg et al., 2011), and adipose tissue from cows during this period clearly did not manifest the degree of immune cell infiltration observed in obese rodent models (Akter et al., 2012). Nevertheless, potentially important changes in adipose tissue endocrine profiles could affect systemic inflammatory status; for example, animals with greater adipose stores have decreased adiponectin concentrations (Frayn et al., 2003; Häussler et al., 2013). The attenuation of this anti-inflammatory signal may be an important, often overlooked effect of increased adiposity. The mechanistic effect of adipose tissue on metabolic disorders in dairy cows remains unclear, but endocrine factors other than TNF α are perhaps better candidates for continued investigation.

CONCLUSIONS

In contrast to our initial hypothesis, continuous administration of TNF α into a subcutaneous adipose tissue depot did not significantly alter systemic nutrient metabolism. Furthermore, TNF α treatment had limited effects on the inflammatory mediators measured, and several changes in adipose tissue and liver suggested a shift to a less inflammatory state. These findings are consistent with the concept that continuous low-dose TNF α administration activates compensatory mechanisms to induce tolerance to TNF α and enhance anti-inflammatory factors. Compared against previous results, these findings also suggest that repeated bolus administration of TNF α induces much more dramatic effects on nutrient metabolism than continuous infusion of the same dose.

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