Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice

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Submitted 29 August 2011; accepted in final form 27 July 2012

Wu H, Chen Y, Winnall WR, Phillips DJ, Hedger MP. Acute regulation of activin A and its binding protein, follistatin, in serum and tissues following lipopolysaccharide treatment of adult male mice. Am J Physiol Regul Integr Comp Physiol 303: R665–R675, 2012. First published August 1, 2012; doi:10.1152/ajpregu.00478.2011.—Activin A, a member of the transforming growth factor-β family, increases in the circulation within 1 h after administration of bacterial LPS. To clarify the origins of this rapid increase, the distribution of activin A and its binding protein, follistatin, and their production following LPS treatment, were assessed in adult male mice. In untreated mice, activin A was detectable in all 23 tissues examined, with highest mRNA expression (as measured by quantitative RT-PCR) found in the liver, and the largest concentration of activin A protein (by ELISA) was found in the bone marrow. Likewise, follistatin mRNA and protein were present in all tissues, with highest expression in the vas deferens. Activin A and follistatin mRNA did not increase significantly in any tissue within the first hour after LPS, but activin A protein decreased by 35% in the bone marrow and increased 5-fold in the lung. No significant changes were observed in any other tissue. Activin A reached a peak in the circulation 1 h following LPS, and then declined. Cycloheximide, an inhibitor of protein translation, reduced this increase of activin A by more than 50%. Actinomycin D, an inhibitor of mRNA transcription, had no effect. Circulating follistatin did not increase until 4 h after LPS and was not affected by either inhibitor. These data indicate that the rapid increase in circulating activin A during LPS-induced inflammation is regulated at the posttranscriptional level, apparently from newly translated and stored protein, and implicate bone marrow-derived cells, and, in particular, neutrophils, as a significant source of this preformed activin A.

activin a; follistatin; tumor necrosis factor-α; lipopolysaccharide; inflammation

ACTIVIN A IS A DISULFIDE-LINKED homodimeric protein and a member of the transforming growth factor-β (TGF-β) superfamily of growth and development cytokines (10). It is widely produced and distributed in the body and has been shown to play critical roles in embryo development, liver function, reproduction, and immunoregulation. Activin A is also involved in inflammation, and it has been implicated in the progression of many inflammatory diseases, including rheumatoid arthritis, asthma, and meningitis (10). Serum and tissue concentrations of activin A increase during infection and inflammation, and elevated circulating levels of activin A are predictive of death in human and experimental models of septicemia (13, 20). Follistatin, which is structurally unrelated to the TGF-β superfamily, binds activin A with high affinity and blocks the ability of activin A to bind to its receptor and initiate downstream signaling (36). Animal studies show that injection of exogenous follistatin can improve survival follow-
stored protein or de novo synthesis. These data will be able to inform future studies on the role of activin A in inflammation, as well as the potential for follistatin to act as a specific therapeutic agent for controlling activin A actions in inflammation and its consequences.

MATERIALS AND METHODS

Animals

Adult male mice (8—10 wk old; 18—22 g) of the C57BL/6J strain were used in all studies. Procedures involving animals were conducted in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care of Scientific Purposes (1997) and were approved by the Monash Medical Centre Animal Ethics Committee.

Reagents

Solutions of actinomycin D, cycloheximide, and LPS (E. coli serotype 0127:B8) were freshly prepared at a final concentration of 1 mg/ml in 0.9% saline on the day of the experiment. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Tissue Distribution Study

Experiment 1. Five control mice were injected (i.p.) with saline alone (0.1 ml), and 5 mice were injected with 100 μg LPS in an equivalent volume of saline. Mice were euthanized 1 h after injection, and the following tissues were collected: flank skin, lymph nodes (pool of axillary, mediastenial, cervical, brachial, mesenteric, and inguinal nodes), hind-limb skeletal muscle, brain cortex, eye, lung, heart, thymus, liver, spleen, pancreas, small intestine, adrenal, kidney, bladder, vas deferens, testis, epididymis, seminal vesicle, ventral prostate, thyroid, and femoral bone. Bone marrow-derived cells were collected by syringe from the femurs by injection of PBS and centrifuged at 5,000 g for 5 min at 4°C. Blood was obtained by cardiac puncture and stored at 4°C overnight. The following day, the serum was collected by centrifugation at 5,000 g for 10 min at room temperature, then transferred to a fresh tube, and stored at [minus]20°C until assay. Tissues were stored at [minus]80°C before being processed for protein assays and mRNA measurement or were immersion-fixed in formalin or Bouin’s fixative (for testes only), processed on a Leica ASP300 vacuum processor (Leica, Solms, Germany) for embedding in paraffin, and sectioned (5 μm) using a Leica RM2135 microtome onto Superfrost Plus glass slides (Menzel-Glaser, Braunschweig, Germany). Whole bone samples were decalcified overnight in 20% formic acid prior to processing. Slides for staining were incubated in a 60°C oven for 20 min and stained with Harris hematoxylin and 1% aqueous eosin (Amber Scientific, Midvale, Western Australia) before coverslipping with Neo-mount (Merck, Darmstadt, Germany).

