Regulation of mammary parenchymal growth by the fat pad in prepubertal dairy heifers: role of inflammation-related proteins

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Abstract

In prepubertal heifers, the mammary parenchyma consists of epithelial and myoepithelial cells growing within a mammary fat pad (MFP). The MFP produces IGF-I that stimulates epithelial cell proliferation. In other species, adipose tissue expansion induces inflammation-related proteins (IRP), such as tumor necrosis factor α (TNFα), interleukin (IL)-6, IL-1β transforming growth factor β, monocyte chemoattractant protein 1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1). The MFP production of IRP may influence mammary development because they impair not only insulin but also IGF-I actions. Moreover, the MFP expansion seen with development and increased nutrition coincides with reduced parenchymal growth. Our first objective was to identify IRP capable of altering proliferation of bovine mammary epithelial cells. TNFα, but neither IL-6, IL-1β MCP-1 nor PAI-1, inhibited basal and IGF-I-stimulated proliferation in MAC-T cells and primary cells isolated from heifers. Our second objective was to determine whether MFP expression of IRP changed in a manner consistent with inhibition of parenchymal growth. MFP expression was measured from 100 to 350 kg body weight (experiment 1) or at 240 kg body weight (experiment 2) in dairy heifers offered restricted or high planes of nutrition. In experiment 1, neither nutrition nor development altered MFP expression of TNFα. Nutrition increased MCP-1 and PAI-1 but only before MFP expansion and after cessation of allometric parenchymal growth. In experiment 2, nutrition increased TNFα and PAI-1, but not MCP-1. Thus, MFP expansion increases IRP production in cattle, but this is unlikely to contribute to reduced parenchymal growth observed with development or increased nutrition.

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Introduction

In rodents and humans, increased adiposity induces adipose tissue production of the proinflammatory cytokines tumor necrosis factor α (TNFα), interleukin (IL)-6, and IL-1β (Wellen & Hotamisligil 2005, Tilg & Moschen 2006). These cytokines interfere with insulin signaling in adipose tissue, liver, and muscle, leading to the development of whole-body insulin resistance (Wellen & Hotamisligil 2005, Shoelson *et al.* 2006). All three cytokines also impair insulin-like growth factor-I (IGF-I)-stimulated proliferation of human mammary epithelial cells (Shen *et al.* 2002). Mechanistically, these inhibitory actions occur because inflammatory cytokines attenuate the activity and abundance of signaling elements shared by both insulin and IGF-I (e.g. insulin receptor substrate-proteins (IRS) proteins; Rui *et al.* 2002, Wellen & Hotamisligil 2005).

Adipose tissue expansion also induces the production of other proteins associated with inflammation, including transforming growth factor β (TGFβ1), monocyte chemoattractant protein 1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1; Tilg & Moschen 2006). TGFβ1 reduces serum-stimulated proliferation of bovine mammary epithelial cells (Woodward *et al.* 1995, Purup *et al.* 2000*a*). MCP-1 is a chemoattractant molecule that recruits monocytes to adipose tissue (Chen *et al.* 2005, Kanda *et al.* 2006). PAI-1 not only inhibits the activity of urokinase and tissue plasminogen activator proteins (uPA and tPA), which generate plasmin, but also modulates the biological activities of proteins such as vitronectin and IGF-I-binding protein 5 (Stefansson & Lawrence 1996, Maile *et al.* 2006, Sorrell *et al.* 2006). MCP-1 and PAI-1 have been shown to attenuate insulin signaling and cell growth in non-bovine systems (Lopez-Alemany *et al.* 2003, Kanda *et al.* 2006, Kortlever *et al.* 2006, Sell *et al.* 2006).

The ability of adipose tissue to produce inflammation-related proteins (IRP) could be relevant to the growth of the mammary parenchymal compartment of prepubertal dairy heifers. At this stage of development, the parenchyma consists of epithelial and myoepithelial cells growing as multi-layered duct-like structures within the mammary fat pad (MFP). This arrangement maximizes epithelial cell exposure to essential mitogens produced by the MFP, such as IGF-I (Walden *et al.* 1998, Kleinberg *et al.* 2000, Meyer *et al.* 2006*a*, Connor *et al.* 2007). In theory, it would also facilitate growth inhibition if MFP expansion triggered the synthesis of IRP. This possibility is

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suggested by situations where indices of parenchymal and MFP growth are inversely related. Specifically, expansion of the MFP in rapidly growing dairy heifers is associated with decreased parenchymal growth when assessed at a similar body weight (Sejrsen *et al.* 1982, Capuco *et al.* 1995). Moreover, cessation of allometric parenchymal growth occurs near 300 kg body weight when the MFP has expanded significantly (Meyer *et al.* 2006*b*).

These observations raise the possibility that MFP expansion induces the synthesis of IRP, which then impair mammary epithelial cell growth. Our first objective was to identify which IRP, previously shown to be produced by human or rodent adipose tissue, could inhibit proliferation of bovine mammary epithelial cells *in vitro*. Our second objective was to verify physiological relevance for the inhibitors identified *in vitro*. We did so by determining whether MFP expression of these factors was inversely related with indices of parenchymal growth in prepubertal dairy heifers.

Materials and Methods

[^{3}H] thymidine incorporation in bovine mammary epithelial cells

The MAC-T bovine mammary epithelial cell line was established from primary bovine mammary alveolar cells by immortalization with the SV-40 large T antigen (Huynh *et al.* 1991). MAC-T cells were routinely grown at 37 °C with 5% CO_2 in basal Dulbecco's modified Eagle's medium (DMEM with 4·5 g/l glucose containing 20 U/ml penicillin, 2·4 nM glutamine) supplemented with 10% fetal calf serum and 5 μg/ml insulin (Thorn *et al.* 2006). For proliferation assays, MAC-T cells were plated at 1×10^4 cells/cm^2 into 48-well plates and cultured for 48 h. Cells were washed twice with PBS and incubated in basal DMEM for 24 h. After 24 h, the media was changed to basal DMEM supplemented with [methyl-^{3}H] thymidine (1 μCi/well; MP Biomedicals, Irvine, CA, USA) and hormones for 18 h as indicated in figure legends.

Primary mammary epithelial cell (pMEC) organoids were obtained from two independent isolations by digestion of the parenchymal mammary compartment of prepubertal Friesian heifers, as described previously (Purup *et al.* 2001). Frozen stocks of pMEC were thawed as needed in basal medium 199 (M199 containing 2·6 g/l BSA, 5 mg/l transferrin, 1 mg/l reduced glutathione, 1 mg/l soybean trypsin inhibitor, 1 μg/l selenium, 0·2% penicillin, and streptomycin antibiotic solution) and kept at 37 °C with 5% CO_2. For cell proliferation assays, the two independent cell isolates were mixed and seeded in basal M199 supplemented with 10 μg/l insulin (Sigma) into three-dimensional gels, as described previously (Weber *et al.* 1999). After 24 h, cells were incubated for 4 days with basal M199 supplemented with insulin and indicated hormones (see figure legends). Media were changed every 2 days and [methyl-^{3}H] thymidine was added for the last 24 h of the culture period.

For both cell systems, all treatments were performed in triplicate. Thymidine incorporation was measured, as previously reported (Thorn *et al.* 2006). Human recombinant proteins tested were TNFα (PeproTech Ltd, Rocky Hill, NJ, USA), IL-6 (PeproTech), IL-1β (PeproTech), MCP-1 (PeproTech), the stable and constitutively active form of PAI-1 described by Czekay *et al.* (2003) (Calbiochem, San Diego, CA, USA), and IGF-I (National Institute of Diabetes & Digestive & Kidney Diseases, Bethesda, MA, USA for MAC-T; Austral Biologicals, San Ramon, CA, USA for pMEC).

Prepubertal heifer experiments

Mammary adipose tissue was obtained from two experiments performed in prepubertal dairy heifers. All experimental procedures were conducted with the approval of the local Institutional Animal Care and Use Committee (Cornell University or Danish Animal Experimentation Inspectorate). In the first experiment performed at Cornell University, Holstein dairy heifers were randomly assigned to a restricted (R) or high (H) plane of nutrition beginning at 10 days of age to attain an average daily gain (ADG) of 650 or 950 g/day respectively, as previously described (Meyer *et al.* 2006*c*). Heifers in each treatment group were killed at 100, 150, 200, 250, 300, and 350 kg body weight ($n=6$ per group). In the second experiment performed at the Danish Institute of Agricultural Sciences, Holstein–Friesian dairy heifers were randomly allocated at 42 days of age to a R or H plane of nutrition ($n=12$ per treatment) to attain an ADG of 700 or 1200 g/day respectively, as described previously (Thorn *et al.* 2006). Heifers in both treatments were killed at 240 kg body weight. In both experiments, mammary gland weights were recorded and MFP samples were collected and snap frozen in liquid nitrogen.

Measurement of cytokine gene expression

Representative samples (200 mg) of MFP tissue were homogenized with 1 ml Qiazol (Qiagen). Total RNA was isolated and purified using RNeasy Mini columns and on-column RNase-free DNase treatment (Qiagen) following the manufacturer's protocol. Quantity and integrity of RNA was determined using the RNA Nano Lab Chip Kit (Agilent; Palo Alto, CA, USA). Reverse transcription reactions were performed with 2 μg RNA, 500 ng random primers (Invitrogen), and ImPromII reverse transcriptase (Promega) in a 20 μl volume.

Gene expression was measured by real-time PCR. TNFα transcripts were detected with a Taqman probe assay, whereas TGFβ1, MCP-1, PAI-1, and 18S transcripts were detected with a SYBR green assay (Table 1). A previously validated assay was used for leptin (Thorn *et al.* 2006). Reactions were performed in duplicate in a 25 μl volume using Perfect Real Time 2× Premix with supplied ROX dye (Takara; Madison, WI, USA) for leptin, 2× Universal Mix (Applied Biosystems Inc., Foster City, CA, USA) for TNFα, and Power SYBR Mix (Applied Biosystems Inc.) for other assays. Reactions contained 500 nM of each primer, 100 nM probe (for leptin and TNFα assays), and diluted cDNA (20 ng reverse-transcribed RNA, except 2 ng for 18S). The average S.D. for sample C_T was 0·10. To analyze the data, a relative standard

Table 1 Real-time PCR primer and probe sequences

	Sequence[a]
Transcript	
TNFα	F′: GGTCCTCTTCTCAAGCCTCAAG
	R′: AGCTGCCCCGGAGAGTTG
	Probe: FAM-CGGTAGCCCACGTTGTAGCCGACA-TAMRA
TGFβ1	F′: GGCCCTGCCCTTACATCTG
	R′: CCGGGTTGTGCTGGTTGTAC
MCP-1	F′: CACCAGCAGCAAGTGTCCTA
	R′: GCTTGGGGTCTGCACATAAC
PAI-1	F′: AGCTGACCACAGGAGGAGAA
	R′: TCCTTGTACAGTCGGTGGAA
18S	F′: GATCCATTGGAGGGCAAGTCT
	R′: GCAGCAACTTTAATATACGCTATTGG

[a]For each transcript, the sequence of the forward primer (F′), reverse primer (R′), and probe are given in the 5′ to 3′ direction. FAM, carboxyfluorescein; TAMRA, carboxytetramethylrhodamine.

curve was generated for each transcript using pooled cDNA prepared from prepubertal mammary parenchyma and MFP. The relative standard curve consisted of six serial twofold dilutions of the pooled cDNA. Amplification was linear and efficient across the range of standards for each assay (efficiency for all assays was $>0{\cdot}9$, based on efficiency $=10^{(-1/\text{slope})}-1$, where slope is obtained from regression of C_T versus log input). Unknown sample expression was then determined from the standard curve, adjusted for 18S, and expressed as a fold difference as indicated in the figure legends.

Statistical analysis

Analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). When testing a single dose, thymidine incorporation data from the MAC-T and pMEC experiments were analyzed separately with the fixed effects of IRP (TNFα, IL-6, IL-1β, MCP-1, or PAI-1), IGF-I, their interaction (INT), and experiment (block). For the dose–response experiments, thymidine incorporation data in basal and IGF-I-stimulated conditions were analyzed separately from the MAC-T and pMEC experiments. The model included the fixed effect of treatment dose and experiment (block). Treatment dose differences were detected using multiple comparison tests with a Tukey adjustment. Gene expression data were analyzed with a mixed model accounting for nutrition, body weight at slaughter, and their INT in the first experiment, or only the effect of nutrition in the second experiment. Statistical significance was declared at $P<0{\cdot}05$.

Results

Effect of IRP on the proliferation of bovine mammary epithelial cells

The effects of IRP were first evaluated in the transformed mammary epithelial cell line, MAC-T, using thymidine incorporation as an index of proliferation. These cells are responsive to IGF-I as evidenced by a two to threefold increase in proliferation ($P<0{\cdot}001$, Fig. 1). Treatment of the MAC-T cells with 10 ng/ml TNFα-reduced proliferation by 50% under basal growth conditions and 70% in the presence of IGF-I (INT $P<0{\cdot}001$, Fig. 1). In contrast, the proinflammatory cytokines IL-6 or IL-1β, the chemoattractant MCP-1, and the inhibitor of plasminogen activation PAI-1 did not alter thymidine incorporation under basal or IGF-I-stimulated growth conditions even if used at high concentrations (50–100 ng/ml, Fig. 1).

We also determined whether these IRP had similar effects on primary mammary epithelial cells isolated from prepubertal dairy heifers (pMEC cells). Proliferation in these cells is also IGF-I dependent, with an almost twofold increase in thymidine incorporation when incubated with IGF-I at the concentration of 10 ng/ml ($P<0{\cdot}001$, Fig. 2). As seen with the MAC-T cells,

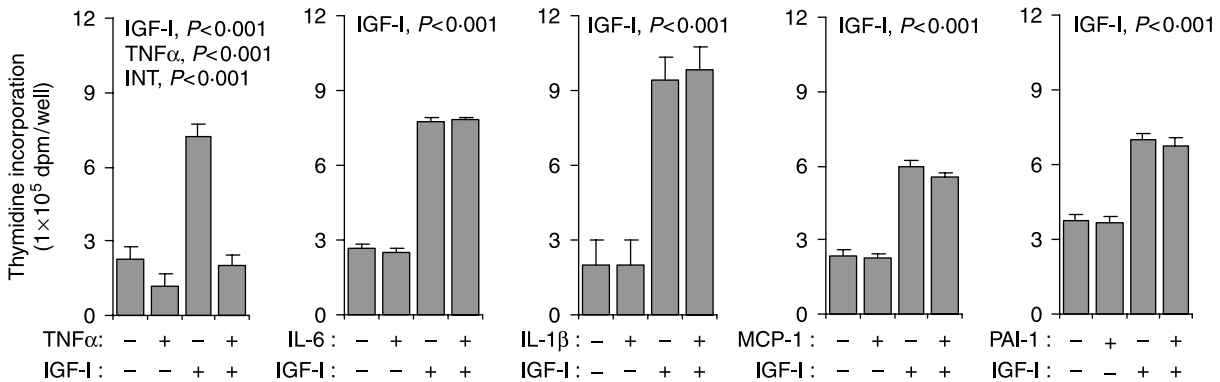

Figure 1 Effect of inflammation-related proteins on [^{3}H] thymidine incorporation in MAC-T cells. MAC-T were plated and grown in complete media for 48 h and then washed and incubated in basal media for 24 h. Cells were incubated under the same conditions in the absence or presence of TNFα (10 ng/ml), IL-6 (50 ng/ml), IL-1β (100 ng/ml), MCP-1 (100 ng/ml), PAI-1 (100 ng/ml), and IGF-I (10 ng/ml). Hormone treatments and [^{3}H] thymidine were added for 18 h. Mean ± S.E.M. is shown for each treatment. The effects of IGF-I, inflammation-related protein (TNFα, IL-6, IL-1β, MCP-1, or PAI-1), and their interaction (INT) are reported when significant ($P<0{\cdot}05$). Data are representative of two or three experiments.

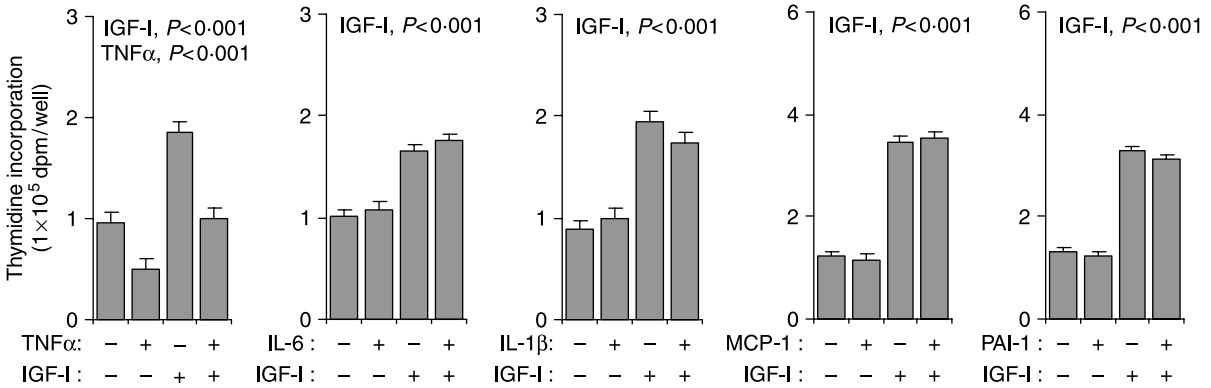

Figure 2 Effect of inflammation-related proteins on [^{3}H] thymidine incorporation in pMEC cells. pMEC were plated and incubated in basal media supplemented with insulin for 24 h. Cells were incubated under the same conditions in the absence or presence of TNFα, (10 ng/ml), IL-6 (100 ng/ml), IL-1β, (100 ng/ml), MCP-1 (100 ng/ml), PAI-1 (100 ng/ml), and IGF-I (10 ng/ml). Cells were incubated with hormone treatments for 4 days with a media change every 2 days, and [^{3}H] thymidine was added for the last 24 h of the culture period. Mean ± S.E.M. is shown for each treatment. The effects of IGF-I and inflammation-related protein (TNFα, IL-6, IL-1β, MCP-1, or PAI-1) are reported when significant ($P<0·05$). Data are representative of two or three experiments.

a 10 ng/ml dose of TNFα caused a 50% reduction in thymidine incorporation under both basal and IGF-I-stimulated conditions ($P<0·001$, Fig. 2), whereas 100 ng/ml doses of IL-6, IL-1β, PAI-1, and MCP-I had no effect.

Next, we characterized the dose-dependent effect of TNFα treatment on proliferation. The MAC-T and pMEC cells were incubated with increasing concentrations of TNFα (0, 0·01, 0·1, 1, and 10 ng/ml) under basal and IGF-I-stimulated conditions. The minimally effective dose of TNFα in MAC-T cells was 1 ng/ml. This dose reduced basal and IGF-I-mediated thymidine incorporation by 30–35% (Fig. 3A). In pMEC, the minimally effective concentration was 10 ng/ml under basal conditions and 1 ng/ml in the presence of IGF-I (Fig. 3B). These results demonstrate that a low dose of TNFα is sufficient to attenuate IGF-I-stimulated mammary epithelial cell proliferation.

Effects of development and nutrition on MFP production of IRP in prepubertal heifers

To determine whether the expression of IRP with effects on cell proliferation varies in a manner consistent with diet-induced changes in mammary parenchymal mass, we measured the expression of these factors in the MFP of prepubertal heifers growing at a rate of 650 (R) or 950 g/day (H) (Meyer *et al.* 2006*b*). The mass of the MFP increased over eightfold in these heifers across the 100–350 kg body weight interval and was 60% higher in the H than in the R heifers at 350 kg body weight (1126 g in H versus 710 g in R heifers). The H heifers also had less DNA per unit of MFP mass than R heifers (Meyer *et al.* 2006*b*). TNFα expression in the MFP, however, was unaffected by plane of nutrition or body weight (Fig. 4). We also measured TGFβ1 expression because it is increased in the serum of rapidly growing heifers and has been shown to inhibit MAC-T and pMEC proliferation (Woodward *et al.* 1995, Purup *et al.* 2000*a*,*b*). TGFβ1 expression was not affected by development or nutrition (Fig. 4).

IL-6 and IL-1β expression was not evaluated because these cytokines failed to alter proliferation *in vitro*. We did, however, measure the expression of MCP-1 and PAI-1 because their activities could depend on factors that were not present *in vitro*, such as the presence of macrophages for MCP-1 and extracellular matrix proteins for PAI-1 (Lopez-Alemany *et al.* 2003, Kanda *et al.* 2006, Tilg & Moschen 2006). The expression of PAI-1 and MCP-1 was higher in H heifers at 100 kg of body weight but expression then decreased and remained similar between R and H heifers until 350 kg of body weight. At 350 kg of body weight, PAI-1 expression was increased over threefold and MCP-1 expression was increased over eightfold in H compared with R heifers (Fig. 4). Both plane of nutrition and body weight increased leptin expression (Fig. 4), demonstrating that adipose tissue expansion has positive effects on MFP leptin gene expression, as it does in other adipose depots.

To test the effect of greater MFP expansion in the mammary gland, we analyzed MFP tissue obtained from heifers growing at a rate of 750 (R) or 1202 g/day (H) between 42 days of age and slaughter at 240 kg (Thorn *et al.* 2006). MFP mass was over three times greater in H than in R heifers (1749 g in H versus 474 g in R heifers) and leptin expression was increased by threefold, as we previously reported (Thorn *et al.* 2006). Expression of TNFα and PAI-1 was 50 and 300% higher in the MFP of H than in R heifers, whereas expression of TGFβ1 and MCP-1 did not differ significantly (Fig. 5).

Discussion

In rodents, prepubertal expansion of the mammary epithelial compartment proceeds only in the presence of the MFP (Couldrey *et al.* 2002). This requirement reflects in part the ability of the MFP to synthesize growth factors such as IGF-I, which then drive epithelial cell proliferation (Walden *et al.* 1998, Kleinberg *et al.* 2000). Consistent with this model,

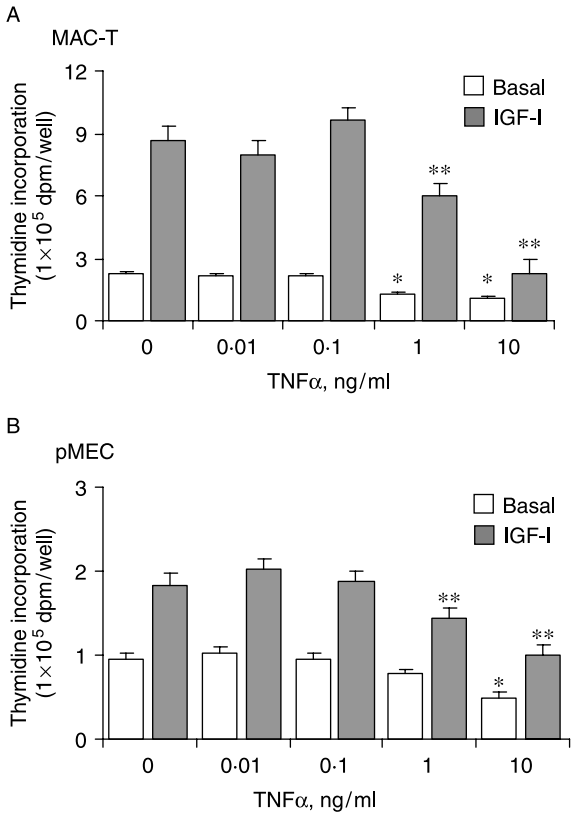

Figure 3 TNFα dose–response in bovine mammary epithelial cells. (A) MAC-T were plated and grown in complete media for 48 h and then washed and incubated in basal media for 24 h. pMEC were plated and incubated in basal media supplemented with insulin for 24 h. Cells were incubated in basal media supplemented with 0, 0·01, 0·1, 1·0, and 10 ng/ml of TNFα in the absence of presence of IGF-I (10 ng/ml). For MAC-T cells, hormone treatments and [^{3}H] thymidine were added for 18 h. (B) For pMEC, cells were incubated with hormone treatments for 4 day with a media change every 2 day, and [^{3}H] thymidine was added for the last 24 h of the culture period. Mean±S.E.M. is shown for each treatment. Data are representative of two to four experiments. *P<0·05 compared with cells grown in basal media alone. **P<0·05 compared with cells grown in the presence of IGF-I alone.

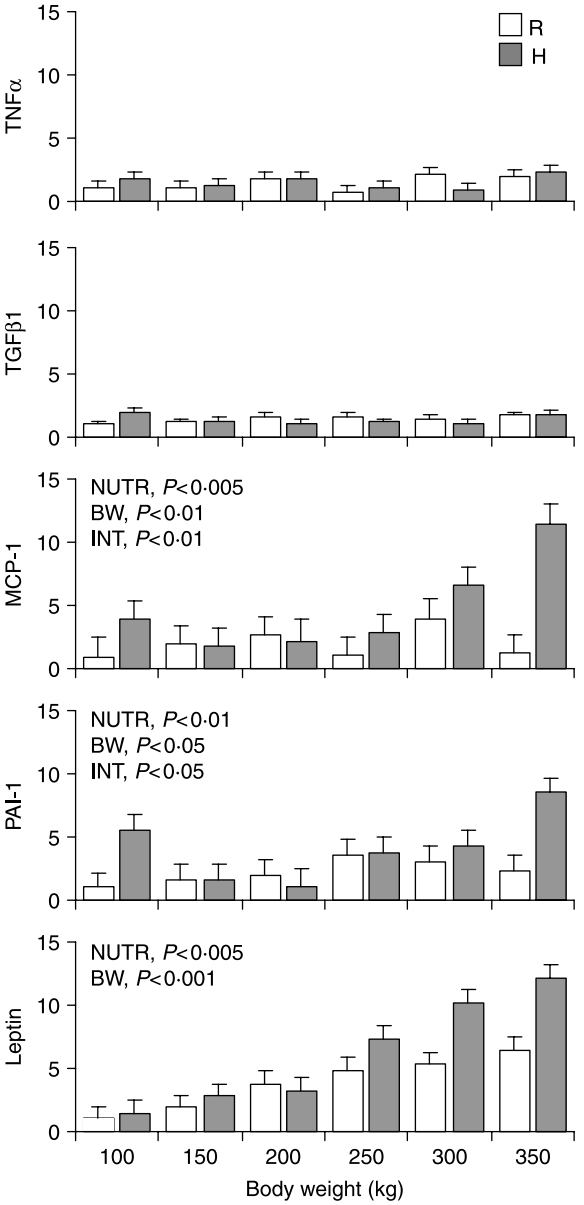

Figure 4 Effect of nutrition and body weight on the mRNA abundance of inflammation-related proteins and leptin in the mammary fat pad of prepubertal dairy heifers. Dairy heifers were offered a restricted (R) or high (H) plane nutrition between 10 days of age and slaughter at 50 kg intervals from 100 to 350 kg body weight (six animals per treatment). Total RNA was extracted from the mammary fat pad and analyzed by real-time PCR for the abundance of TNFα, TGFβ1, MCP-1, PAI-1, and leptin. Results are expressed relative to the mean expression level in the MFP of the 100 kg group on the R plane of nutrition. The effects of nutrition (NUTR), body weight (BW), and their interaction (INT) are reported when significant. Mean±S.E.M. is shown for each treatment.

prepubertal ductal growth is impaired in IGF-I null mice, but proceeds normally in mice retaining MFP IGF-I production even if deficient in plasma IGF-I (Ruan & Kleinberg 1999, Richards *et al.* 2004). Current evidence suggests a similar role for the MFP in prepubertal cattle. IGF-I in the mammary gland is produced in the MFP in response to systemic factors such as estrogen (Berry *et al.* 2003, Meyer *et al.* 2006*a*). Furthermore, the highest density of proliferating epithelial cells is found at the interface with the MFP (Ellis *et al.* 2000, Capuco *et al.* 2002). Recent data in rodents and humans have also shown that adipose tissue produces potent inhibitors of IGF-I actions such as TNFα, IL-6, and IL-1β and other factors that could modulate IGF-I action such as MCP-1 and PAI-1 (Shen *et al.* 2002, Lopez-Alemany *et al.* 2003, Wellen & Hotamisligil 2005, Sell *et al.* 2006). TGFβ1 is also

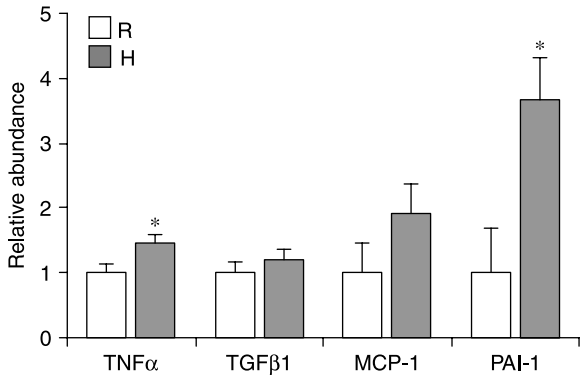

Figure 5 Effect of nutrition on the mRNA abundance of inflammation-related proteins in the mammary fat pad of prepubertal dairy heifers. Dairy heifers were offered a restricted (R) or high (H) plane nutrition between 42 days of age and slaughter at 240 kg (12 animals per treatment). Total RNA was extracted from the mammary fat pad and analyzed by real-time PCR for the abundance of TNFα, TGFβ1, MCP-1, and PAI-1. Results are expressed relative to the mean expression level in the MFP of the R heifers. Mean ± S.E.M. is shown for each treatment. *$P<0{\cdot}05$ relative to expression in R heifers.

expressed in adipose tissue and serum levels are increased in rapidly growing heifers (Purup *et al.* 2000*b*). TGFβ1 has been shown to inhibit bovine mammary epithelial cell proliferation (Woodward *et al.* 1995, Purup *et al.* 2000*a*).

Consistent with the possibility that an IRP could modulate IGF-I-dependent parenchymal growth in dairy heifers, we found that a low dose of TNFα reduced basal and IGF-I-stimulated proliferation in both MAC-T and pMEC cells. TNFα has previously been reported to reduce serum-stimulated proliferation in mammary epithelial cells (Okada *et al.* 1999). Our results suggest that at least a portion of this effect of TNFα is due to the inhibition of IGF-I actions. Our results are also consistent with those showing that TNFα inhibited basal and IGF-I-mediated proliferation in human mammary epithelial cells (Shen *et al.* 2004). Unlike others, however, we found that high doses of IL-6 and IL-1β (50 and 100 ng/ml respectively) did not affect basal or IGF-I-stimulated proliferation of bovine mammary cells. These results are not likely to reflect a lack of activity of human IL-6 and IL-1β in bovine cells or their use at sub-effective concentrations. Identical or even lower doses of both cytokines elicited other responses in mammary epithelial cells and chondrocytes of bovine origin (Okada *et al.* 1999, Attur *et al.* 2000, Andriamanalijaona *et al.* 2003, Thorn *et al.* 2006). Similarly, human MCP-1 and PAI-1 are bioactive in rodents and thus likely to react in bovine cells (Sell *et al.* 2006, Sorrell *et al.* 2006). Our results indicate that TNFα is unique among the adipose-derived IRP we tested in its ability to inhibit IGF-I-mediated proliferation *in vitro* in bovine mammary epithelial cells.

In dairy heifers, development and nutrition have been shown to impact indices of mammary parenchymal growth. Specifically, the parenchyma grows allometrically for most of the prepubertal period until heifers reach ~300 kg of body weight when growth slows to an isometric rate (Meyer *et al.* 2006*c*). In the case of nutrition, diets supporting daily growth rates in excess of 700 g/day are associated with reduced parenchymal mass when assessed in heifers at a similar body weight (Capuco *et al.* 1995, Sejrsen *et al.* 2000, Meyer *et al.* 2006*b*). Meyer *et al.* (2006*b*,*c*) showed that increased nutrient intake reduced mammary parenchymal mass predominantly by shortening the period of allometric growth. This finding did not exclude the possibility of other negative effects, such as inhibition of IGF-I-mediated cell proliferation by MFP-derived IRP. Indeed, adipose tissue expansion in humans and rodents results in increased production of TNFα, IL-6, TGFβ1, MCP-1, and PAI-1 (Wellen & Hotamisligil 2005, Tilg & Moschen 2006). All of these proteins have been shown to reduce insulin or IGF-I actions (Shen *et al.* 2002, Lopez-Alemany *et al.* 2003, Sartipy & Loskutoff 2003, Sell *et al.* 2006). This may explain why indices of mammary parenchymal growth are reduced with development and increased nutrient intake, even though the MFP IGF-I expression is unchanged when these reductions are detected (Meyer *et al.* 2007). Our *in vivo* data, however, offer little support for such a mechanism. Specifically, neither TNFα nor TGFβ1 expression was affected by development or nutrition in the first experiment. Increased nutrient intake did increase MCP-1 and PAI-1 expression, but in a manner that is inconsistent with the hypothesis. The H heifers had increased expression of MCP-1 and PAI-I at 100 kg body weight in the absence of MFP expansion and in conjunction with a higher rate of epithelial cell proliferation than R heifers, and again at 350 kg body weight when epithelial cell proliferation was unaffected by nutrient intake (Meyer *et al.* 2006*b*,*c*). Moreover, MCP-1 and PAI-I expression did not increase at 300 kg body weight when allometric growth ceased (Meyer *et al.* 2006*c*). Significant increases in TNFα and PAI-1, but not TGFβ1 or MCP-1, were observed in the second experiment, where the MFP had a greater degree of expansion in response to increased nutrient intake. Overall, these data show that MFP expansion does increase the production of IRP in dairy cattle, but give little support to the idea that these factors contribute to the reduced parenchymal growth observed with development or increased nutrient intake.

It is interesting to compare these results with those obtained with leptin, another cytokine hypothesized to mediate the negative effects of the expanding MFP on mammary parenchymal growth (Silva *et al.* 2002). Leptin expression in the MFP increases with development and nutrient intake. Quantitative real-time PCR assays have shown that the signaling form of the leptin receptor (Ob-Rb) is undetectable in MAC-T and pMEC cells and negligible in mammary parenchyma when compared with the hypothalamus, a recognized leptin target tissue (Thorn *et al.* 2006, 2007). Moreover, leptin is unable to induce signaling events or alter basal and IGF-I-stimulated proliferation in cultured bovine mammary epithelial cells (Thorn *et al.* 2006). Thus, despite an expression profile in the MFP that is consistent with modulation of parenchymal growth, leptin is unable to do so by acting directly on epithelial cells. In contrast, TNFα and TGFβ1 are potent inhibitors of IGF-I-mediated proliferation *in vitro*, but are unlikely to explain

reduced parenchymal growth because neither development nor nutrition consistently altered their expression in the MFP.

In conclusion, TNFα has potent inhibitory effects on mammary epithelial cell proliferation *in vitro*. MFP expression of TNFα, TGFβ1, MCP-1, and PAI-1 was static around the time when allometric parenchymal growth ceases. Moreover, increased nutrient intake reduced mammary parenchymal mass in both the absence and the presence of increased TNFα and PAI-1 expressions. Overall, these data suggest that MFP production of IRP is unlikely to play a role in terminating the allometric growth phase of the mammary parenchyma or in reducing parenchymal mass in heifers fed high planes of nutrition.

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