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Regulation of Acute Phase Gene Expression Following Surgery and Endotoxin Administration in the Anesthetized Pig

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Background: **The hepatic acute phase response (APR) reflects an organism's integrated response to stress. This APR results in augmented synthesis and secretion of specific procoagulants and antiproteases and a complementary decrease in the synthesis and secretion of several constitutive proteins, such as albumin. The cytokines tumor necrosis factor (TNF) or interleukin-6 (IL-6) have been identified as proximal mediators of the APR in response to endotoxin stress. The authors hypothesized that TNF, IL-6, or both would be the proximal mediators of the APR in response to anesthesia and surgical stress.**

Methods: **The effects of a standardized surgical stress on the APR in pigs under general anesthesia with sodium pentobarbital and ketamine hydrochloride was investigated. Acute phase gene transcription was assayed in nuclei from serial liver biopsies obtained before and after 2.5 h of surgical stress, and after endotoxin administration. Tumor necrosis factor and IL-6 mRNA levels in this liver tissue were examined by Northern blot hybridization, and simultaneous plasma levels of these cytokines were measured using bioassays.**

Results: **The transcription rates of three positive acute phase genes—chymotrypsin inhibitor, inter-α-trypsin inhibitor and β-fibrinogen—increased seven-, six-, and twofold, respectively ($P < 0.05$), and the transcription rate of albumin, a negative acute phase gene, decreased to 34% of baseline ($P < 0.01$) during the 2.5 h of anesthesia and surgical stress. During this initial 2.5 h, plasma concentrations of TNF and IL-6 did not change. Hepatic IL-6 mRNA expression was never observed, and TNF mRNA expression was undetectable in six of seven pigs. Subsequent 10-μg/kg endotoxin administration caused 20- and 100-fold increases in plasma concentrations of TNF and IL-6, respectively ($P < 0.01$), and were associated with substantial hepatic expression of the TNF and IL-6 mRNAs. These increments in cytokines were not associated with any further increase in the acute phase gene transcription rates. Thus, the APR was initially regulated at the transcriptional level during surgical stress independent of, and not augmentable by, an endotoxin-provoked increase in either plasma levels or hepatic mRNA expression of TNF or IL-6.**

Conclusions: **Surgical stress induced hepatic acute phase gene transcription within 2.5 h in the absence of either systemic or local (hepatic) increases in TNF or IL-6. Subsequent endotoxin-induced increases in TNF or IL-6 did not alter this surgical stress-induced acute phase gene transcription. (Key words: Anesthesia. Molecular biology: gene expression. Stress, surgical: acute phase protein; interleukin-6; tumor necrosis factor.)**

ACUTE phase proteins are specific plasma proteins synthesized predominantly in the liver.[1–3] Their plasma levels are modulated within 12–24 h in response to surgical trauma, infection, or inflammation, and many are thought to limit injury by acting as protease inhibitors and clotting factors.

Induction of many of the acute phase proteins can be mediated by inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1 (IL-1), or tumor necrosis factor (TNF). *In vivo* administration of these cytokines increases acute phase protein plasma levels.[4,5] This increase is preceded by an increase in hepatic acute phase mRNA levels during acute inflammation or lipopolysaccharide (LPS) administration in the rat.[6,7] *In vitro* studies have demonstrated that IL-6 can induce mRNA expression and production of acute phase proteins in primary cultured hepatocytes and hepatoma cells.[8–11] In animal models of sepsis using *Escherichia coli* endotoxin or lipopolysaccharide (LPS) administration, TNF is rapidly produced and secreted, followed by secretion of IL-6.[7,8,12] Humans respond to an endotoxin challenge by increasing their circulating IL-6 level (peaking in 2–4 h) and subsequently incrementing their circulating C-reactive protein (an acute phase protein) within 20 h of endotoxin administration.[13]

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A substantial body of information links IL-6 (acting *via* receptors on hepatocytes) to the induction of the acute phase response;[9] however, the *in vivo* correlates of these events over time in intact whole animals during anesthesia and surgical stress have not been studied. We initially hypothesized that TNF and IL-6 would be common proximal mediators of the acute phase response to both surgical and inflammatory stresses.

We, therefore, studied: 1) the transcription rate for hepatic acute phase genes; and 2) TNF and IL-6 expression in pigs after anesthesia and surgical stress. These results were compared in the same pigs with the effects of a subsequent further inflammatory stress produced by the administration of endotoxin.

Materials and Methods

Experimental Protocols

Animals. Seven- to 10-week-old prepubertal female pigs (21.6–30.5 kg) were studied under sterile and nonpyrogenic conditions. Prepubertal pigs were employed to obviate potential effects of cyclic estrogen variation on hepatic gene expression. The experimental protocol is summarized in figure 1. Anesthesia was induced with 10 mg/kg intramuscular sodium pentobarbital and 20 mg/kg intramuscular ketamine hydrochloride, and maintained with 1–2 mg/kg intravenous sodium pentobarbital administration. A heating pad was used to maintain body temperature. A tracheotomy was performed and the lungs were ventilated with oxygen-enriched room air using a volume ventilator. Venous access was obtained by cannulating the left jugular vein. Lactated Ringer's solution with 5% dextrose was continuously infused at a rate of 18–36 $ml \cdot kg^{-1} \cdot h^{-1}$. A laparotomy was rapidly performed and the first liver biopsy, consisting of 3–5 g of tissue, was obtained immediately. After this, the left carotid artery was cannulated to measure blood pressure and obtain blood samples. A thermodilution pulmonary artery catheter was placed *via* the right jugular vein, and its placement was confirmed by typical pressure waveforms. Appropriately sized ultrasound transit-time flow probes[14] (Transonic, Ithaca, NY) were placed around portal vein and hepatic artery during the next 60 min. The animals were then observed for another 60 min with no further surgical intervention to assure that the preparation was hemodynamically stable. The second liver biopsy was then taken 2.5 h after the first biopsy from a site at least 3 cm from the original biopsy site.

After the second liver biopsy, either 10 μg/kg endotoxin (*E. coli* 055:B5, Difco, Detroit, MI), or saline was administrated intravenously as a bolus over 1 min. Subsequent liver biopsies (3.5–5 g) were obtained every hour for the next 4 h, each as far as possible from any other previous biopsy site, such that a total of six biopsies were obtained during the entire experiment. After each biopsy, 3 g of liver tissue were rapidly plunged into 0.14 M NaCl and 10 mM Tris-HCl (*p*H 7.4) at 4° C for preparation of the nuclei, and the remaining tissue was homogenized in guanidinium thiocyanate solution for preparation of total RNA and frozen, or quickly frozen in liquid nitrogen and stored in a −80° C freezer.

Arterial blood pressure was monitored continuously once available during the study. All the physiologic

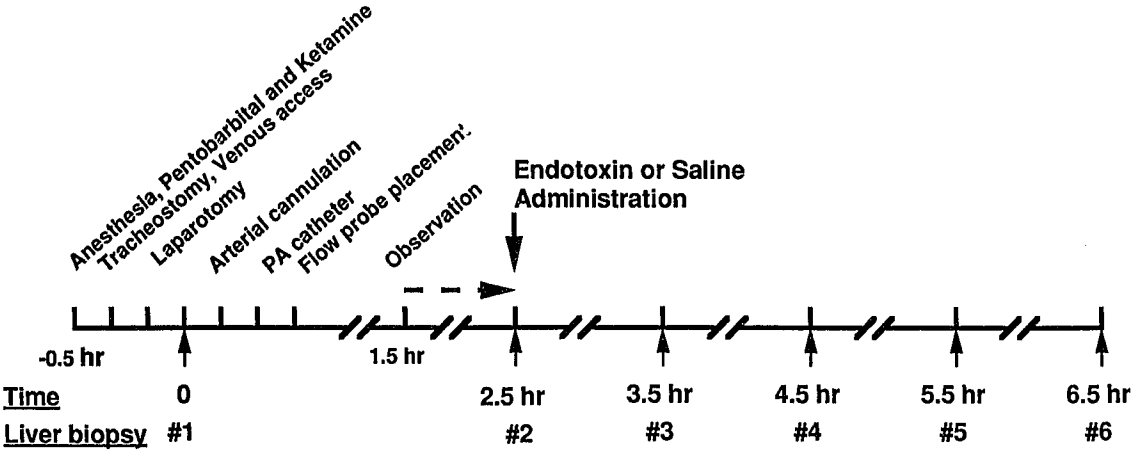

Fig. 1. The experimental protocol. All subsequent figures when referring to time correspond to the time sequence denoted here. PA = pulmonary artery.

parameters and arterial blood gases (Radiometer, Copenhagen) were measured at the end of the observation period and every 30 min after endotoxin or saline administration. Arterial blood was obtained before each liver biopsy for measurement of plasma cytokine levels and plasma liver associated enzyme activity. Alkaline phosphatase, lactate dehydrogenase, aspartate transaminase, and alanine aminotransferase were measured by an Olympus AU5000 (Tokyo, Japan) in a commercial laboratory (SmithKline Beecham, Owings Mills, MD).

The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions.

In Vitro *Transcription in Isolated Hepatic Nuclei*

Nuclei were isolated by centrifugation through a sucrose gradient, as previously described.[15] The tissue was minced finely on a glass plate and homogenized using a Potter-Elvehjem homogenizer in 4.5 ml of a solution containing 0.32 M sucrose, 3.0 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM ethlendiamin tetra acetic acid (EDTA), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM Tris[hydroxymethyl]-aminomethane (Tris)-HCl (*p*H 8.0). The homogenate was filtered through gauze and the filtered homogenate was diluted with ×2.5 volumes of a solution containing 2.0 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM PMSF, and 50 mM Tris-HCl (*p*H 8.0). This same solution (2.5 ml) was placed in a 12-ml ultracentrifuge tube and 7.5 ml of diluted homogenate was overlaid. Samples were centrifuged for 15 min at 100,000 ×g at 4° C. The supernatant was carefully aspirated and the nuclear pellet was resuspended in 200 μl of glycerol storage buffer containing 25% glycerol, 5.0 mM magnesium acetate, 5.0 mM dithiothreitol (DTT), and 50 mM Tris-HCl (*p*H 8.0). Samples were stored at −80° C until assayed.

In vitro transcription was performed as described by Greenberg and Bender.[16] Duplicate aliquots of every nuclear suspension were counted in a hemocytometer. Repeated counts of fields containing >100 nuclei typically varied by not greater than 5%, and never greater than 10%. Transcriptional activity was normalized to the number of nuclei. Nuclei ($7.2–14.4 \times 10^7$ per 200 μl) were incubated with 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 170 μCi [α-^{32}P]UTP, and a reaction buffer containing 5 mM Tris-HCl (*p*H 8.0), 2.5 mM $MgCl_2$, 2.5 mM DTT, and 0.15 M KCl for 30 min at 30° C. RNA was extracted using guanidinium thiocyanate solution, acidic phenol, and chloroform/isoamylalcohol (49:1). The extracted RNA was precipitated by isopropanol and dissolved in TES (10 mM N-tris[hydroxy-methyl]methyl-2-aminoethan sulfonic acid; *p*H 7.4), 1 mM EDTA, and 0.2% sodium dodecyl sulfate (SDS). Then the RNA was precipitated by ethanol with sodium acetate (*p*H 5.2) and dissolved in TES with 0.3 M NaCl.

The following porcine cDNA clones were used: albumin (ALB), chymotrypsin inhibitor (CTI), inter-α-trypsin inhibitor (αTI), and β-fibrinogen (βFIB). These are among 12 hepatic genes previously shown to be induced in pigs after resuscitation from tamponade-induced shock.[17] The plasmids were linearized by digestion with *EcoR*I. Linearized DNA (5 μg) was spotted onto nitrocellulose membrane using a slot blot apparatus, and was cross-linked by UV and baked at 80° C for 1 h. Nitrocellulose membrane was prehybridized with 1 ml of TES with 0.3 M NaCl and 1 ml of RNA hybridization solution containing 50% formamide, 5 × SSC, 2.5 × Denhart's solution 0.2 mg/ml salmon sperm single strand DNA, 1 mM EDTA, and 1% SDS at 68° C for 1.5 h. Subsequently, filters were placed in TES with 0.3 M NaCl solution containing the radioactive labeled RNA for 36 h at 68° C. After hybridization, the filters were washed twice in 2 × SSC for 2 h at 68° C, soaked in 2 × SSC and 0.1 mg/ml RNase A for 30 min at 37° C, and then washed once more in 2 × SSC at 37° C for 1 h. The bound radioactivity was quantitated using a betascope (Betagen, Walham, MA), which is a computer-linked planar array of β counters. The counting fields were of uniform size and proportional to the size of the slots in which the cDNA was immobilized. (This methodology circumvents an important limitation of autoradiography; namely, the nonlinear response of the film outside a narrow range of photon exposure.) The background was measured and subtracted from each raw count, and then the transcriptional activity of each gene at each time point was expressed as the proportion of that gene's transcriptional activity at time zero. For illustration purposes, autoradiography was also performed by exposing the filters for 5–7 days at −80° C.

Isolation of Hepatic RNA and the Northern Blot Hybridization

Total RNA from porcine liver (n = 7: four endotoxin pigs, three sham pigs) was isolated according to the method of Chomczynski and Sacchi.[18] Frozen liver tissue was homogenized in 4 M guanidinium thiocyanate containing 25 mM sodium citrate (*p*H 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol. Total RNA was ex-

tracted using phenol and chloroform/isoamylalcohol (49:1), then precipitated in isopropanol. The pellet was rinsed once with 75% ethanol and dissolved in TES. Optical density of a small aliquot of the RNA solution was measured at 260 and 280 nm to determine yield and purity.

RNA samples (20 μg) were dried by vacuum centrifugation and denatured by heating at 65° C for 15 min in the presence of 15 μl of gel-loading buffer containing 50% formamide, 2.2 M formaldehyde, 50 mM 3-[N-morpholino]-propanesulfonic acid (MOPS; *p*H 7.0), sucrose, and bromophenol blue. RNA samples were separated by electrophoresis on 1% agarose gel containing 50 mM MOPS (*p*H 7.0) and 2.2 M formaldehyde, and using 50 mM MOPS (*p*H 7.0) and 1 mM EDTA as the running buffer. Visual inspection of the ethidium bromide-stained gel confirmed the absence of significant degradation (28S band twice as bright as 18S) and uniformly of lane-to-lane loading. RNA was transferred to nylon membrane (Genescreen plus; Du Pont, Boston, MA) by capillary action using 10 × SSC solution and cross-linked by exposure to UV light. The filter was prehybridized for 1.5 h and hybridized overnight in hybridization solution. Porcine cytokine cDNA probes were cleaved from the plasmid using the appropriate restriction enzymes. cDNA probes (100 ng) were labeled with [α-^{32}P]CTP (100 μCi) using the random priming method. Prehybridization was carried out for 1.5 h in a buffer solution of 50% formamide, 5 × SSC, 2.5 × Denhart's solution 0.2 mg/ml salmon sperm single strand DNA, 1 mM EDTA, and 1% SDS at 68° C. The ^{32}P-labelled cDNA probe was added, and, after overnight hybridization, the filter was washed twice with 50 mM Tris-HCl (*p*H 7.7), 2.5 mM EDTA, 1 M NaCl, and 1% SDS at 55° C for 2 h, washed twice with 2 × SSPE and 0.1% SDS at 55° C for 1.5 h, and then exposed to x-ray film for 2 days at −80° C. The blots were stripped of their radioactivity by boiling in preparation for reprobing with other cDNAs.

Measurement of Plasma TNF and IL-6

Plasma TNF bioactivity was assayed using the L929 fibroblast cell line to measure cytotoxicity in actinomycin D-treated cells.[19] L929 cells were maintained in continuous culture and were harvested by trypsinizing the monolayer cultures. One hundred-microliter aliquots containing 1×10^4 washed cells were seeded on 96-well culture plates and were incubated for 48 h at 37° C. One hundred-microliter volumes of sample or standard TNF were added to the cells and serially diluted twofold across the plate. Each well then received 50 μl containing 4 μg/ml actinomycin-D, and the plates were reincubated for an additional 18 h. The surviving cells were quantitated by staining with 2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromic (MTT) for 4 h.[20] The plates were washed twice with phosphate buffered saline and solubilized with acid-isopropanol, and the optical density was read at 570 and 630 nm. The levels of TNF in experimental samples were determined from a standard curve derived from the assay of standard TNF.

Plasma IL-6 bioactivity was measured by cell proliferation assay using B9 cells.[21] B9 cells in RPMI 1640 and 1% fetal bovine serum were added to serially diluted plasma samples or standardized IL-6 (Endogen, Boston, MA) on 96-well microtiter plates. The cells were incubated for 3 days at 37° C before assessing remaining cell viability by the MTT colorimetric method.[20]

Statistics

Graphic values are expressed as mean ± SEM. A value of $P < 0.05$ was accepted as significant.

Physiologic parameters and plasma levels of TNF and IL-6 were evaluated by one-way and two-way ANOVA for repeated measurements with Scheffe's F-test for within-group comparisons over time when appropriate. Plasma enzyme levels at time 0 and 2.5 h were compared by paired *t* tests. Plasma enzyme levels for each treatment group (endotoxin or saline sham) were compared by one-way ANOVA for repeated measurements for change over time between 2.5 and 6.5 h. Transcription rates were compared first between 0 and 2.5 h by paired *t* tests. Transcription rates for each treatment group (endotoxin or saline sham) were compared by one-way ANOVA for repeated measurements for change over time between 2.5 and 6.5 h. Saline sham *versus* endotoxin treatments were compared by two-way ANOVA.

Results

Effects of Anesthesia and Surgical Stress on Acute Phase Protein Gene Transcription

Physiologic Conditions. Arterial blood pressure remained stable and within normal limits during the experimental period before the second biopsy. All measured physiologic parameters at the time of the

second biopsy were within normal limits and are presented in table 1. Arterial p_{O_2}, p_{CO_2}, and *p*H were 332.6 ± 17.8 mmHg, 38.5 ± 3.7 mmHg, and 7.43 ± 0.03, respectively. Plasma liver associated enzyme activity, which, if elevated, would have suggested acute liver injury, did not increase during the first 2.5 h of the experimental period (table 2).

Acute Phase Gene Transcription. There was no statistically significant change in total transcription rate before and after surgery, 2.83 ± 0.73 and 2.26 ± 0.44 dpm/nucleus, respectively (N = 9). Specific transcriptional rates of the genes encoding albumin, chymotrypsin inhibitor, inter-α-trypsin inhibitor, and β-fibrinogen were determined by hybridizing the radiolabelled nuclear transcripts to cDNAs coding for these four genes immobilized on filter membranes. A representative autoradiograph of such a nuclear runoff assay is shown in figure 2. Quantitative results of the nuclear runoff transcription assays are shown in figure 3. Albumin transcription rate was decreased to 34 ± 10% of time 0. The transcription rate of chymotrypsin inhibitor, inter-α-trypsin inhibitor, and β-fibrinogen increased approximately seven-, six-, and twofold, respectively, *versus* the rate at time 0. To exclude the initial hepatic biopsy as the cause of the acute phase gene transcription observed at 2.5 h, three additional pigs were subjected to the same protocol without the early (time 0) biopsy. These three pigs demonstrated levels of acute phase transcription indistinguishable from that observed in the nine animals reported here in detail (data not shown).

mRNA Expression and Plasma Levels of TNF and IL-6. mRNA of IL-6 was not detected at all by Northern blot hybridization in hepatic tissue. mRNA of TNF was faintly detected in only one of seven pigs at time 2.5 h. Plasma TNF levels were less than 20 μ/ml and IL-6 levels were less than 150 μ/ml at times 0 and 2.5 h. Neither plasma level increased during this time period.

Table 1. Physiologic Parameters of Biopsy at 2.5 h

Parameters	
Mean arterial pressure	106 ± 4 mmHg
Mean pulmonary arterial pressure	13.3 ± 0.7 mmHg
Right atrial pressure	5.8 ± 0.3 mmHg
Pulmonary wedge pressure	4.6 ± 0.4 mmHg
Cardiac output	4.03 ± 0.24 l/min
Heart rate	137 ± 6 beats/min
Body temperature	37.1 ± 0.3° C
Portal vein blood flow	0.541 ± 0.063 l/min
Hepatic artery blood flow	0.247 ± 0.033 l/min

Data represent mean ± SEM; n = 9.

Table 2. Plasma Enzyme Activities

Plasma Enzyme	Activity (U/l)	
	Time 0	2.5 h
Alkaline phosphatase	175 ± 11	164 ± 12
Lactate dehydrogenase	925 ± 79	854 ± 112
Aspartate transaminase	101 ± 14	117 ± 18
Alanine aminotransferase	45 ± 3	39 ± 2

Data represent mean ± SEM; n = 8.

Effects of Administration of Endotoxin on Acute Phase Gene Transcription

Physiologic Conditions. The physiologic parameters after the second biopsy and subsequent to endotoxin or saline administration (sham) are shown in figure 4. There was no significant change in mean arterial blood pressure in sham pigs during the experimental period. The bolus injection of 10 μg/kg endotoxin produced a transient increase in blood pressure within 0.5

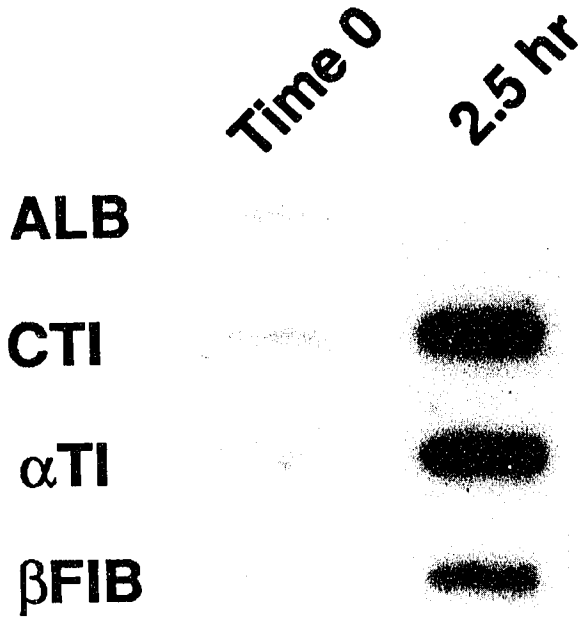

Fig. 2. Representative results of nuclear runoff assay with isolated pig hepatic nuclei at time 0 and 2.5 h. RNA purified from isolated nuclei, which had been incubated with radioactive RNA precursors, was hybridized with cDNAs complementary to mRNAs for albumin (ALB), chymotrypsin inhibitor (CTI), inter-α-trypsin inhibitor (αTI), and β-fibrinogen (βFIB).

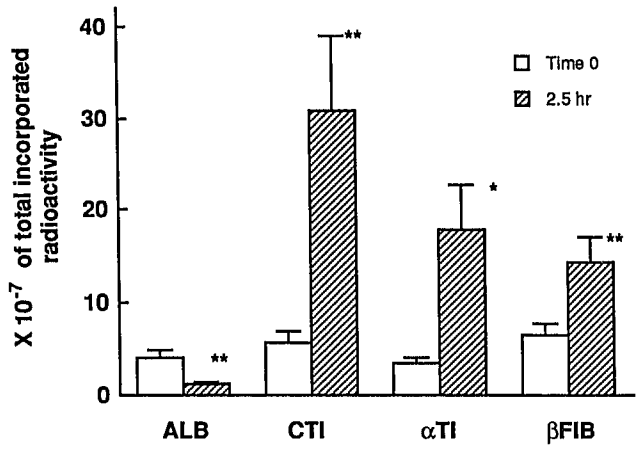

Fig. 3. Gene-specific transcription rate for albumin (ALB), chymotrypsin inhibitor (CTI), inter-α-trypsin inhibitor (αTI), and β-fibrinogen (βFIB) during surgical stress at time 0 and 2.5 h. Transcription rate was expressed as $\times 10^{-7}$ of total incorporated radioactivity. Each column with vertical bars shows mean ± SEM (n = 9). Significantly different from time 0 by paired *t* test; $^{**}P < 0.01$, $^{*}P < 0.05$.

h. Within 2 h after endotoxin administration, blood pressure gradually decreased to 70 ± 6 mmHg and remained at this level for the rest of the study period. Right atrial (Pra) and mean pulmonary artery (Ppa) pressures did not change; however, pulmonary wedge pressures (Ppaw) decreased over time ($P < 0.05$) in the sham pigs. After endotoxin, Ppa increased acutely and then decreased to a value that was still higher than the sham control group. Pra decreased over time ($P < 0.05$); however, Ppaw did not change. Cardiac output (CO) decreased 0.5 h after endotoxin, increased slightly, and then fell over time. Cardiac output gradually decreased in sham pigs during the experimental period ($P < 0.01$), but was still greater than the endotoxin-treated pigs ($P < 0.05$).

Neither portal vein blood flow (Qpv) nor hepatic artery blood flow (Qha) changed in the sham pigs over time. After endotoxin, Qpv decreased acutely with no further change over time, while Qha demonstrated a biphasic response with a substantial acute decrease at 0.5 h, followed by a return to baseline.

There were insignificant changes in heart rate, p_{O_2} and p_{CO_2}, and hematocrit in both endotoxin and sham groups. Body temperature slightly increased in sham pigs. *p*H decreased after endotoxin administration (table 3).

Alkaline phosphatase activity increased 3 h after endotoxin administration, while aspartate transaminase activity increased in both sham and endotoxin pigs over the experimental period (table 4).

Acute Phase Protein Gene Transcription. A representative autoradiograph of a nuclear runoff assay is shown in figure 5. Transcription rates, relative to the immediate preendotoxin transcription rate, are shown in figure 6. Relative transcription rates were calculated based on the time 2.5 h transcription rate. After the increase in transcription rates during the initial 2.5 h of the experiment, neither the endotoxin nor the sham pigs showed any further increase in either the transcription rate of acute phase proteins or the total transcription rate. There was no detectable transcription of the albumin mRNA at time 2.5 h or thereafter.

mRNA Expression and Plasma Levels of TNF and IL-6. Both IL-6 and TNF mRNA were detected at 1 and 2 h after endotoxin administration (*i.e.*, times 3.5 and 4.5 h; fig. 7). Plasma TNF bioactivity was detected at 1 and 2 h after endotoxin (*i.e.*, times 3.5 and 4.5 h; fig. 8A). Although IL-6 levels did not change in the sham pigs, a three-orders-of-magnitude increase in IL-6 levels was observed in the endotoxin pigs (fig. 8B).

Discussion

The goals of this study were: 1) to demonstrate, in an intact animal, quantitative acute phase gene transcription during anesthesia and surgical stress, and to compare these results with a subsequent administration of endotoxin producing an inflammatory stress; and 2) to determine, *in vivo,* if TNF or IL-6 levels correlated with acute phase gene transcription during surgical and inflammatory stresses over time.

Gene expression involves many events that culminate in protein synthesis and secretion. It is convenient to divide these events into three categories: pretranscriptional, transcriptional, and posttranscriptional. Pretranscriptional events include signal transduction, second messenger activation, and the activation of transcriptional factors specific to the regulatory region of each specific gene. Transcriptional events include the initiation of RNA synthesis, elongation of the nascent RNA chain, and termination of RNA synthesis. Posttranscriptional events include processing of the RNA transcript into messenger RNA (mRNA), translation of that mRNA into protein, protein modification, and protein export.

We focused on the transcription phase of acute phase gene expression because *in vitro* studies suggest that the secretion of acute phase proteins from cultured

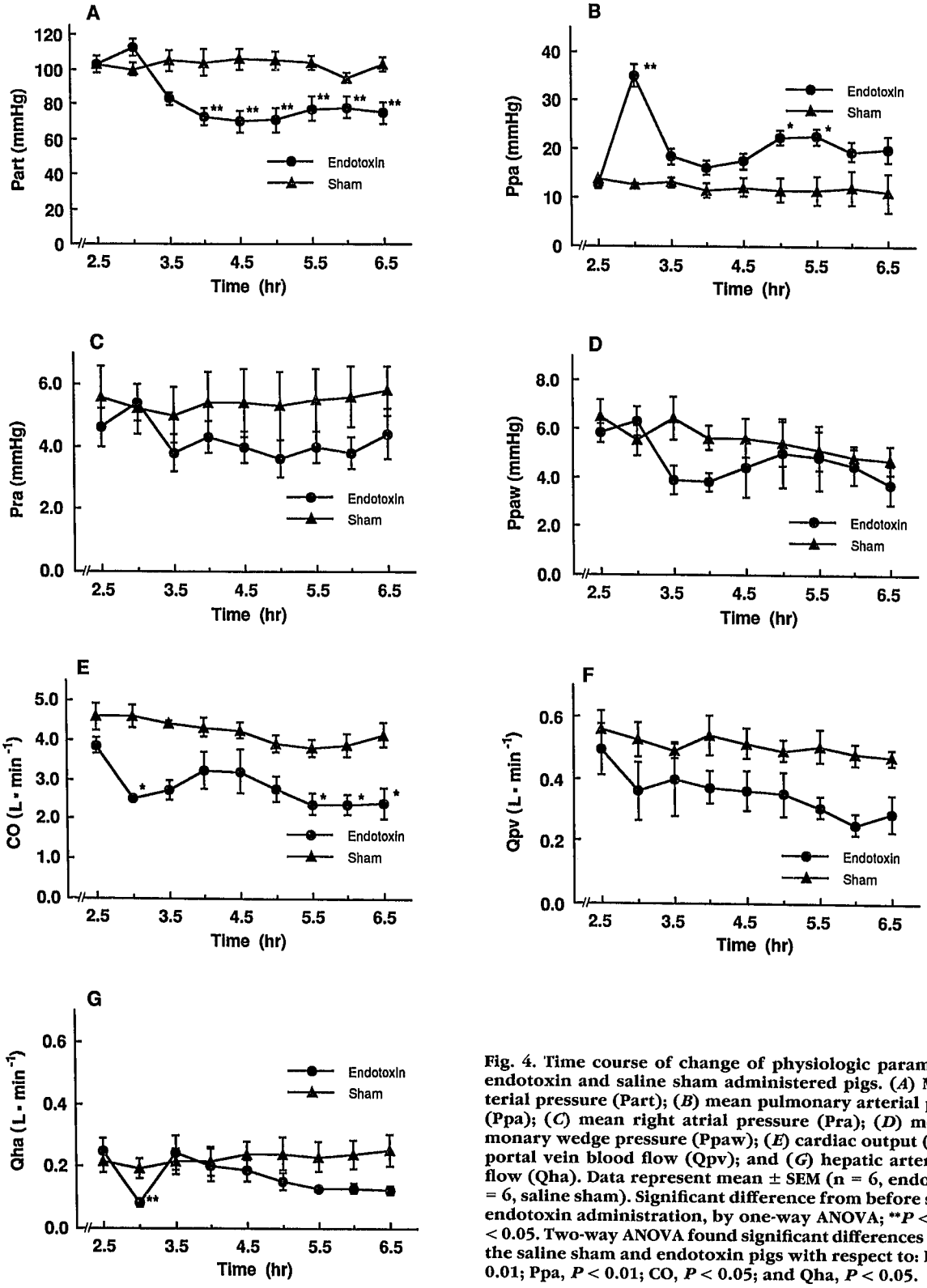

Fig. 4. Time course of change of physiologic parameters in endotoxin and saline sham administered pigs. (*A*) Mean arterial pressure (Part); (*B*) mean pulmonary arterial pressure (Ppa); (*C*) mean right atrial pressure (Pra); (*D*) mean pulmonary wedge pressure (Ppaw); (*E*) cardiac output (CO); (*F*) portal vein blood flow (Qpv); and (*G*) hepatic artery blood flow (Qha). Data represent mean ± SEM (n = 6, endotoxin; n = 6, saline sham). Significant difference from before saline or endotoxin administration, by one-way ANOVA; **$P < 0.01$, *$P < 0.05$. Two-way ANOVA found significant differences between the saline sham and endotoxin pigs with respect to: Part, $P < 0.01$; Ppa, $P < 0.01$; CO, $P < 0.05$; and Qha, $P < 0.05$.

Table 3. Physiologic Parameters before and after Endotoxin or Saline Sham Treatment

Parameter	Treatment	Pretreatment 2.5 h	Posttreatment 6.5 h
Heart rate (beats · min^{-1})	Endotoxin	137 ± 6	157 ± 7
	Sham	145 ± 10	158 ± 9
Body temperature (° C)	Endotoxin	37.3 ± 0.5	37.7 ± 0.7
	Sham	37.6 ± 0.4	38.5 ± 0.5*
P_{O_2} (mmHg)	Endotoxin	350.1 ± 22.6	274.0 ± 38.4
	Sham	308.0 ± 16.5	291.2 ± 40.8
P_{CO_2} (mmHg)	Endotoxin	36.9 ± 4.8	49.2 ± 3.9
	Sham	42.3 ± 3.2	43.4 ± 3.9
*p*H	Endotoxin	7.46 ± 0.04	7.29 ± 0.03†
	Sham	7.37 ± 0.01	7.39 ± 0.05
Hematocrit (%)	Endotoxin	30 ± 2	27 ± 3
	Sham	30 ± 1	28 ± 1
Crystalloid administered (ml · kg^{-1} · h^{-1})	Endotoxin	17.4 ± 1.8	36.1 ± 3.6†
	Sham	17.2 ± 1.4	31.6 ± 3.0†

Data represent mean ± SEM. Endotoxin pigs, n = 6; sham pigs, n = 6.

* Significant difference from pretreatment values by one-way analysis of variance, $P < 0.05$.

† Significant difference from pretreatment values by one-way analysis of variance, $p < 0.01$.

cells is proportional to mRNA, and that acute phase mRNA content is typically regulated by synthesis (and not by degradation) of mRNA.[22] We used the nuclear runoff technique (as opposed to Northern blots) because the former is blind to preexisting mRNA. This nuclear runoff technique takes advantage of the fact that isolated nuclei will elongate (but not initiate) RNA transcripts; therefore, incorporation of a radioactive RNA precursor is proportional to the transcriptional activity of each gene at the time the nuclei are isolated.

In contrast to the acute phase genes, regulation of the expression of the cytokine TNF is posttranscriptional, and depends primarily on the high degradation rate of that specific mRNA. The initial increment in TNF expression after endotoxin exposure is not reflected in increased transcription rate, but, rather, is caused by a sudden drop in the degradation rate of the specific mRNA. In contrast to acute phase genes, the most appropriate assay for *early* changes in TNF gene expression is the Northern blot.

These assays do not account for the potential contributions of changes in translational and posttranslational events to global changes in gene expression; however, *in vitro* studies suggest that such contributions are minor.[8–11]

Acute inflammation induced in rats, by either the subcutaneous injection of turpentine or the administration of endotoxin, raises hepatic acute phase gene transcription, acute phase mRNA levels, and plasma levels of acute phase proteins.[3,6,7] Both *in vivo* and *in vitro* studies have demonstrated that TNF and IL-6 can induce both transcription and translation of the acute phase genes.[5,8,9,23,24] In turn, TNF and IL-6 are induced by the administration of endotoxin in animal and human studies, resulting in high plasma levels of those cytokines.[7,13,25] In both animal and human studies, observed increases in plasma levels of these cytokines have been associated with prolonged surgical procedures, substantial physiologic stress, or substantial tissue injury.[26–32]

In trying to understand the etiology of the APR in an intact animal, it, therefore, seemed reasonable to separate the effects of a moderate surgical stress (which produces little, if any, TNF or IL-6) from an inflam-

Table 4. Plasma Enzyme Activities before and after Endotoxin or Sham Saline Administration

Enzyme	Treatment	Pretreatment 2.5 h	Posttreatment Activity (U/l) 3.5 h	4.5 h	5.5 h	6.5 h
Alkaline phosphatase	Endotoxin	178 ± 20	188 ± 19	187 ± 23	238 ± 27*	225 ± 27
	Sham	178 ± 8	182 ± 19	177 ± 18	164 ± 13	158 ± 12
Lactate dehydrogenase	Endotoxin	702 ± 76	830 ± 106	659 ± 108	687 ± 111	661 ± 97
	Sham	971 ± 113	1012 ± 94	1191 ± 107†	1083 ± 87	895 ± 57
Aspartate transaminase	Endotoxin	91 ± 16	119 ± 25	134 ± 16	148 ± 33	210 ± 49*
	Sham	102 ± 26	116 ± 26	157 ± 33†	223 ± 29*	233 ± 41*
Alanine aminotransferase	Endotoxin	39 ± 3	43 ± 2	33 ± 4	33 ± 4	29 ± 3
	Sham	41 ± 4	42 ± 4	43 ± 4	41 ± 4	39 ± 3

Data represent mean ± SEM. Endotoxin pigs, n = 6; sham pigs, n = 6.

* Significant difference from pretreatment values by one-way analysis of variance, $P < 0.01$.

† Significant difference from pretreatment values by one-way analysis of variance, $P < 0.05$.

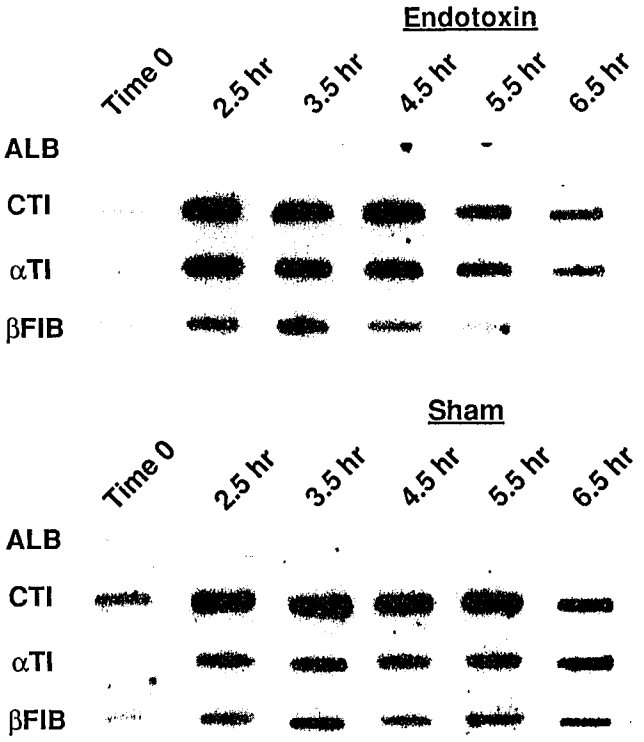

Fig. 5. Representative results of nuclear runoff assay with isolated pig hepatic nuclei at baseline (time 0), after surgical stress (time 2.5 h), and after the administration of saline or endotoxin at 2.5 h (time 3.5–6.5 h). Runoff RNA was hybridized with cDNAs complementary to mRNAs for albumin (ALB), chymotrypsin inhibitor (CTI), inter-α-trypsin inhibitor (αTI), and β-fibrinogen (βFIB).

matory stimulus known to produce large increases in TNF and IL-6. We expected that both the cytokine levels and acute phase transcription would be low after surgical stress, and both would increase after endotoxin administration. The anticipated linkage between cytokine levels and gene transcription was based on studies implicating Kupffer cells and IL-6 as crucial to the hepatic APR. To our surprise, maximal acute phase gene transcription occurred in the absence of detectable changes in either hepatic gene expression or plasma levels of the cytokines, TNF and IL-6. Moreover, no further change in acute phase protein transcription rate was observed after endotoxin administration associated with large increases in plasma levels and hepatic mRNA expression of TNF and IL-6. There are data showing that endotoxin reacts with endothelial cells and monocytes to indirectly promote acute phase gene transcription, *via* a CD14 surface receptor triggered by LPS and a lipopolysaccharide binding protein.[33,34] One such indirect mechanism is expression of immediate-early genes (such as *jun-fos*) binding of those transcription factors to the AP-1 site present in the promoters of many acute phase genes and accelerating transcription.[35]

One possible explanation is that hepatic production of inflammatory mediators below the level of detection of our assays may account for the increment in acute phase gene transcription. Activated Kupffer cells, acting in a paracrine fashion, could induce the APR in adjacent hepatocytes by producing and releasing undetectable levels of IL-6.[36–38] There are limits to the ability of Northern hybridization to detect small increases in mRNA levels, particularly when the RNA is obtained from tissues that contain mixed cell population. However, we have demonstrated, in this report and in our previous report,[39] that the anesthetized pig rapidly secretes large quantities of TNF and IL-6 in response to endotoxin. Moreover, this increase correlates with massive increases in hepatic cytokine transcripts, which are readily detectable by routine Northern hybridization. If this explanation—local cytokine production undetectable by our methods—accounts for the increment in acute phase transcription, then the increment in local cytokine concentration required to trigger acute phase transcription must be miniscule compared with the cytokine production potential of the liver.

An alternative possibility favored by us is that the APR is more complex than previously assumed. Although TNF or IL-6 may be able to induce the APR, other mediators may also be able to induce, independently or *via* synergistic interactions with low levels of IL-6, a substantial APR. For example, during stress, plasma concentrations of epinephrine, ACTH, and glucocorticoid increase rapidly. In rats, epinephrine induces IL-6, as well as the APR.[40] In both rat and human hepatoma cell lines, glucocorticoids markedly enhance the APR to IL-6.[23] Our findings correlate with the recent observation in rats by Billiar *et al.*, who noted that either endotoxin or turpentine stimulated an increase in hepatocyte fibrinogen synthesis, but that increased circulating levels of IL-6 and TNF could be found only after endotoxin.[37]

Indeed, almost all of the previous animal studies in this field have been performed in rats, which are substantially more resistant to the physiologic effects of endotoxin administration on a mg/kg basis[7,41] than either pigs or humans.[42–44] Even under nonsterile conditions, rats can be surgically instrumented without increases in plasma cytokine levels.[31] In contrast, in pilot studies in anesthetized pigs, we found that

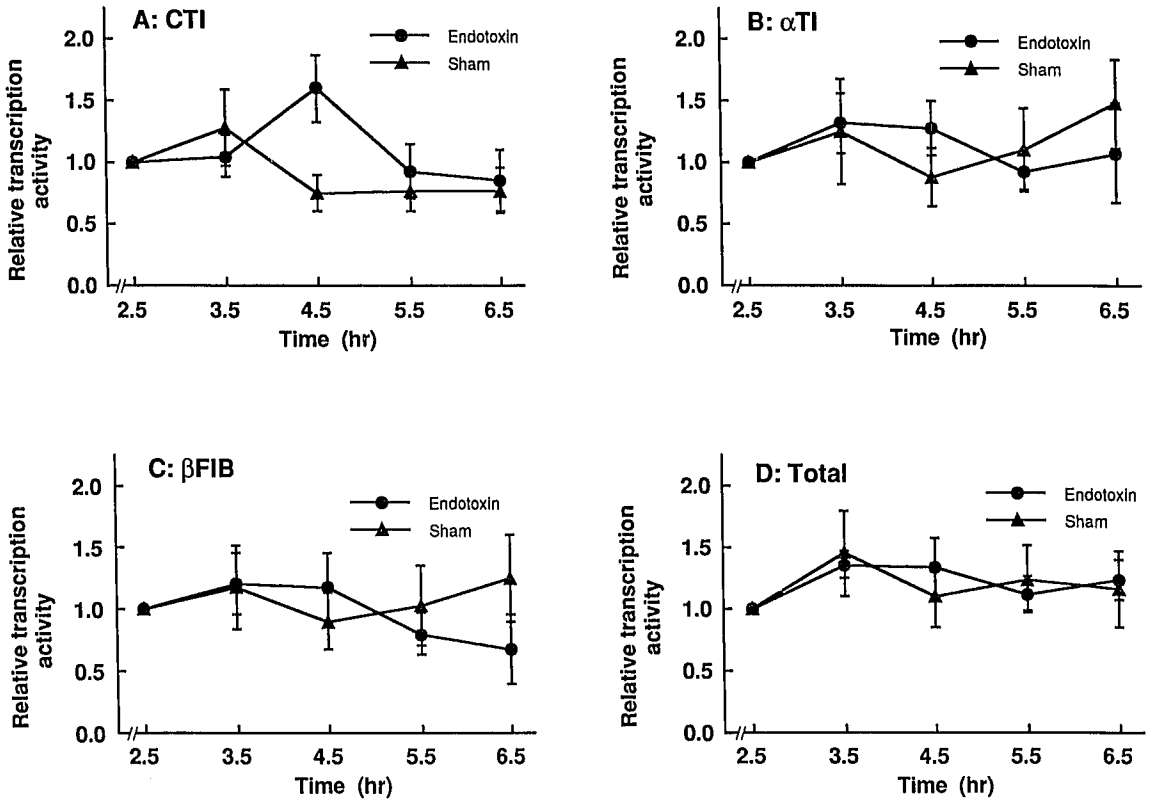

Fig. 6. Relative transcription rates of chymotrypsin inhibitor (CTI, *A*), inter-α-trypsin inhibitor (αTI, *B*), β-fibrinogen (βFIB, *C*), and total transcription rate (Total, *D*) after endotoxin or saline administration. Rates were calculated based on radioactivity before either endotoxin or saline administration. No statistical differences were found within each treatment group over this time period by one-way ANOVA. Two-way ANOVA found significant differences between the saline sham and endotoxin pigs for CTI only.

even minor surgical procedures under similar nonsterile conditions resulted in large increases in plasma TNF and IL-6 levels (unpublished results). In a subsequent study in anesthetized pigs under sterile and nonpyrogenic conditions, minor surgical manipulation caused no, or minimal, changes in TNF or IL-6 during 4 h of observation. In contrast, large increases in the plasma levels of the same cytokines were observed soon after administration of a minimal amount of endotoxin (1 μg/kg).[39]

We found that a particular anesthetic and moderate surgical stress increased the mRNA transcription rate for hepatic acute phase mRNAs without associated increases in plasma levels or hepatic mRNA expression of TNF and IL-6. The study design did not include separate arms to evaluate the independent effects of either anesthetic depth or different anesthetic agents. A deeper anesthetic level can blunt β-adrenergic stimulation, which has been associated, in rats, with increases in IL-6 levels and induction of the APR.[40] Ketamine is well known to increase sympathetic tone, and may conceivably have contributed to our observations.

In aggregate, these results suggest that surgical stress regulates transcription of genes involved in the APR, and that more than one pathway may be involved. The induction of gene expression (or, in the case of albumin, its transcriptional repression) occurs rapidly with surgical stress. Transcription of acute phase genes may have elements of an all-or-none response, or may, perhaps, exhibit a ceiling effect, because a large increase in TNF and IL-6 does not enhance transcription of acute phase genes that are already expressing well above normal levels. Furthermore, because clinical experience correlates increases in the APR with increasing degrees of stress, our results indicate that an important component of the control of this response rests in post-transcriptional regulation of gene expression.

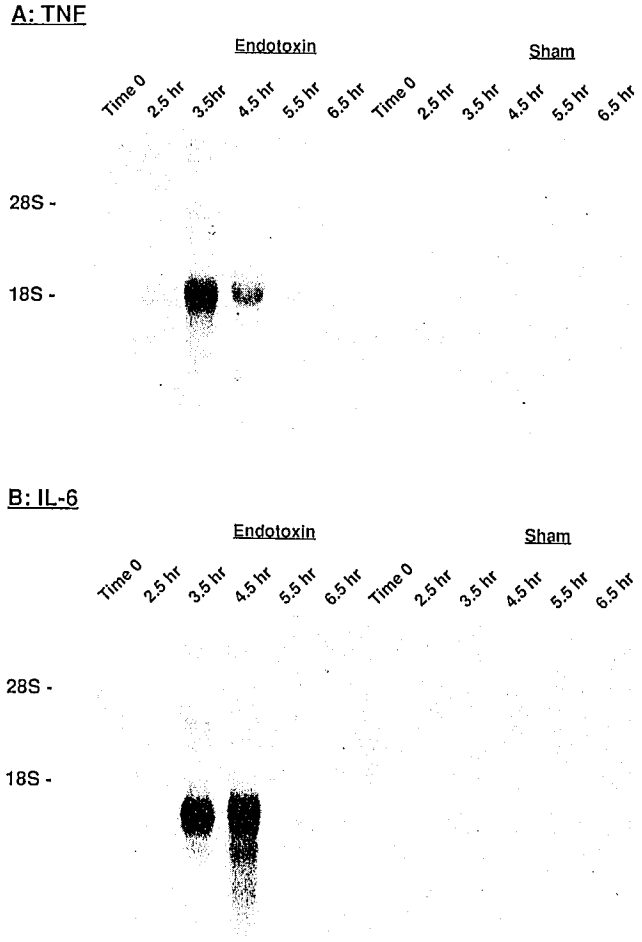

Fig. 7. mRNA expression of tumor necrosis factor (TNF, *A*) and interleukin-6 (IL-6, *B*) during surgical stress (time 0 and 2.5 h) and after either endotoxin or sham saline administration. This particular pig was the only one of seven accumulating any detectable TNF transcripts before endotoxin administration.

Critique of Methods

In vitro transcription rates were determined by nuclear runoff assay. Hepatic nuclei were isolated and allowed to incorporate radioactive RNA precursors directly into nascent RNA chains *in vitro*. In this assay, newly synthesized RNA chains initiated *in vivo* at the time of liver biopsy were completed *in vitro*. This technique has a number of advantages over other techniques, including the Northern blot hybridization used to measure hepatic cytokine mRNA levels, in that it measures only those genes being expressed at the moment of tissue sampling, and is extremely sensitive to small changes in gene expression, because previously formed mRNA still present in the cell is not measured. Although transcription is not the sole arbiter of protein synthesis and secretion, programmatic changes induced in cells by stress can be manifested by acute changes in transcription with or without pre- or posttranscriptional changes. Others report that increases in mRNA precede increases in the translation of acute phase genes.[8–11]

Finding a control gene constitutively expressed and not affected by the stimuli in our protocol to quantitatively normalize hybridization remains problematic. Neither actin[45,46] nor tubulin (unpublished results) appear to be appropriate for this control comparison in the presence of endotoxin. Although many others have historically used these two genes as "controls,"

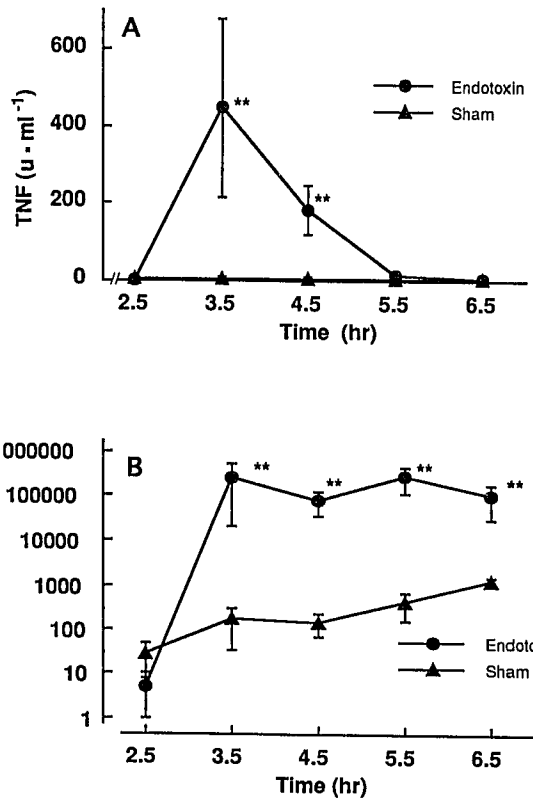

Fig. 8. Plasma concentration of tumor necrosis factor (TNF, *A*) and interleukin-6 (IL-6, *B*) after endotoxin or saline sham administration. IL-6 is shown on a logarithmic scale because of the four-order-of-magnitude increase in plasma concentration. Significant difference from baseline value before endotoxin or saline administration, **$P < 0.01$ by one-way ANOVA. Two-way ANOVA found significant differences between the saline sham and endotoxin pigs with respect to: TNF, $P < 0.01$, and IL-6, $P < 0.05$.

their mRNA accumulation may not be stable after exposure to endotoxin.

In pilot studies for our initial investigations using the nuclear runoff technique,[15] we observed consistent transcriptional activity from multiple samples obtained from a single liver at the same time. Implicit in our findings of highly consistent levels of transcriptional activity at time 0 in an n = 9 is demonstration of minimal variation in our measurement techniques.

The hepatic tissue sampled contained multiple types of hepatic and blood cells; it is technically impossible to both separate the cell types and perform the nuclear runoff assay without risk of inducing further (artifactual) alterations in gene expression. However, based on an extensive published literature of both *in vivo* and *in vitro* studies, it seems reasonable to assume that hepatocytes are the predominant site of expression of acute phase proteins, while the macrophage related Kupffer cells are predominantly responsible for expression of any cytokines.[4,37,47]

The importance of adequate separation of the biopsy sites should be emphasized. By definition, obtaining a sample of liver tissue for analysis causes tissue injury. Pilot studies showed that inadequate separation of the sites (<1 cm) resulted in a local induction of the expression of another gene associated with ischemia reperfusion injury,[15] which was not detected in biopsies from more remote (>3 cm) sites. So long as a full 3 cm of grossly undisturbed tissue separated biopsy sites, there was no change either in transcription or mRNA accumulation when compared with widely separated (*e.g.*, 15 cm) sites. Moreover, we noted that the induction of the acute phase transcription occurred at 2.5 h in three additional animals subjected to the anesthesia and surgical instrumentation in which we deliberately avoided the time 0 biopsy. These animals were not included in the statistical analysis, and were studied solely to exclude the small possibility that the time 0 biopsy may somehow have artifactually induced acute phase transcription.

IL-6 mRNA was not detected in hepatic tissue during anesthesia and surgical stress alone, and TNF mRNA was found, albeit in trace amount, in only one of seven pigs. However, both mRNAs accumulated massively and rapidly after endotoxin administration. Recently, IL-11 and Oncostatin M have been reported to induce acute phase proteins in both rat and human hepatoma cells or in primary cultured hepatocytes.[48,49] It is possible that these recently described cytokines, or extremely small amounts of local IL-6 not detected by our techniques, could be involved in induction of the APR.

In conclusion, the acute phase response is initially regulated at the transcriptional level at an early time during surgical stress. However, acute phase transcription occurred in the absence of increases in plasma TNF or IL-6 and in the absence of hepatic cytokine gene expression. In contrast, deliberate induction of TNF and IL-6 by endotoxin administration resulted in detectable hepatic transcripts and plasma levels of TNF and IL-6, but without further increase in acute phase transcription. Although we cannot exclude the possibility that amounts of TNF and IL-6 below the detection levels of our assays may play a role in surgical stress induced acute phase transcription, these data collectively suggest that the acute phase response may be triggered either by undetectable levels of circulating IL-6 or by TNF- and IL-6-independent mechanisms, and, moreover, that a superimposed cytokine stimulus does not cause an increase in acute phase transcription over and above simple surgical stress.

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