Experiment 2. Seven control mice were injected intraperitoneally with saline alone (0.1 ml), and 7 mice were injected with 100 μg ip LPS in an equivalent volume of saline. Mice were euthanized 1 h after injection, and the bone marrow-derived cells and liver were collected, as in experiment 1. Blood obtained by cardiac puncture was collected into 1.5-ml Eppendorf tubes containing 15 U heparin and centrifuged at 3,000 g for 10 min at room temperature. The plasma fraction and leukocytes were collected by careful aspiration. All tissues and cells were stored at [minus]80°C until processed for protein assays and mRNA measurement.

Regulation of Activin A and Follistatin by LPS

Experiment 3. Mice were divided into the following treatment groups: injection of 100 μg ip LPS alone (6 mice/time point), injection of cycloheximide (5 mg/kg ip) 60 min prior to an injection of 100 μg ip LPS (6 mice/time point), injection of actinomycin D (5 mg/kg ip), 60 min prior to an injection of 100 μg LPS (4 mice/time point), or 0.1 ml saline alone (4 mice/time point). The concentrations of cycloheximide and actinomycin D used were based on those used by Zhang et al. (45). Blood and liver samples were collected at 0, 0.5, 1, 2, 3, 5, and 7 h after injection of LPS or saline (with the 0-h group acting as control), as described in experiment 1. Mice were monitored throughout the experimental procedure, and mice exhibiting evidence of severe distress were euthanized. As a result, in the actinomycin D with LPS treatment groups, two of the mice at 5 h and all of the mice at 7 h were removed from the experiment.

Experiment 4. Mice were injected intraperitoneally with saline alone or 100 μg ip LPS, with or without a prior injection of cycloheximide (5 mg/kg) 60 min earlier (6 mice/treatment group). Bone marrow-derived cells and serum were collected and processed for assay, as described in experiment 1.

Tissue Extraction for Total Protein Quantification

Tissue and cell samples were homogenized in PBS containing protease inhibitors (EMD Biosciences, La Jolla, CA). After homogenization, samples were centrifuged at 10,000 g for 10 min at 4°C, and the supernatants were collected. Total protein was measured in the extracts using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), as per the manufacturer’s instruction. The sensitivity of the assay was 1 μg/ml.

Immunosassays

Activin A was measured by a two-site ELISA (Oxford Bioservices, Cherwell, Oxfordshire, UK), using human recombinant activin A as a standard (17). This assay measures both free and follistatin-bound activin A dimers and has no significant cross reaction with other isoforms of activin or TGF-β. The mean assay sensitivity was 12 pg/ml. The mean intra-assay and inter-assay coefficients of variation (CVs) were 5.0% and 6.7%, respectively.

Follistatin was measured by RIA, using human recombinant follistatin 288 as standard and tracer, as described previously (24). This assay measures total follistatin. The mean assay sensitivity was 0.85 ng/ml. The mean intra-assay and inter-assay CVs were 12.1% and 5.0%, respectively.

TNF-α was measured by a Quantikine mouse TNF-α ELISA (R&D Systems, Minneapolis, MN), using mouse recombinant TNF-α as a standard (28). The sensitivity of the assay was 12 pg/ml. The mean intra-assay and inter-assay CVs were 6.8% and 8.4%, respectively.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR

Tissue samples were processed and assessed for mRNA expression levels using established methods, which have been described in detail previously (6, 41). All tissues for mRNA measurements were stored at [minus]80°C. Total RNA was extracted and purified from homogenized tissue by lysis in 1 ml TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. After extraction, RNA solutions were treated with DNase to remove genomic DNA using a DNA-free kit (Ambion, Austin, TX). All data in each experiment were determined from samples of RNA extracted at exactly the same time under identical conditions to eliminate variation in mRNA extraction efficiency as a result of procedural differences.

Oligo-dT-primed cDNA was synthesized from 1 μg of RNA using the Superscript kit (Invitrogen). The amount of mRNA used to make cDNA was equalized for all tissues to minimize errors due to differences arising from small amounts of starting mRNA. Sample cDNAs were diluted 1:10 in diethylpyrocarbonate-treated water immediately prior to PCR reactions, and 2 μl of this diluted cDNA was added to each reaction. For each tissue cDNA, a nonreverse-transcribed control was produced to act as an internal negative control. A phagemid-
derived RNA “spike” was added to each reaction as an external standard control, as previously described (6, 41). Gene expression was measured by quantitative PCR (qPCR) using a Bio-Rad iQ5 system (Bio-Rad, Hercules, CA) and the Fast-Start DNA Master SYBR-Green system (Roche, Mannheim, Germany). The primers for the activin βA-subunit were 5'-TGGAGTGTGATGGCAAGGTC-3' (forward) and 5'-AGCCACACTCCTCCACA-TC-3' (reverse); for total follistatin (i.e., to detect both Fst288 and Fst315 variant mRNA), the primers were 5'-CCACTTGTGTGGTAGTGATCAG-3' (forward) and 5'-AGCTTCCTTCATGGCACACT-3' (reverse); for IL-1β, the primers were 5'-CCAGGATGAGGACCAGAGACCAAGCA-3' (forward) and 5'-CCCGACCATTGCTGTTC-3' (reverse); and for TNF-α, the primers were 5'-TCATCGTTC-TATGCGCCAGAC-3' (forward) and 5'-CCCTTGGAAGAGACCTGGGAGT-3' (reverse). In addition to a blank negative control and nonreverse-transcribed cDNA negative control, the external standard RNA spike was used to normalize reaction efficiency, to avoid the variability of other housekeeping genes from different tissues (6). This spike was detected at the same level in each tissue, and all data were analyzed by relative quantitation to the RNA spike, using the $2^{-ΔΔCt}$ method. The efficiency of each reaction was between 87% and 110%, and the patterns of gene expression were highly reproducible. Data are presented as relative expression compared with liver as the reference tissue.

Fig. 1. Tissue distribution of activin βA-subunit mRNA, activin A, follistatin, and activin A:follistatin ratio in untreated mice. A: mean threshold copy number for activin A mRNA measured by quantitative PCR (mean ± SD; n = 5 samples) in multiple runs. Threshold copy number is inversely proportional to mRNA concentration in the tissue, which means that the highest threshold copy number indicates the lowest amount of mRNA. B: activin A levels in tissues relative to total protein (mean ± SD; n = 3). C: follistatin levels in tissues relative to total protein (mean ± SD; n = 3). D: ratio of activin A to follistatin in tissues, as an indicator of relative levels of free activin A. Note that the ratio value is based on immunoactivity estimates, and not absolute mass values, for activin A and follistatin. a,b,c,dValues with letters that differ are significantly different.
**Immunohistochemistry for Activin A**

Activin A immunohistochemistry was performed on cytospin preparations of bone marrow-derived cells and 5-μm paraffin sections of lung tissue and decalcified whole bone, as previously described (4, 25). A mouse monoclonal antibody (E4) directed toward the βA subunit of activin A was used as a primary antibody (17). An isotype-matched mouse antibody (IgG2b) was used as a negative control.

**Statistical Analyses**

One-way and two-way ANOVA were used to determine significant differences between groups, following suitable transformation to normalize the data and equalize variance, in conjunction with a Dunnett’s multiple comparisons of means test (Prism 5, GraphPad Software, San Diego, CA). All values are presented as mean ± SD.

**RESULTS**

**Tissue Distribution of Activin A and Follistatin in Adult Male Mice**

Activin βA-subunit mRNA was detectable in every tissue examined by qPCR in normal control mice (Fig. 1A). It was not possible to assay all tissues in a single qPCR run for direct quantitative comparison; however, multiple qPCR analyses of the mean crossing threshold (Ct) for each tissue, which is inversely related to the amount of mRNA present, indicated that the liver had the highest βA-subunit mRNA expression (i.e., lowest threshold crossing point; 22.6 ± 0.9; n = 5), while the seminal vesicles and prostate had the lowest βA-subunit mRNA expression (i.e., highest threshold crossing points; 35.4 ± 4.5 and 37.3 ± 3.2, respectively) (Fig. 1A). The thyroid

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**Fig. 2. Comparison of mRNA expression levels in liver, vas deferens, testis, bone marrow, prostate, seminal vesicles, and lung of control mice and mice injected with LPS 1 h previously. A and B: activin βA-subunit. C and D: follistatin. E and F: TNF-α: left panels are representative qPCR comparisons incorporating the liver (highest activin A mRNA expression) and seminal vesicles (lowest activin A mRNA expression), the male reproductive tract tissues, and bone marrow-derived cells, measured in a single qPCR run (y-axis is linear). Right panels are a direct qPCR comparison of liver and lung, measured in a single qPCR run (y-axis is log10). All data are presented as means ± SD; n = 5 samples, except prostate (n = 7). There was no significant difference between control and LPS treatment expression levels for either activin βA-subunit or follistatin, and tissues with same letter superscript were not significantly different by two-way ANOVA. NS, not significantly different from control at P > 0.05. Comparisons for TNF-α between control and LPS-treatment expression levels: *P < 0.05, **P < 0.01, ***P < 0.001 compared with equivalent tissue control group.**
tissues during a single qPCR run established that there was a reference standard tissue for all subsequent analyses. Comparing relative mRNA concentrations in a selection of tissues during a single qPCR run established that there was a 180-fold difference in βA-subunit gene expression between the liver (highest expression, lowest Ct) and the seminal vesicles (lowest expression, highest Ct) in control mice and that all other tissue expression levels fell between these limits (Fig. 2, A and B). In spite of these large differences in βA-subunit mRNA expression, there was no significant difference between activin A protein concentrations in most tissue extracts, with the exception of bone marrow-derived cells, which contained activin A at levels at least 10-fold higher than the majority of other tissues in control animals (Fig. 1B). Levels of activin A in thyroid and total blood leukocyte samples were below the limit of assay detection (<0.005 ng/mg protein).

Follistatin mRNA was observed in all tissues but displayed lower levels and less between-tissue variation of expression compared with the activin βA-subunit mRNA (Ct range: 27.7−38.6). In control mice, the vasa deferens showed the highest follistatin mRNA expression, which was at least 10 to 15 times higher than any other tissue examined (Fig. 2, C and D). The concentration of follistatin protein was similar in most tissues, with the exception of the vas deferens and prostate, which had significantly higher levels of follistatin protein than all other tissues, with the exception of the skin and seminal vesicle (Fig. 1C). When activin A levels were expressed as a ratio of activin A:follistatin, as an index of activin A bioavailability in the tissue, bone marrow-derived cell activin A was still seen to be considerably higher (∼10-fold) than in all other tissues (Fig. 1D).

These data demonstrated that, in spite of a wide range of βA-subunit mRNA expression, activin A protein was relatively evenly distributed in most tissues, together with its binding protein, follistatin. The clear exception was the bone marrow-derived cells, where both total activin A and the activin A/follistatin ratio were much higher than in all other tissues, even though these cells did not display exceptionally high βA-subunit mRNA expression.

### Tissue Responses to LPS Administration

There was no significant increase in either activin βA-subunit mRNA (Fig. 2, A and B) or follistatin mRNA (Fig. 2, C and D) 1 h following LPS administration, in any of the tissues or isolated cell preparations (total blood leukocytes and bone marrow-derived cells) examined. In contrast, after 1 h of LPS stimulation, expression of TNF-α (Fig. 2, E and F) and IL-1β (data not shown), mRNA was significantly increased in all tissues and cell preparations.

One hour after LPS administration, the lung samples showed a five-fold increase in activin A protein concentration and the activin A/follistatin ratio, while the bone marrow cells showed a 35% decrease in content (Fig. 3, A and C). There was no change in activin A concentrations 1 h after LPS treatment in any other tissue or in total blood leukocytes. Follistatin protein was slightly increased in lymph node, but not in any other tissue, 1 h after LPS treatment (Fig. 3B).

These data indicate levels of activin A and follistatin do not change in most tissues within 1 h of LPS administration, consistent with the lack of change in mRNA; however, there is a significant decline in activin A in the bone marrow and a corresponding increase in the lung. This suggests that there may be a net transfer of activin A from the bone marrow to the lungs within 1 h after LPS treatment.

### Regulation of Activin A and Follistatin in Serum

Following LPS administration, serum activin A increased rapidly in the circulation and reached a peak concentration at 1 h, then gradually declined, but was still elevated 7 h later.
Follistatin also increased in the circulation after LPS, but this occurred much later, reaching a peak concentration at 5 h (Fig. 4, C and D). The release of TNF-α into the circulation coincided with the release of activin A but declined much more rapidly (Fig. 4, E and F).

Injection of the protein synthesis inhibitor, cycloheximide (5 mg/kg), 1 h before LPS reduced activin A concentrations in the circulation by more than 50% (Fig. 4A), had no effect on follistatin (Fig. 4C), and delayed the peak release of TNF-α by 1 h without changing the apparent size of the peak (Fig. 4E). Administration of cycloheximide alone to mice was followed by a declining trend (i.e., not statistically significant) in activin A concentrations in the blood (Fig. 4G) and had no effect on either follistatin or TNF-α levels over 7 h (data not shown). Cycloheximide had no additional effect on the decline in activin A in bone marrow-derived cells 1 h after LPS treatment but reduced activin A concentrations by more than 60% in bone marrow-derived cells from control mice (Fig. 5).

Injection of actinomycin D (5 mg/kg), an mRNA transcription inhibitor, had no significant effect on the initial rise in activin A after LPS treatment but caused a gradual increase in activin A in the blood 3 h after LPS treatment (Fig. 4B).
Actinomycin D had no effect on follistatin release after LPS (Fig. 4D) but caused both a delay and an increase in the duration of the peak of TNF-α/H9251 after LPS (Fig. 4F). Administration of actinomycin D alone to mice had no effect on activin A (Fig. 4H), follistatin, or TNF-α (data not shown) within 5 h.

Although activin A-subunit gene expression in the liver was not stimulated in the first hour after LPS administration, there was a significant decline in expression in the liver (tissue with highest level of activin A mRNA) by 4 h after LPS treatment (Fig. 6A). In contrast with activin A, follistatin mRNA increased gradually after LPS treatment (Fig. 6B), and TNF-α mRNA expression was rapidly induced by LPS (Fig. 6C).

Cycloheximide caused a transient increase in the expression of all three genes following LPS treatment (Fig. 6, A-C). Actinomycin D had no effect on activin βA-subunit expression but blocked the induction of follistatin and TNF-α mRNA by LPS (Fig. 6, B and C).

The fact that the rapid release of activin A into the blood following LPS administration could be substantially blocked by cotreatment with the protein synthesis inhibitor (cycloheximide), but not by an inhibitor of mRNA transcription (actinomycin D), was consistent with the observation that LPS had no acute stimulatory effect on activin βA-subunit mRNA expression in any tissue. Altogether, these data indicate that activin A levels in the serum are regulated at the posttranscriptional level under both basal and LPS-stimulation conditions. On the other hand, both follistatin and TNF-α appear to be primarily regulated by LPS at the transcriptional level, under the same conditions, but over a different time frame.

**Immunohistochemical Localization of Activin A in Lung and Bone Marrow**

Immunohistochemical examination of the bone marrow cells collected from control mice indicated that the only cells containing significant activin A protein were the neutrophil precursors (band neutrophils, segmented neutrophils, and ring-form myelocytes) (Fig. 7A). Activin A tended to be perinuclear in its distribution in these isolated cells. Following LPS treatment, the number of neutrophil precursors in bone marrow samples declined from 46 ± 3% of total cells (in control mice, n = 4) to 19 ± 2% of total cells (in LPS-treated mice, n = 4), although many mononuclear cells in these samples now contained activin A protein as well (Fig. 7B). In the lungs from control mice, activin A was present in the broncheolar and alveolar epithelial cells and alveolar macrophages (Fig. 7D). After LPS treatment, this pattern of staining persisted, but...
numerous neutrophils accumulated in the lungs, and these cells were also strongly activin A positive (Fig. 7E).

In sections of intact bone from normal mice, approximately half of the cells in the bone marrow were positive for activin A (Fig. 8A). The majority of these activin A-containing cells possessed the distinctive polymorphonuclear morphology of bone marrow neutrophil precursors (band neutrophils, segmented neutrophils, and some ring-form myelocytes), but labeled megakaryocytes, osteoclasts, chondrocytes, endothelial cells, barrier cells, and bone-lining cells were also observed. By contrast, the majority of mononuclear cells within the bone marrow, which includes the mononuclear myelocytes, rubricytes, and lymphocytes, were activin A-negative, although small numbers of labeled mononuclear cells were observed. After treatment with LPS, the number of activin A-positive cells in the bone marrow declined dramatically (to around 20% of total cells), and the majority of the remaining activin A-containing cells were of mononuclear cell morphology (Fig. 8B). These observations are indicative of a loss of activin A from the bone marrow due to extravasation of neutrophils containing prestored activin A after LPS treatment but are also consistent with release of activin A stored in other bone cell types as well.

Altogether, these data suggest that a significant proportion of the increase in activin A protein levels in the lungs following LPS administration may be due to translocation of neutrophils containing preformed activin A from the bone marrow. Accordingly, the bone marrow shows a reduction in activin A content concurrent with the reduction in neutrophil precursors in this tissue.

DISCUSSION

The major aim of this study was to investigate the source and regulation of activin A and its binding protein, follistatin, during acute LPS-induced inflammation. The results of the distribution of activin A mRNA and protein across 23 tissues and cell preparations in the adult male mouse show that, under normal conditions, the liver has the highest mRNA expression, but contains relatively little activin A protein, while bone marrow cells have the highest endogenous protein levels, but relatively low mRNA expression. Thus, there seems to be no close correlation between the putative sites of production and actual tissue content, suggesting that activin A is subject to a dynamic pattern of secretion and storage. Further complicating the issue is the fact that follistatin is likewise widely distributed. At least some of the activin A in some of the tissues may be assumed to be already bound to follistatin, either in solution or bound to cell-surface proteoglycans (36). When all tissues are considered, most tissues show a similar ratio of activin A to follistatin, with the notable exception of the bone marrow cells, which show at least a five-fold higher activin A:follistatin ratio than other tissues. This implicates the bone marrow as a major potential source of stored free activin A (i.e., unbound to follistatin) in the normal adult mouse. The inverse implication, that a significant proportion of the activin A present in other
tissues is actually bound to follistatin, and, hence, may be inactive, also merits consideration.

In contrast to the large response of other rapidly induced inflammatory cytokines, in this case TNF-α and IL-1β, there was no significant increase of activin A mRNA levels in any of the tissues examined within 1 h following LPS challenge. Large variability in mRNA levels is a characteristic of qPCR studies, particularly when dealing with genes that are expressed at low levels, such as activin A, and undoubtedly reflects real differences in mRNA in the source tissues. In the case of the bone marrow and liver, the wide variation and subsequent overlap in mRNA levels in controls and treated samples make it doubtful that these represent meaningful increases or decreases. This was in spite of the fact that serum activin A levels increased at least four-fold at the same time, suggesting that the activin A protein response to LPS stimulation occurs post-mRNA production. This hypothesis was supported by the observation that an inhibitor of mRNA synthesis, actinomycin D, had no significant inhibitory effect on activin A levels in serum after LPS treatment, at a dose that inhibited the increase in both TNF-α and IL-1β.

Post-mRNA regulation could involve new protein translation from the preexisting mRNA in tissues with high levels of endogenous mRNA expression, such as the liver. The other potential source could be the release of prestored activin A protein from cells and tissues. The observation that the protein synthesis inhibitor, cycloheximide, reduced activin A levels following LPS treatment by about 50%, suggest that both mechanisms may be involved. Among the tissues examined, only bone marrow cells, which had the highest activin A levels under normal conditions, had reduced activin A protein 1 h after LPS injection. The only other site that showed a significant change in activin A protein in the first hour after LPS treatment was the lung, with a five-fold increase. These observations suggest that bone marrow could be a major source of released prestored activin A in the serum and in the lungs during acute inflammation induced by LPS, although less significant release of activin A from other tissues as well cannot be excluded.

The failure of activin A gene expression to be immediately up-regulated by inflammatory stimuli has been observed before, for example, in endothelial cells and testicular Sertoli cells (26, 40). This is probably related to the fact that, unlike other rapid response inflammatory cytokines, such as TNF-α and IL-1β, the activin A promoter lacks a response element for the crucial proinflammatory transcription factor, NF-κB, and is more responsive to stress-related signaling pathways, activated via the MAP kinases, with potentially greater latency (10). On the other hand, LPS was able to induce activin A mRNA expression in the liver within 1 h in the presence of cycloheximide, a treatment that also enhanced TNF-α and follistatin expression in response to LPS, implying that protein synthesis-dependent inhibitory regulation may also be involved. