

# Underlying Disease Stress Augments Plasma and Tissue Adrenomedullin (AM) Responses to Endotoxin: Colocalized Increases in AM and Inducible Nitric Oxide Synthase within Pancreatic Islets\*

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## ABSTRACT

Rapid onset metabolic impairments accompany the initiation of the acute phase response to many disease stresses, whereas more chronic metabolic perturbations may prolong the recovery period. In the present experiment the application of a mild endotoxin challenge [lipopolysaccharide (LPS)] alone or additive to a chronic subclinical parasitic infection (*Sarcocystis cruzi*; LPS + PI) in calves was used as a model to investigate and define a dynamic axis coordinated between adrenomedullin (AM) and nitric oxide in response to immune challenge. Plasma AM and NO<sub>2</sub>/NO<sub>3</sub> concentration responses after LPS (0.45 µg/kg, iv) were rapid in onset and of higher magnitude and longer duration in PI + LPS calves than in those challenged with LPS alone. The post-LPS increase in plasma insulin was significantly greater in PI + LPS than in LPS; following refeeding of calves, insulin secretion was most blunted in PI + LPS calves, consistent with the inhibitory effects of NO and AM on insulin secretion. A more chronic response to the immune challenge (organ specific) was in evidence in tissues harvested 24 h after LPS challenge. Where lung and liver showed no immunostaining for inducible nitric oxide (iNOS), iNOS immunostaining was present in the pancreas, localized to islets only. The percentages of iNOS-immunopositive cells in islets were 1.7%, 21%, 6.7%, and 24% for control (C; saline infused), PI, LPS, and PI + LPS calves, respectively. AM immunostaining was not evident in the

liver and was present, but not differentially affected by treatment, in airway epithelium in the lung. The number of islet cells with positive immunostaining for AM was increased in LPS, PI, and PI + LPS calves. The percentages of AM-immunopositive cells in islets were 8%, 27%, 20%, and 33% for C, PI, LPS, and PI + LPS, respectively. Immunostaining for AM and iNOS was colocalized with cells positive for pancreatic polypeptide. By triple label confocal fluorescence immunocytochemistry, colocalization of intense AM and iNOS immunostaining was confirmed in peripheral islet cells. A weaker, more diffuse iNOS signal was also apparent in insulin-containing cells in PI + LPS. We conclude that chronic low level infection potentiates the severity of metabolic perturbations that arise with additive sudden onset immune challenge, as can occur with bacterial toxins. These metabolic disturbances are reflected in and possibly mediated by early onset increases in plasma tumor necrosis factor-α, insulin, and AM and up-regulated iNOS activity. These acute complications rapidly progress into a more chronic state characterized by diminished insulin response to feeding stimulus and colocalized increases in pancreatic islet AM and iNOS. The pancreatic responses in AM and iNOS may play a major role in mediating prolonged disturbances in nutrient use by tissues through their influences on temporal patterns of pancreatic hormone secretion during chronic illness. (*Endocrinology* 140: 5402–5411, 1999)

**C**HANGES IN metabolism and organ perfusion during the acute phase response of infection are mediated through an elaborate cascade of cytokines, hormones, catecholamines, PGs, and inorganic messengers such as nitric oxide (NO) (1–6). Adrenomedullin (AM) is a newly discovered pluripotent peptide with functions in autocrine growth regulation, development, metabolism, and cardiovascular performance (7–12). The involvement of AM in the response

to severe disease stress has been implied through measured increases in circulating plasma concentrations of AM and tissue messenger RNA (mRNA) levels for AM during sepsis and after endotoxin challenge (13–16). The major focus of these earlier studies was directed toward potential cardiovascular effects of AM and NO as contributors to severe acute multiple organ failure (lung, liver, and intestine) that was terminal within 3 h of endotoxin [lipopolysaccharide (LPS)] administration.

During the acute phase response, cytokine-driven induction of nitric oxide synthase (iNOS) increases NO production in many cell types (1). The use of arginine analogs to disrupt NOS generation of NO also attenuates the actions of AM (13, 14), further suggesting a link between AM and NO in the regulation of cellular processes. Although several cardiovascular organ perfusion functions of AM have been linked to NO-dependent mechanisms (13, 17) during lethal endotoxemia, few, if any, studies have examined the systemic and

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localized changes in AM and iNOS in low level disease states, and none has explored the involvement of AM in the metabolic perturbations seen in disease stress.

Recently, we suggested that AM plays a significant role in regulating metabolism through AM's ability to modulate insulin responses *in vivo* and *in vitro* (8). The aims of the present study were to 1) determine whether AM is affected by nonlethal, chronic parasitic disease that has underlying elements of insulin secretion impairment and is further altered in response to challenge with low level endotoxin when additive to this chronic disease, and 2) identify the tissue site(s) where AM and iNOS responses might be localized.

## Materials and Methods

### Animal model

We used a protozoan infection (18) (*Sarcocystis cruzi*) as well as a bolus LPS challenge in the young calf to investigate the impact of acute, chronic, and additive low level disease stresses on changes in plasma and tissue AM. Four-month-old Holstein calves were used in the study because of the well defined timing and response of animals of this age to this infection or LPS challenge level (19–21). Twenty Holstein male calves were obtained at birth and reared in individual, parasite-free pens. At approximately 115 kg, 10 random calves were moved to a designated remote infection facility and inoculated orally with 30,000–50,000 sporulated oocyst of *Sarcocystis cruzi* (18). After 1 week, infected calves were returned to their individual pens. All calves were monitored daily for clinical signs of infection, including general appearance and behavior, feed intake, and rectal temperature. Four treatment groups ( $n = 5$  each) were created: noninfected, saline-challenged controls (C); parasite-infected (saline-challenged, PI); noninfected, LPS-challenged (LPS; 0.45  $\mu\text{g}/\text{kg}$ , iv; *Escherichia coli* 055:B5 serotype, Sigma Chemical Co., St. Louis, MO); and infected, LPS-challenged (PI + LPS). Parasitized calves were inoculated with *S. cruzi* 31 days before administration of either saline or LPS; at this point animals are past the acute phase response of the infection as the parasite has erupted from the endothelium and begun to encyst in the tissues.

On the day immediately preceding the LPS and saline challenges, each animal's feed was removed at 1600 h. New feed was offered each animal at 1400 h on the day of challenge, 6 h after administration of the challenge. Refeeding after fasting was used as a physiological stimulus for food-induced insulin release. Feed intake after refeeding was measured in individual animals.

### Blood sampling and tissue collection

On days 29–30 postinfection, a sterile Teflon cannula (Abbocath, Abbott, North Chicago, IL) was inserted into a jugular vein of each calf for blood sampling and administration of endotoxin or saline. After an initial baseline blood sample (8 ml, EDTA anticoagulant), calves were injected with either 3 ml pyrogen-free saline or LPS. Additional blood samples were obtained via cannula up through 24 h relative to the administration of LPS. After the last blood sample, calves were moved to the abattoir, at which time they were killed by captive bolt and exsanguination. Samples of liver, lung, and pancreas were obtained and immediately placed into Bouin's fixative or frozen in liquid nitrogen and maintained at  $-80\text{ }^{\circ}\text{C}$ .

### Analytical

*Hormones, cytokines, and metabolites.* Insulin (3), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) (3, 22), and AM (23) were measured by validated RIAs. Plasma concentrations of nitrite were measured using standard methods in the Greiss reaction after enzymatic conversion of plasma nitrate to nitrite with bacterial nitrate reductase (22). Glucose was measured using a solid phase membrane glucose oxidase-based analyzer (3) (YSI, Inc., Yellow Springs, OH).

*Western blot confirmation of AM and iNOS.* Extracts of the pancreatic tissue were prepared by thawing the tissue on ice and homogenizing accu-

rately weighed samples in ice-cold homogenization buffer [5:1, vol/wt; 50 mM HEPES, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.2, containing soy trypsin inhibitor (10  $\mu\text{g}/\text{ml}$ ), antipain, pepstatin, aprotinin, leupeptin (1  $\mu\text{g}/\text{ml}$ ), and phenylmethylsulfonyl fluoride (100  $\mu\text{g}/\text{ml}$ )] using a Polytron (Brinkmann Instruments, Inc., Westbury, NY) in two bursts of 20 sec each at maximum speed. The homogenate was centrifuged at  $100,000 \times g$  for 30 min, and the supernatant was collected. Homogenate supernatant protein content was measured using the bicinchoninic acid procedure (Pierce Chemical Co., Rockford, IL) after trichloroacetic acid precipitation and NaOH resolubilizing of the extract protein.

For iNOS Western blots, protein from pancreas extracts (100  $\mu\text{g}/\text{well}$ ) was electrophoretically separated on 3–8% gradient polyacrylamide Tris-acetate gels (Novex, San Diego, CA) 120 V for 1.5 h under reducing conditions. Proteins in the gels were transferred to nitrocellulose by semioist transfer blotting. Nonspecific binding was blocked by incubating the nitrocellulose overnight with 5% nonfat milk containing 0.1% Tween-20 in PBS at 4 C. The membrane was probed for iNOS immunoreactivity with rabbit antimouse macrophage iNOS specific for the carboxyl-terminal NADPH binding region (Transduction Laboratories, Inc., Lexington, KY). Protein bands were visualized by chemiluminescence excitation of autoradiographic film using an avidin-biotin-peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) and ECL Plus Western blotting reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Band intensities were quantified by densitometry using the ChemImager 4000 (Alpha Inntech Co., San Leandro, CA).

Western blot analysis of AM was performed on the same pancreatic extracts using 10–20% acrylamide gradient Tricine gels as previously performed by Miller *et al.* (7).

### Immunocytochemistry

*Light microscopy.* For immunohistochemistry, Bouin's fixed tissues from each calf were embedded in paraffin, sectioned at 6  $\mu\text{m}$ , and mounted on poly-L-lysine-coated glass slides (8). After deparaffinizing in xylene, tissue sections were rehydrated, blocked with normal goat serum, and incubated overnight at 4 C with either antimouse macrophage iNOS (Transduction Laboratories, Inc.; 5  $\mu\text{g}/\text{ml}$  anti-iNOS) or rabbit anti-AM [serum 2343 (8); 1:1000 dilution]. Initial determinations of cell-specific localizations of AM and iNOS were obtained using serial cut sections of the paraffin-embedded tissues from calves challenged with infection and LPS. The serial sections were immunostained for AM, iNOS, somatostatin, glucagon, and pancreatic polypeptide. Immunoreactivity was visualized using the avidin-biotin horseradish peroxidase complex method (Vector Laboratories, Inc.), followed by a light nuclear counterstain with Gill's hematoxylin. Percentages of AM- and iNOS-immunopositive cells within islets were obtained by direct counting of cells in multiple islets in 20 fields under a  $\times 20$  objective.

*Confocal microscopy.* Colocalizations were further studied using confocal microscopy. Paraffin sections were dewaxed and rehydrated through a graded ethanol series. Sections were blocked with normal donkey serum (1:30 in PBS) for 30 min and then incubated overnight at 4 C in a mixture of three antibodies: guinea pig antibovine insulin (CAPPE/Labs, Inc., King of Prussia, PA) at 1:2000 dilution, mouse monoclonal anti-iNOS (Transduction Laboratories, Inc.) at 1:200 dilution, and rabbit anti-AM at 1:1000 dilution. The second layer consisted on a mixture of Cy5-antiguinea pig (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), Bodipy antirabbit (Molecular Probes, Inc., Eugene, OR), and biotinylated horse antimouse serum (Vector Laboratories, Inc.), each at a final dilution of 1:200. A third layer containing lissamine-rhodamine-streptavidin (Jackson Immuno-Research Laboratories, Inc.) at 1:200 dilution was used to detect iNOS. Sections were observed with a Carl Zeiss confocal microscope (New York, NY) equipped with four lasers.

### Statistical analysis

Data were statistically analyzed using regression analysis in the general linear models (24) procedure of SAS (SAS Institute, Cary, NC) with nonorthogonal contrasts to differentiate among the effects of LPS, infection, and additivity. Area under the concentration-time curve responses for hormones and metabolites were estimated by trapezoidal summation of the total area with the specific response presented as the

response above baseline after subtraction of the associated baseline area after time zero.

## Results

At the level of infection used in the present study, calf responses to the parasite were minimal. Of 10 calves infected with *Sarcocystis*, only 4 displayed minor elevations in rectal temperatures and transient 15–20% reductions in voluntary feed intake beginning 24–25 days postinfection, consistent with the anticipated onset of the acute phase response of this infection. These clinical signs resolved back to normal within 2–4 days. There were no other observable significant clinical signs in the infected calves at the time the saline or LPS challenges were administered. Endotoxin challenge resulted in transient increases in rectal temperature, mild lethargy, and increased respiratory rate and heart rate that normalized within 4–6 h.

The immune challenge model used in this study clearly demonstrated that some aspects of the infection-response process in the host can be compartmentalized into two distinct phases. When a host is challenged by immune stimuli that elicit the customary acute phase response, an early onset phase initiates, marked by and easily quantified through rapid, large changes in plasma concentrations of hormones, and further significantly affected by the additivity of concurrent stresses. Secondarily, a more chronic, subtle phase can be delineated when a quantification of the response is shifted to tissue-specific markers of metabolic perturbation and perturbed regulation of hormone secretion normally regulated by physiological secretagogues such as feeding.

Concentrations of glucose, nitrate/nitrite, hormones, and TNF $\alpha$  in plasma were measured in samples collected through the 24-h point after LPS challenge. Mean plasma concentrations of glucose were not different between experimental animal groups before LPS administration (Table 1). After LPS, plasma glucose concentrations changed in a biphasic pattern; the immediate period of hyperglycemia peaked at 1 h after LPS and was numerically higher in LPS + PI than in LPS alone, but the difference was not statistically significant. By 3 h post-LPS the hypoglycemic period ensued. In calves challenged with saline, plasma glucose concentrations remained stable throughout the postchallenge sam-

**TABLE 1.** Changes in plasma concentrations of glucose after the administration of saline or endotoxin (LPS; 0.45  $\mu\text{g}/\text{kg}$ , iv) to noninfected calves and calves infected with *Sarcocystis cruzi*

Time After endotoxin (h)	Control	LPS only	Infected only	Infection + LPS
0	99 $\pm$ 3.5 <sup>a</sup>	103 $\pm$ 2.3	95 $\pm$ 3.1	98 $\pm$ 2.2
1	95 $\pm$ 2.9 <sup>a</sup>	126 $\pm$ 14 <sup>b</sup>	96 $\pm$ 2.4 <sup>a</sup>	139 $\pm$ 21.5 <sup>b</sup>
2	95 $\pm$ 3.3 <sup>a</sup>	96 $\pm$ 7.1	99 $\pm$ 3.8 <sup>a</sup>	101 $\pm$ 12.1
3	96 $\pm$ 3.6 <sup>a</sup>	52 $\pm$ 6.2 <sup>c</sup>	101 $\pm$ 4.0 <sup>a</sup>	55 $\pm$ 5.2 <sup>c</sup>
4	93 $\pm$ 3.1	67 $\pm$ 3.7 <sup>c</sup>	97 $\pm$ 1.1	63 $\pm$ 6.6 <sup>c</sup>
6	99 $\pm$ 1.9	73 $\pm$ 1.7 <sup>c</sup>	90 $\pm$ 2.8	68 $\pm$ 2.7 <sup>c</sup>
8	92 $\pm$ 1.9	79 $\pm$ 1.2 <sup>c</sup>	87 $\pm$ 3.2	75 $\pm$ 7.5 <sup>c</sup>
12	94 $\pm$ 2.5	98 $\pm$ 2.8	94 $\pm$ 4.3	87 $\pm$ 5.5
24	107 $\pm$ 2.3	97 $\pm$ 1.8	99 $\pm$ 3.3	96 $\pm$ 3.5

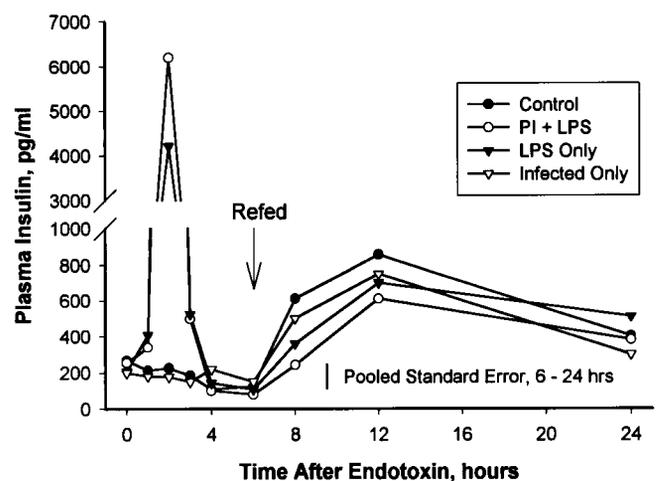
Values represent mean plasma concentrations  $\pm$  SEM of glucose (milligrams per dl; n = 5/group).

<sup>b,c</sup> Within a column, values significantly different from the time zero value; across rows, values significantly different between animal treatment groups with different letters: <sup>b</sup>  $P < 0.02$ ; <sup>c</sup>  $P < 0.05$ .

pling period and were not affected by refeeding at the 6 h point. In calves challenged with LPS, plasma glucose responses were at a nadir at the 3 h point after LPS challenge and slowly returned to prechallenge concentrations by 12 h.

Plasma concentrations of insulin (Fig. 1) changed after LPS in a pattern consistent with the temporal and directional changes in glucose. The immediate increases in plasma insulin concentrations peaked 2 h after LPS. The initial increase was greatest in PI + LPS, greater ( $P < 0.05$ ) than that measured in calves challenged with LPS alone. Coincident with the hypoglycemic period, plasma insulin levels declined below the time zero baseline in PI + LPS and LPS, reaching a nadir at 6 h after LPS. Plasma insulin concentrations were minimally perturbed in saline-treated calves (C and PI) and not affected by the time without access to food. Plasma insulin increased in all calves after refeeding at the 6 h point in the challenge protocol; the amount of feed consumed by calves was not different between treatment groups. However, in parasitized calves challenged with LPS, the insulin response to refeeding was numerically lower in concentration at several time points than that measured in C, PI, and LPS calves and significantly lower when calculated as an area response index than that in C and PI.

Plasma TNF $\alpha$  levels increased in all calves challenged with LPS, with numerically greater responses occurring in PI + LPS calves than in calves receiving LPS only (Table 2). Plasma TNF responses to LPS were not statistically different between LPS and PI + LPS largely due to animal to animal variation in response. Baseline concentrations of plasma nitrate and nitrite tended to be greater at time zero in infected animals ( $P < 0.08$  vs. noninfected). However, increases in plasma nitrate and nitrite concentrations greater than baseline, as a measure of NO production by iNOS, were observed only in PI + LPS ( $P < 0.02$ ; Fig. 2), suggesting that calves harboring the underlying infection were more sensitive or responsive to LPS as an inducer of iNOS. When the values were expressed and normalized as the area under the response curve



**FIG. 1.** Mean plasma concentrations of insulin as affected by saline or endotoxin (0.45  $\mu\text{g}/\text{kg}$ , iv) in normal and parasitized (*S. cruzi*) calves (n = 5/group). After refeeding 6 h after endotoxin or saline challenge, increases in plasma insulin over the ensuing 6 h were blunted by responses to endotoxin alone (33% decrease vs. control;  $P < 0.05$ ) and further blunted by endotoxin in the presence of infection (41% decrease vs. control;  $P < 0.02$ ).

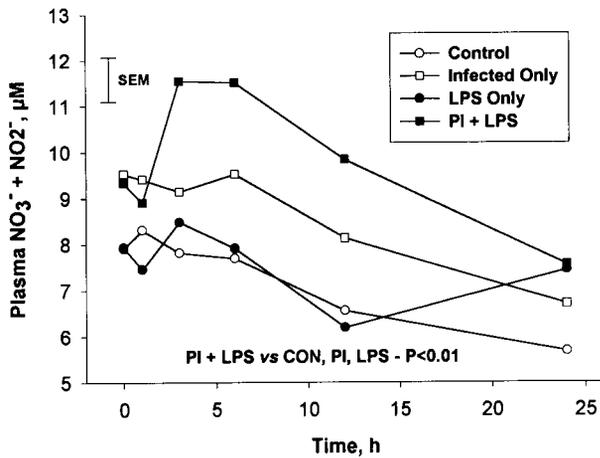
**TABLE 2.** Changes in plasma concentrations of TNF $\alpha$  after the administration of saline or endotoxin (LPS; 0.45  $\mu$ g/kg, iv) to noninfected calves and calves infected with *Sarcocystis cruzi*

Time after endotoxin (h)	Control	LPS only	Infected only	Infection + LPS
0	103 $\pm$ 11	132 $\pm$ 14	115 $\pm$ 9	162 $\pm$ 15
1	154 $\pm$ 11 <sup>a</sup>	6644 $\pm$ 2615 <sup>b</sup>	145 $\pm$ 8 <sup>a</sup>	11780 $\pm$ 5140 <sup>b</sup>
2	138 $\pm$ 9 <sup>a</sup>	2850 $\pm$ 645 <sup>b</sup>	139 $\pm$ 9 <sup>a</sup>	5126 $\pm$ 223 <sup>b</sup>
3	110 $\pm$ 10 <sup>a</sup>	268 $\pm$ 67 <sup>c</sup>	101 $\pm$ 10 <sup>a</sup>	568 $\pm$ 223 <sup>c</sup>
4	80 $\pm$ 10	130 $\pm$ 39	110 $\pm$ 11	215 $\pm$ 56
6	91 $\pm$ 9	128 $\pm$ 24	123 $\pm$ 11	153 $\pm$ 15

Values represent mean plasma concentrations  $\pm$  SEM of TNF $\alpha$  (picograms per ml; n = 5/group).

<sup>a</sup> For the given time,  $P < 0.05$  between columns with different letter superscripts.

<sup>b,c</sup> Within a column, values significantly different from the time zero value; across rows, values significantly different between animal treatment groups with different letter: <sup>b</sup>  $P < 0.001$ ; <sup>c</sup>  $P < 0.01$ .

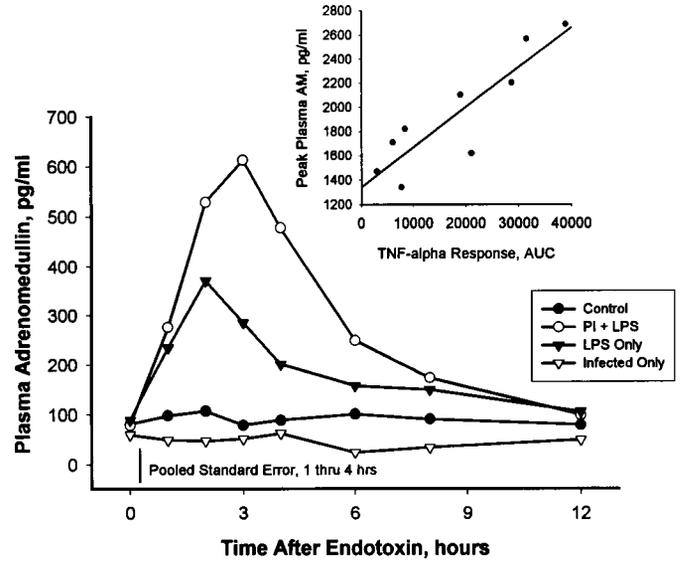


**FIG. 2.** Plasma concentrations of total nitrite (NO<sub>2</sub>/NO<sub>3</sub>) as affected by saline or endotoxin (0.45  $\mu$ g/kg, iv) in normal and parasitized (*S. cruzi*) calves. Values represent the mean percentage of the time zero prechallenge concentrations for each calf. Only responses in infected calves challenged with LPS were significant ( $P < 0.02$ ).

with the baseline subtracted, responses after LPS were only present and significant in PI + LPS ( $P < 0.001$ ).

Plasma concentrations of AM (Fig. 3) were increased by LPS in all calves challenged ( $P < 0.001$ ). Plasma AM levels did not change over time in control or infected calves. Peak concentrations of AM were present at 2 and 3 h, respectively, in LPS and PI + LPS calves. The mean AM response area was augmented (41%) in calves harboring parasites compared with that in noninfected calves after the administration of LPS ( $P < 0.01$ ). Regression analysis demonstrated that the increase in AM was linearly related ( $r^2 = 0.89$ ;  $P < 0.01$ ) to the magnitude of the TNF responses measured in calves receiving LPS.

Lung and liver, traditionally thought of as acutely responsive shock organs in endotoxemia, were devoid of iNOS immunostaining (Fig. 4, a–d). In the lung, positive immunostaining for AM was associated with epithelial cells from all animals. AM immunostaining was principally located in bronchi and terminal bronchiolar structures. In the liver, a marginal degree of positive immunostaining for AM was present only in bile duct cells. Neither the intensity nor the relative proportion of AM-staining cells was affected by in-



**FIG. 3.** Mean plasma concentrations of AM as affected by saline or endotoxin (0.45  $\mu$ g/kg, iv) challenge in normal and parasitized (*S. cruzi*) calves. Endotoxin challenge *per se* significantly increased AM in all challenged calves ( $P [lt]0.001$ ). The mean time to peak (4 vs. 2 h;  $P < 0.01$ ) and mean peak concentrations (610 vs. 381 pg/ml;  $P < 0.01$ ) of AM were significantly greater after endotoxin challenge in infected calves compared with noninfected calves. The inset illustrates a significant positive correlation ( $r^2 = 0.89$ ;  $P < 0.01$ ) between the area under the concentration-time response curve for TNF and the peak plasma AM concentrations in endotoxin-challenged calves.

fection or LPS in lung or liver at the time of tissue harvest (results not shown).

Prominent immunostaining for iNOS was apparent only in pancreatic islet cells after challenge with LPS (Fig. 4f); iNOS immunoreactivity was rarely seen in the pancreatic islet cells of control animals (Fig. 4e). The number of positive cells and the intensity of staining also significantly increased in parasitized animals (Fig. 4, g and h). The pattern of AM immunoreactivity in the pancreas was similar to that observed for the intense peripheral islet iNOS immunostaining (Fig. 4, j–m). Colocalization analysis using serial sections of pancreas revealed that intense iNOS staining was colocalized in islet cells with AM and pancreatic polypeptide, apart from SSN and glucagon-secreting cells (Fig. 4, N–R).

Western blot analysis of pancreatic homogenate proteins for iNOS demonstrated protein bands migrating at a relative molecular mass of 117 kDa under the reducing conditions employed. A moderate degree of band density was present in all control calves (Fig. 5). However, the band intensity increased up to 3.5-fold in samples from animals with underlying infection challenged with LPS. The relative intensity of the iNOS bands for each animal tested was significantly correlated ( $r^2 = 0.64$ ;  $P < 0.02$ ) with the increasing percentage of islet cells staining positive for iNOS between treatments. Western blot analysis of the pancreatic extracts indicated that AM was present largely as the higher molecular mass precursor forms, pro-AM and prepro-AM, with relative molecular weights of 14 and 18 kDa, respectively (data not shown).

Less than 2% of all islet cells stained positive for the presence of iNOS in control calves. The percentage of cells staining positive for iNOS increased 3-, 10-, and 12-fold ( $P < 0.02$ )

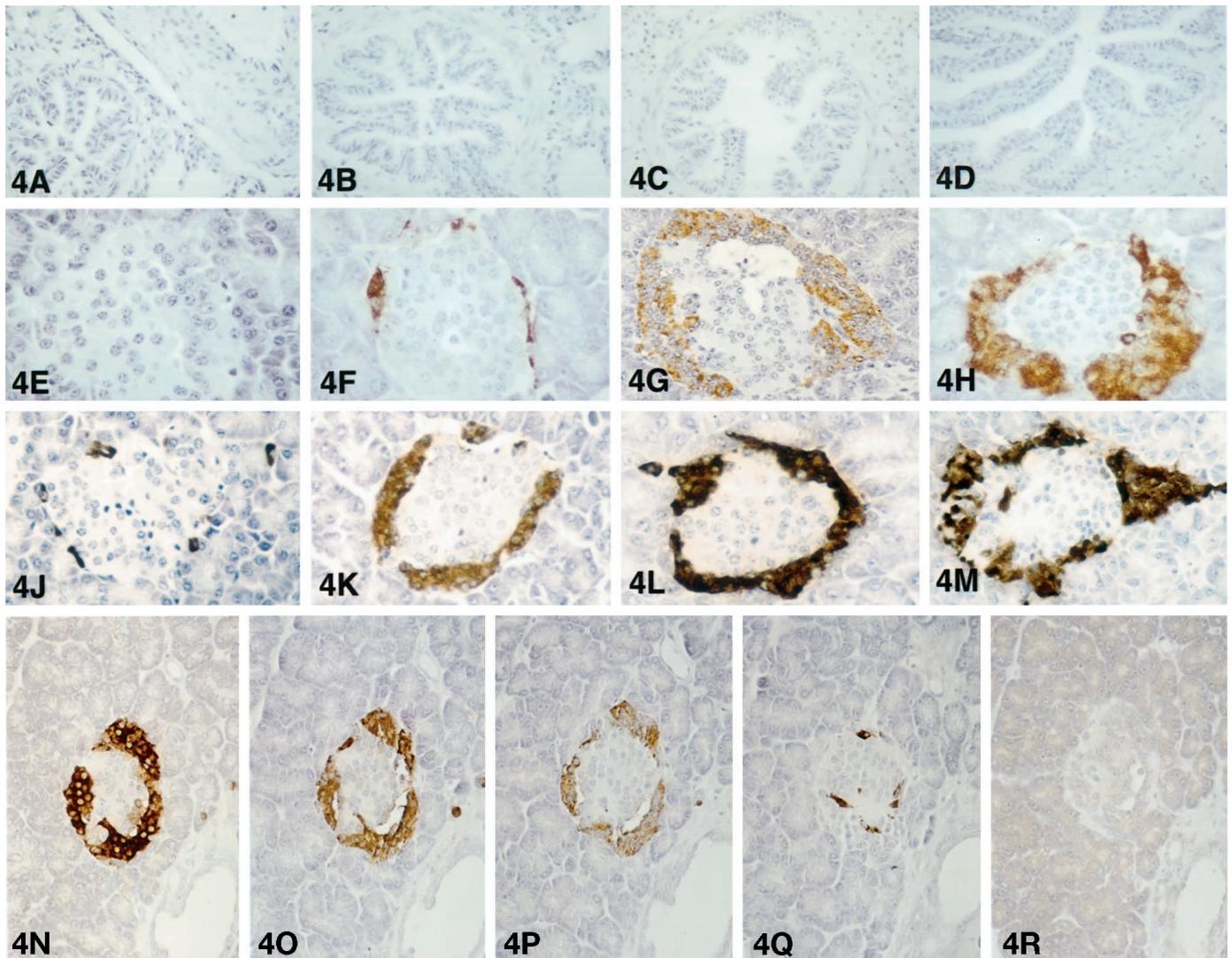


FIG. 4. Lung (A–D) and pancreas (E–H) harvested 24 h after endotoxin (0.45  $\mu\text{g}/\text{kg}$ , iv) or saline challenge presented different patterns of immunoreactive iNOS. J–M, Immunohistochemical localization of AM in pancreatic islets. The following treatments were given: control (A, E, and J), endotoxin-challenged (B, F, and K), *Sarcocystis* parasitized (C, G, and L), and parasitized and endotoxin-challenged (D, H, and M). Staining of serial sections suggests that iNOS (P) is coexpressed in cells containing AM (N) and pancreatic polypeptide (O), but not somatostatin (Q) or glucagon (R).

in response to LPS alone, infection, and the combination of LPS and infection, respectively. The increase was approximately equal in PI and PI + LPS calves (21–24%; Fig. 6), but the overall intensity of the staining was darker in PI + LPS (Fig. 4) than in PI alone, suggesting a greater protein induction response per cell in the combined stress challenge group of animals. Islet areas of all calf treatment groups had some positive immunostaining for AM, which was localized exclusively to peripheral cells of the islets. Approximately 8% of islet cells were positive for the presence of immunoreactive AM in control calves. There was a 3-fold increase in the proportion of immunoreactive AM cells after LPS ( $P < 0.02$ ) and also a 2.3-fold increase in association with infection ( $P < 0.05$ ). The largest percentage of AM-positive cells was observed in subclinically infected calves challenged with LPS (4.5-fold increase over the control value;  $P < 0.01$ ). Morphological evaluation of the hematoxylin-stained or TNF-immunostained sections suggested that there was little or no in-

filtration of the islets with circulating immune cells capable of producing AM or iNOS in cell numbers sufficient to account for the significant increase in the percentages observed.

Confirmation of the cell-staining patterns and colocalization of AM and iNOS in islet cells was obtained by confocal immunofluorescence microscopy (Fig. 7). The increased number of cells immunopositive for AM and iNOS as affected by LPS and infection was again markedly evident in the peripheral islet cells. The color shift associated with the blending of the green fluorescence of AM staining and the red fluorescence of the iNOS staining resolved the yellow color of the iNOS and AM colocalization in distinct cells. iNOS immunostaining in the confocal study revealed two patterns of increased expression of iNOS in islets. Where the majority of iNOS increase was localized to the peripheral islet cells, as was the case with the intense horseradish peroxidase staining, an additional pattern of fluorochrome staining was present among the interior cells of the islets. This faint lo-

FIG. 5. Western blot image of iNOS (A, top panel) obtained after electrophoretic separation of pancreatic homogenate proteins by PAGE and band intensity quantification for the autoradiogram (B, bottom panel) for the iNOS protein in each lane. Data represent the pancreas homogenate iNOS band pattern for seven random calves receiving various treatments.

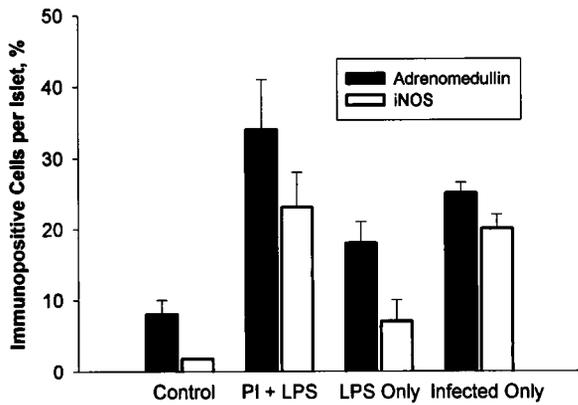
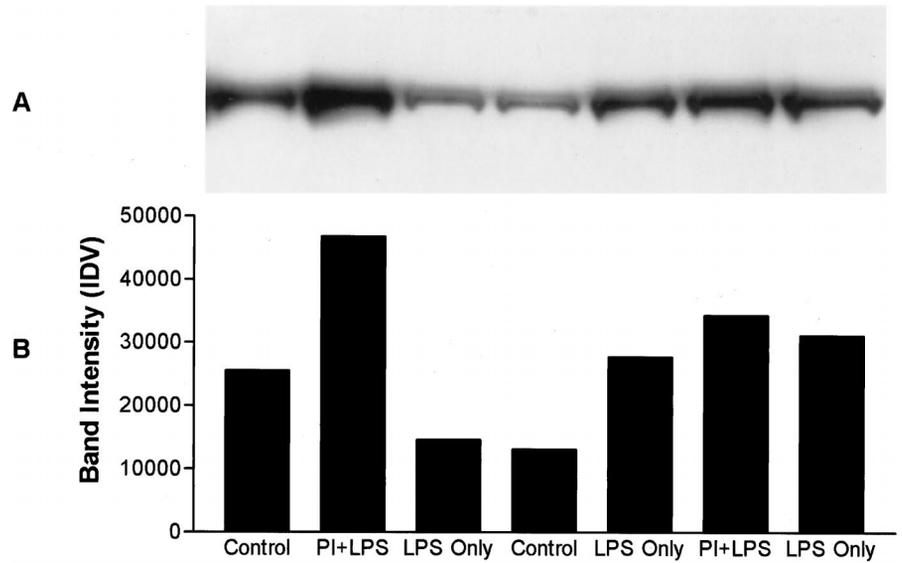


FIG. 6. Percentage of islet cells immunostained for iNOS or AM. Overall effect of endotoxin challenge *per se* to increase the percentage of AM- and iNOS-positive cells,  $P < 0.02$ ; effect of infection alone to increase AM- and iNOS-positive cells,  $P < 0.01$ ; effects of infection to further augment AM and iNOS responses to endotoxin challenge: AM,  $P < 0.05$ ; iNOS,  $P = \text{NS}$ .

calization of iNOS in insulin-containing  $\beta$ -cells was present only in sections from calves in which the treatment combination of LPS and infection was present.

### Discussion

The overall results of this research suggest that two phases of host response to LPS can be resolved. The initial phase is represented by an abrupt and transient change in metabolic regulation reflected in and defined by pronounced changes in plasma insulin, glucose, TNF, AM, and nitrate and nitrite concentrations. Typically, the observed acute changes in TNF are in part causative of pathology as a key trigger to the cascade of cytokine, PG, catecholamine, and hormone effectors elaborated during the immune response to LPS. However, many of the other hormone and cytokine responses observed during this period may also reflect attempts by the host to enact homeostatic resolution of metabolic disturbances. The later phase of the response is characterized by longer lasting perturbations apparent in specific organs that

disturb the capacity for the body to correct disease-related metabolic imbalances in part due to over- or underreactive signal transduction pathways that are disrupted by inflammatory-type responses.

Although the coordination and relationship between the early acute response and the late phase chronic response need to be further explored, the linear correlation of TNF and AM responses to the LPS challenge are consistent with literature citations suggesting a cytokine-driven mechanism for the increase in circulating plasma AM after LPS challenge within the early phase of the response (13–16). With regard to a potential source for the AM measured in plasma after LPS challenge, Isumi *et al.* (25) suggested that the AM response to cytokines released post-LPS in vascular endothelial and smooth muscle cells is sufficient to account for the measured increases in plasma AM, which peak 3–4 h after LPS.

Although the acute AM response is easily measured in plasma AM changes from baseline, it appears that the more chronic phase of the host response to these immune stimuli may be more of a localized paracrine nature not readily apparent in terms of plasma concentrations of AM. Western blot analysis of extracts of calf pancreas indicated that immunoreactive AM was present in the higher molecular mass precursor forms rather than the 6-kDa form to which biological activity is ascribed. The absence of 6-kDa AM is not uncommon, in that authentic AM is rapidly secreted from sites of processing but accumulates in cell culture fluid (7, 25), in temporal agreement with the induction of AM mRNA after stimulation.

The coordinated increase in pancreatic islet AM and iNOS can be implicated as a key factor participating in the pancreatic response to disease that complicates insulin and glucose homeostasis. These data suggest for the first time that the pancreas develops a shock organ-type response in calves challenged with LPS or infection that resolves to a coordinated localized increase in both iNOS and AM. Interpreting the immunohistochemical data raises some interesting questions regarding whether the pancreatic response to either low level infection or LPS is more sensitive to disease stress factors that affect pancreatic hormone secretion regulation or

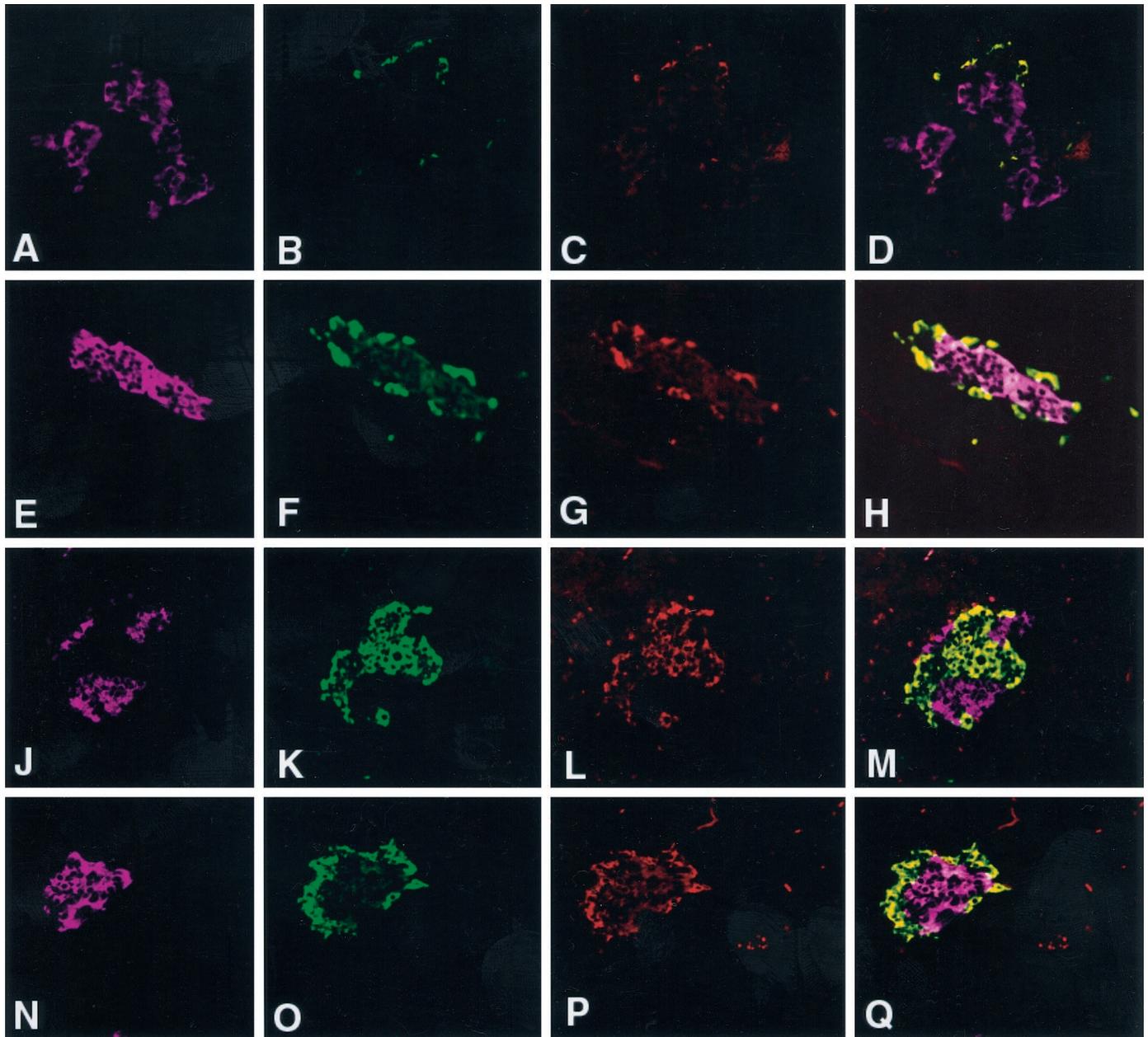


FIG. 7. Fluorescent confocal microscopy localization of AM, iNOS, and insulin in pancreatic islets of control (A–D), parasite-infected (E–H), LPS-treated (J–M), and parasite and LPS combination (N–Q) animals. *Purple* staining (A, E, J, and N) detects insulin, *green* (B, F, K, and O) localizes AM, and *red* (C, G, L, and P) labels iNOS. A composition of the three colors is presented in the *last column* (D, H, M, and Q). Note that colocalization of AM and iNOS produces a *yellow* color.

whether the pancreas has a rather unique capacity to maintain these AM and iNOS responses for a period of time considerably longer than those in the lung or liver. This is particularly striking considering that the dose of LPS per unit BW used in the present study was approximately 1/10,000th the typical dose used to stimulate iNOS or AM responses in several rodent models and was clinically relevant because human sensitivity to LPS is also far greater than that of the rat (22).

In terms of tissue response, the interesting feature about the pancreas appears to be that the AM and iNOS responses are different from those that develop in liver or

lung, the more traditional shock organs in disease. In the present study, pancreas specimens harvested 24 h after LPS challenge still maintained AM- and iNOS-immunopositive staining, whereas the liver parenchyma was negative, and the lung showed no more immunostaining than that normally observed in control animals. These localized paracrine effects are typical of NO-mediated responses due to the chemical properties of tissue diffusion potential and relative half-life of NO (26) and the autocrine and paracrine character of AM actions in development, growth, metabolism, and neoplasia (27). Data from our laboratory (unpublished) demonstrated that doses of LPS

greater than 1.0  $\mu\text{g}/\text{kg}$  administered iv to calves similar to the normal calves used here could cause an up-regulation of immunohistochemically identifiable iNOS in lung epithelia as well as in liver and an increase in AM staining on infiltrating monocytes at 5–6 h postchallenge. This immunostaining subsided within a few hours. The transient expression of these effector molecules in lung and liver is consistent with observations by others on the timing of the induction and loss of mRNA for AM and iNOS in lung, liver, and monocytes (13, 28, 29). The data here suggest that the pancreatic response to inducers of AM and iNOS (LPS in this case) is either more sensitive or of longer duration than that characteristically seen in lung and liver. The contributions that localized changes in cell AM responses make to deleterious (hypotension, metabolic shunting) as well as beneficial (antiapoptotic, antimicrobial peptide) (27) biological processes remain open for debate.

Contained in our hypothesis was the concept that changes in local tissue and pancreatic islet cell function can evolve during low level chronic disease stress and predispose animals to greater pathophysiological responses when further subjected to acute challenges from bacterial toxins. The present report integrates and defines new elements of a pancreatic-immune axis that appear to play a significant role in the metabolic perturbations of disease stress. The coordinated up-regulation of AM and iNOS as components of the pancreatic-immune axis may mediate changes in pancreatic function observed during low level chronic disease stress. These observations complement the mechanism originally suggested by Mandrup-Poulsen *et al.* (30) and extended by McDaniel (31) and others (32) that nitric oxide generation by pancreatic cells limits the release of insulin and contributes to perturbations of the islets. That this response may be particular to specific hormones in the pancreas such as insulin and not simply a generalized occurrence is supported by the facts that 1) plasma glucagon responses after LPS in these calves were neither temporally nor concentration different between LPS and PI + LPS (data not presented); 2) we previously demonstrated that AM inhibited insulin release from isolated cultured islets (8); and 3) recently, NO has been shown to confer aspects of differential hormone regulation in the pancreas by inhibiting insulin and stimulating glucagon secretion in experiments where *N*-nitro-*L*-arginine methyl ester was used to inhibit NOS (33). We had little indication in the present study that the iNOS response was greatly manifest in the  $\beta$ -cells, although light immunostaining was evident in  $\beta$ -cells in calves experiencing both immune stresses. Our data are consistent with the capability for islet cells themselves to generate NO in the iNOS cascade, separate and apart from that which might be generated from inflammatory cells that migrate to or reside in the pancreas (31, 32). Light microscopy and monocyte staining procedures failed to demonstrate the presence of infiltrating immune cells in the pancreas of calves at the time of tissue fixation.

Constitutive forms, in contrast to the inducible form, of NOS have been identified in several cell types in the pancreas, most notably as neuronal NOS colocalized specifi-

cally with somatostatin  $\delta$ -cells (34) and some  $\beta$ -cells (35). However, the present report is the first to suggest that the inducible isoform can be present in the AM/pancreatic polypeptide cells of islets and is coordinately up-regulated with AM by low level parasitic infection as well as LPS in both chronic and acute disease stress scenarios. A connection between AM and NO was initially speculated to exist in the cardiovascular system, *i.e.* the demonstration that the hypotensive effects of AM were blocked in the presence of *N*-nitro-*L*-arginine methyl ester, a competitive inhibitor of NOS (36). Similarly, data from So *et al.* (13) suggested that a significant part of AM function may be mediated by AM's ability to induce NO production via the iNOS pathway. In addition, AM has been implicated as having immunomodulatory capability through its effects on interleukin and TNF $\alpha$  expression in activated macrophages (37) and conversely as demonstrated by the effects of LPS and the LPS-mimetic taxol to stimulate AM mRNA content and peptide release from macrophages (38). Thus, the paracrine/autocrine function of AM in the pancreas may be related to the modulation of NO production within the cells in which AM and iNOS colocalize.

The relative importance of potential endocrine actions of AM (as reflected in the acute rise and fall in plasma after LPS) compared with the impact of paracrine effects (consistent with the increased pancreatic localization of AM in the infected calves) is not clear. Previous data from our laboratory demonstrated that this sarcocystis infection causes a blunting of insulin responses to provocative arginine challenge (39). The lag in insulin response to refeeding most evident in infected calves further challenged with LPS is consistent with the capacity for AM to blunt insulin release as previously demonstrated in isolated cultured islets and *in vivo* after glucose challenge (8). Furthermore, patterns of insulin measured in plasma after LPS treatment were consistent with the concentration and temporal changes in plasma TNF $\alpha$  and glucose during the hyperglycemic and hypoglycemic phases of response. A significant NO response, mirrored in the changes in nitrate measurable in plasma, is similarly consistent with the acute induction of the high output NO pathway mediated through iNOS in the cytokine response cascade after LPS. Issues associated with more chronic changes in plasma glucose after LPS and in association with infection are more difficult to interpret in these experiments. The interpretation of the consequences associated with and mechanisms underlying changes in tissue fluxes as well as plasma concentrations of glucose are made difficult due in no small part to the simultaneous alterations in pancreatic hormone release, hepatic glucose-glycogen turnover, and paradoxical differences in TNF-driven insulin resistance and glucose uptake in muscle (40, 41). Therefore, the plasma concentrations of glucose present during the post-feeding events may or may not be reflective of the tissue-specific impact of the altered endocrine-immune milieu on glucose and energy metabolism, even though significant differences in the insulin response to feeding were present between some treatments.

As reviewed previously (5, 42), the degree to which TNF $\alpha$  and iNOS are up-regulated by infection and LPS is highly

dependent on the intensity of the immune challenge, the nature of the cytokine milieu evoked, and the type of tissue in which the response is studied. The ability of cytokines such as interleukin-1 and TNF $\alpha$  to induce AM and iNOS expression in nonimmune cells, such as vascular endothelial cells (43), is consistent with the observed local islet cell response in the endocrine pancreas. The coordinated amplification and colocalization of AM and iNOS in islet cells as demonstrated in the present study suggests some common functional linking of pancreatic tissue responses to AM and the immunoeffector NO. The data further suggest that the previously observed capacity for AM/NO to inhibit insulin secretion in normal animals may be exacerbated in disease processes that result in localized NO (and AM) up-regulation in the pancreas. Although previous reports illustrated that frank cytotoxicity associated with overproduction of NO in islets as driven by specific cytokine induction can result in  $\beta$ -cell destruction (31), the present report underscores the sensitivity of the pancreas to low level infection and the apparent paracrine mediation of hormone secretion in the pancreas via AM and NO.

Disease stress may participate in and increase the risk for long lasting metabolic disease to develop in the pancreas (44). In essence, the presence of low level and subclinical infection may be sufficient to exacerbate a pathological response to a secondary infection within the pancreas, with the end result being a chronic disease state coincident with a localized organ response. Host resistance and susceptibility to disease vary with the competence of the immune system and are compromised in instances such as human immunodeficiency virus infection, diabetes, and other endocrinopathies and in association with certain therapeutic interventions in cancer. Often, patients suffering from these conditions harbor chronic, occult infections, and when further challenged with a secondary low level infection, present clinically significant signs of multiple organ or metabolic dysfunction, which have been attributed to NO effects (41). With the link established between AM and NO, intervention strategies targeting modulation of both AM and NO relationships to disease may have clinical application and relevance similar to those proposed by others in multiple organ failure associated with sepsis.

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