ABSTRACT: The emerging view is that reduced feed intake, lean muscle accretion, and growth in immunologically challenged pigs is the result of increased cytokine activity, but this has not been directly tested. To begin addressing this issue, 72 crossbred barrows and gilts (11.55 ± .19 kg BW) were not fed for 12 h and then injected i.p. with 0, .5, or 5 μg/kg of Escherichia coli lipopolysaccharide (LPS). Blood was collected by jugular puncture at 0, 2, 4, 8, 12, and 24 h after injection. Plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cortisol, plasma urea nitrogen (PUN), NEFA, and triglycerides were determined. Immunological stress was induced by LPS as indicated by increased secretion of TNF-α, IL-6, and cortisol. In pigs receiving 5 μg/kg of LPS, plasma TNF-α was increased 10-fold at 2 h after injection and was still elevated (P < .01) at 4 h. In these same pigs, plasma concentration of IL-6 was increased at 2 h and peaked at 4 h with levels exceeding baseline values by 200-fold (P < .01). Cortisol was elevated at 2, 4, and 8 h after injection (P < .01). The increased secretion of cytokines and cortisol in pigs injected with 5 μg/kg of LPS was followed by an increase in protein degradation, as evidenced by PUN values that were increased two- and threefold at 8 and 12 h after injection, respectively. However, unlike previous reports in laboratory animal species, plasma glucose, NEFA, and triglycerides were not altered by LPS. Nonetheless, as the period of feed deprivation progressed from 12 to 36 h, plasma NEFA and triglycerides increased (P < .05) and plasma glucose tended to decrease. We believe that immunological challenge induces cytokine synthesis and secretion in swine which, in turn, may induce protein catabolism.

Key Words: Cytokines, Cortisol, Pigs, Lipopolysaccharides

Introduction

Considerable evidence indicates that the metabolic effects characterizing immunological challenge are important for maintaining homeostasis during infection (Klasing and Johnstone, 1991; Adi et al., 1992). Those responses are attributed to cytokines that are released by activated macrophages. The proinflammatory cytokines that have profound metabolic effects in rodents include tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Meimon et al., 1994). Because porcine macrophages produce the same array of cytokines (Murtaugh, 1994), the emerging view is that the reduction in feed intake and growth observed in diseased or immunologically challenged pigs is the result of increased cytokine activity (Kelley et al., 1994).

It is apparent from studies comparing the growth of pigs reared in highly sanitized facilities to those reared in more conventional environments that immunological stress can reduce growth rate and feed efficiency from 10 to 25% (Williams et al., 1993; Coffey and Cromwell, 1995). Although this economically important problem has been tentatively linked to the proinflammatory cytokines, neither their synthesis nor the metabolic changes following immune challenge have been studied in swine. Therefore, the objectives of the present study were to characterize plasma levels of TNF-α, IL-6, cortisol, and several metabolites that are indicative of changes in nutrient metabolism.

Materials and Methods

Animals and Management. Pigs from the University of Illinois Swine Research Center resulting from the cross of PIC (Franklin, KY) Line-26 males and...
Camtborough-15 females were used. They were housed in an environmentally controlled building with constant 24-h lighting. The pigs were maintained three pigs per pen in 1.2-m² raised raised wire floor pens equipped with nipple waterers that allowed free access to water. To allow for adaptation to the environment, pigs were assigned to pens 7 d before experimentation. All procedures and housing were approved by the University of Illinois Committee on Laboratory Animal Care.

Blood Collection and Analysis. Blood samples were collected aseptically from the cranial vena cava region into lithium heparinized syringes and centrifuged promptly (2,000 x g for 10 min at 5°C ). Aliquots of plasma from each pig were stored at −23°C until analysis could be conducted.

Total plasma cortisol was measured using a commercially available 125I RIA kit (ICN Biomedicals, Costa Mesa, CA). Recovery was validated using 20 µL of porcine plasma supplemented with 0, 10, 100, or 1,000 ng/mL of cortisol standards in a 25-µL reaction volume. Recoveries ranged from 82 to 100%. Plasma samples were serially diluted 1:2, 1:4, and 1:8 with the provided diluent to demonstrate parallelism. Intra-assay variation was 2.4%, and sensitivity of the assay was 1.5 ng/mL.

Total plasma TNF-α was measured using a commercially available ELISA specific for porcine TNF-α (Endogen, Cambridge, MA). Plasma samples were assayed in duplicate at either 1:1 or 1:10 dilution. The assay was sensitive to 10 pg/mL of TNF-α and had an intra-assay CV of < 10%.

Total plasma IL-6 was measured using the IL-6 sensitive, 7td1 B-cell hybridoma cell proliferation assay (Wright et al., 1993). Cells were suspended at a sensitive, 7td1 B-cell hybridoma cell proliferation intraassay CV of < 10%.

Blood samples were taken from two barrow and two gilt blocks before initial weight determination and at 2, 4, 8, 12, and 24 h after LPS injection. Blood samples taken before initial weight determination are described as time 0. All pigs were deprived of feed for 12 h before time 0, and they were not given feed during the course of the 24-h postinjection evaluation period.

Statistical Analysis. Data were analyzed as a completely randomized design, and ANOVA was conducted using the GLM procedure of SAS (1992). A factorial arrangement of treatments was used with the following factors: sex (barrow vs gilt), dose (0, .5, or 5 µg/kg BW). The LPS (Escherichia coli serotype K-235; Sigma) was dissolved in sterile .9% (wt/vol) NaCl solution so that injection of .1 mL/kg of solution would achieve the desired dosage. All injections were given i.p. in the lower abdominal region.
Data were then subjected to one- (dose, time) and two-way (dose × time) ANOVA to determine the significance of the main factors and their interactions. When ANOVA revealed a significant effect of dose or a dose × time interaction, differences among treatment means were tested using paired t-tests.

Results

Intraperitoneal injection of LPS increased plasma levels of TNF-α and IL-6. Two-way ANOVA of plasma TNF-α and IL-6 concentrations revealed an effect of dose (P < .01), time (P < .01), and a time × dose interaction (P < .01). Whereas .5 μg/kg LPS did not increase plasma TNF-α, 5 μg/kg LPS induced a 10-fold (P < .01) increase in plasma TNF-α at 2 h after the injection (Figure 1). In these pigs, plasma TNF-α was still elevated at 4 h (P < .01) but returned to baseline by 8 h after injection (Figure 1). Concomitant with the peak in TNF-α, the plasma concentration of IL-6 in pigs receiving 5 μg/kg LPS was increased (P < .01), although .5 μg/kg LPS had no effect on IL-6 levels (Figure 2). Plasma IL-6 levels of pigs receiving 5 μg/kg LPS peaked at 4 h after injection at levels greater than 200-fold (P < .01) those of the controls (Figure 2). Plasma IL-6 levels of LPS-treated pigs returned to normal by 12 h after injection.

Administration of LPS increased the activity of the hypothalamic-pituitary-adrenal axis, as evidenced by increased plasma cortisol levels (Figure 3). Two-way ANOVA of plasma cortisol concentration revealed an effect of time (P < .01) and dose (P < .01) and a time × dose interaction (P < .01). Plasma cortisol before injection was between 2.5 and 3.5 μg/dL, which is consistent with previous observations of nonstressed pigs (Johnson et al., 1994). Both doses of LPS increased (P < .05) plasma cortisol concentrations at 2 h after injection, but the change in plasma cortisol induced by .5 μg/kg was smaller and more transient than that induced by 5 μg/kg LPS. Plasma cortisol concentration of pigs injected with 5 μg/kg LPS peaked at 4 h and remained elevated (P < .05) at 8 h before returning to control levels at 12 h after injection.

Plasma urea nitrogen, an indicator of protein catabolism in feed-deprived animals, was elevated by LPS administration (Figure 4). The PUN levels were increased two- and threefold (P < .05) at 8 and 12 h, respectively, in pigs injected with 5 μg/kg LPS. Administration of .5 μg/kg LPS had no effect on PUN levels. There was a trend (P < .10) for increased PUN levels at 24 h after injection that could be explained by proteolysis typical of an extended period without feed.
Discussion

The present view is that the reduction in feed intake, lean muscle accretion, and growth observed in diseased or immunologically challenged pigs is the result of increased cytokine activity, but this has not been directly tested. As a first step toward addressing this issue, pigs were injected i.p. with three doses of LPS in order to activate the immune system and develop complete dose- and time-response curves for the cytokines TNF-α, IL-6, cortisol, and several blood metabolites that may indicate changes in protein, lipid, and carbohydrate metabolism. The resultant data demonstrate that pigs injected with LPS have increased plasma levels of TNF-α, IL-6, and cortisol. Furthermore, the increase in circulating cytokines and cortisol caused by LPS was followed by a dramatic increase in PUN levels, but not triglycerides or NEFA. Because PUN was elevated by LPS in feed-deprived animals, the present results are interpreted to suggest that immunological challenge increases muscle protein degradation, perhaps via the induction of macrophage-derived cytokines.

There is substantial evidence to suggest that cytokines synthesized in response to challenge by LPS either directly or indirectly cause skeletal muscle protein degradation. In rodents, LPS increases plasma levels of TNF-α, IL-6, and IL-1, and it also increases skeletal muscle protein degradation (Jepson et al., 1986; Fong et al., 1989; Goodman, 1991). This is also the case in birds; increased IL-1-like activity in plasma has been reported in chicks injected with LPS (Klasing et al., 1987). Additionally, when incubated with a crude preparation of IL-1 in vitro, increased proteolysis was observed in skeletal muscle isolated from the chick wing (Klasing et al., 1987). Numerous studies involving injections of recombinant cytokines including TNF-α, IL-1, and IL-6 now confirm that the effects of LPS on protein metabolism are due in large part to the induction of cytokine synthesis.

Intravenous infusion of TNF-α alone or in combination with IL-1 increased skeletal muscle protein...
Table 1. Plasma concentration of various blood metabolites and α-1-acid glycoprotein following a challenge dose of lipopolysaccharide

<table>
<thead>
<tr>
<th>Variable and LPSb, mg/kg</th>
<th>Hour after injection</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>NEFA, meq/dL</td>
<td>0</td>
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<tr>
<td>.5</td>
<td>252.9</td>
<td>728.3</td>
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<td>5</td>
<td>270.5</td>
<td>543.4</td>
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<tr>
<td>Triglyceride, mg/dL</td>
<td>0</td>
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</tr>
<tr>
<td>.5</td>
<td>62.5</td>
<td>42.6</td>
</tr>
<tr>
<td>5</td>
<td>45.6</td>
<td>58.6</td>
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<tr>
<td>Glucose, mg/dL</td>
<td>0</td>
<td>82.2</td>
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<tr>
<td>.5</td>
<td>80.3</td>
<td>67.8</td>
</tr>
<tr>
<td>5</td>
<td>77.2</td>
<td>74.7</td>
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<tr>
<td>α-1-AGP, µg/mLc</td>
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<td>1,117</td>
</tr>
<tr>
<td>.5</td>
<td>1,025</td>
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<tr>
<td>5</td>
<td>1,077</td>
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</tr>
</tbody>
</table>

aData represent least squares means of four pigs; average weight was 11.55 kg.

bLPS = lipopolysaccharide (Escherichia coli, serotype K-235). Three doses of LPS (0, .5, or 5 µg/kg BW) were injected i.p. at time 0 and blood samples were taken at 0, 2, 4, 8, 12, and 24 h following injection.

cα-1-AGP = α-1-acidglycoprotein.

catabolism in rats (Flores et al., 1989). In addition, Goodman (1991) showed that infusion of TNF-α and LPS, but not IL-1, enhanced muscle proteolysis, as evidenced by 3-methyl-L-histidine and tyrosine release from isolated skeletal muscle and an increase in blood urea concentrations. Consistent with the idea that cytokines secreted by activated macrophages induce muscle protein degradation, the increase in PUN at 8 h in pigs given 5 µg/kg of LPS occurred only after peak levels of TNF-α (i.e., at 2 h) and IL-6 (i.e., at 4 h) were observed. The time course of increased plasma cytokines, cortisol, and urea nitrogen after i.p. injection of 5 µg/kg LPS is shown in Figure 5. However, pigs that received .5 µg/kg of LPS did not have increased plasma levels of TNF-α or IL-6, and they did not have increased levels of PUN. Interestingly, we have recently reported that this same dose of LPS induced profound anorexia even in pigs deprived of feed for 12 h (Johnson and von Borell, 1994; Warren et al., 1997). Therefore, it seems reasonable to postulate that increased muscle catabolism, indicated in this study by a profound threefold increase in PUN, was due to the elevated levels of plasma cytokines.

Although the proinflammatory cytokines act directly on skeletal muscle to inhibit protein accretion and accelerate protein degradation, cytokines also induce a variety of other endocrine responses that are likely to induce proteolysis. For example, TNF-α, IL-6, and IL-1 stimulate the hypothalamic-pituitary-adrenal axis. In particular, IL-1 has been shown to stimulate neurons in the hypothalamus to secrete corticotropin-releasing hormone (Berkenbosch et al., 1987). Moreover, infusion of IL-1 receptor antagonist into a lateral ventricle of the rat brain inhibited the expression of corticotropin-releasing hormone mRNA in the hypothalamus following i.p. injection of LPS (Kakucska et al., 1993). The secretion of glucocorticoids is part of a negative feedback loop that regulates the immune system to prevent it from overreacting (Knudson et al., 1987; Bertini et al., 1988; McCallum et al., 1990; Johnson et al., 1996). However, glucocorticoids also produce a variety of tissue-specific metabolic effects. In liver they are anabolic in that they increase gluconeogenesis and protein synthesis, but in muscle and adipose tissue, glucocorticoids are catabolic, inducing proteolysis and lipolysis, respectively (Millward et al., 1985; Lacasa et al., 1988). Because LPS increased plasma cortisol in a time- and dose-dependent fashion in our study, it is plausible that they contributed to the increased PUN following LPS administration.

The amino acids liberated from muscle protein degradation during inflammation are thought to provide fuel for hepatic acute phase protein synthesis (Reeds et al., 1994). The cytokine IL-6 acts directly on hepatocytes to stimulate amino acid uptake and synthesis of a broad array of acute-phase proteins. The synthesis of acute-phase proteins may increase 25% or more following tissue damage or infection. Numerous acute-phase proteins have been identified in pigs, including C-reactive protein, haptoglobin, and α-1-AGP (Itoh et al., 1992; Lampreave et al., 1994). Increased serum haptoglobin concentrations have been reported in pigs infected with Actinobacillus pleuropneumonia (Hall et al., 1992) and elevated plasma C-reactive protein concentrations have been demonstrated in pigs infected with turpentine (Burger et al., 1992). More recently, Williams et al. (1993)
compared α-1-AGP levels of pigs in environments that presumably presented either a high or low level of immune stimulation and reported the level of immune stimulation and plasma concentration of α-1-AGP to be positively correlated. In the present study, however, despite increasing plasma TNF-α, IL-6, and cortisol, LPS did not increase α-1-AGP concentrations. It should be noted that the baseline levels of α-1-AGP for pigs in our study were similar to those reported by Williams et al. (1993) for pigs that presumably had a high level of immune stimulation. Indeed, in their study plasma level of α-1-AGP was used as an indicator of immune system activation. Thus, if pigs in the present study had maximal levels of α-1-AGP before LPS, this may explain why α-1-AGP was not increased by LPS. Alternatively, other investigators reported that α-1-AGP levels were not elevated in the plasma of pigs following the injection of turpentine (Lampreave et al., 1994) or LPS (M. E. Spurlock, personal communication), suggesting that acute immunological stress, induced by turpentine or LPS, does not increase the synthesis of α-1-AGP.

Alterations in lipid and glucose metabolism are also associated with infection. In rodents, TNF-α, IL-6, and IL-1 induce hypertriglyceridemia by decreasing muscle and adipose lipoprotein lipase activity and by increasing the rate of hepatic fatty acid synthesis and their subsequent incorporation into very-low-density lipoproteins (Hardardottir et al., 1994). However, in our study neither triglycerides nor NEFA were elevated by LPS, even though IL-6, which was increased 200-fold, has recently been shown to increase triglycerides in rats (Nonogaki et al., 1995). Species differences in the site of fatty acid synthesis could explain the apparent absence of elevated triglyceride levels in the current study because rats and pigs synthesize fat de novo primarily in liver and adipose tissue, respectively.

Alterations in glucose metabolism are associated with immunological challenges, but the observed changes can be highly variable depending on the severity of insult and the time of sampling (Lang and Spitzer, 1987). In general, immunological stress increases hepatic glucose production via increases in gluconeogenesis and glycogenolysis while at the same time increasing extrahepatic utilization of glucose. The net result of these changes is an early, transient hyperglycemia. Lang et al. (1992) reported elevated plasma glucose concentrations following LPS injection that returned to normal values by 2 h after the injection. It is possible that we did not see altered plasma glucose in our study because the first sampling time was too late to observe this change.

Regarding the effects of feed deprivation per se, plasma concentrations of NEFA and triglycerides increased from 0 to 24 h after injection (12 to 36 h of feed deprivation). Elevated NEFA levels are common in feed-deprived animals as a result of increased adipose tissue lipolysis (Murray et al., 1993). Plasma triglycerides also increase during feed deprivation because plasma NEFA concentrations exceed the capacity of liver to oxidize them. An increase in protein breakdown was also evident at 24 h after injection as indicated by an elevation in PUN. Increased protein degradation is a typical response in early feed-deprived conditions because amino acids are needed as gluconeogenic substrates in the liver.

In conclusion, LPS administration triggered the release of TNF-α, IL-6, and cortisol. Because these molecules have been shown to induce proteolysis and lipolysis in other species, it seems reasonable to postulate that they play an important role in modulating growth in immunologically challenged pigs. More specifically, cytokines may have a direct role in inducing elevated muscle protein catabolism, as evidenced by the elevated PUN concentrations observed herein.

**Implications**

The present study indicates that stimulation of the pig's immune system results in increased plasma levels of cytokines and cortisol. These metabolically active molecules may be involved in the reduction of feed intake, lean muscle accretion, and growth in diseased or immunologically challenged pigs. Because the increase in cytokines and cortisol was followed by a profound increase in plasma urea nitrogen, these data support the idea that immunological challenge induces skeletal muscle protein degradation. Therefore, we suggest that understanding how cytokines alter intermediary metabolism in growing animals is a prerequisite to understanding why sick or immune-challenged animals do not grow well.
Literature Cited


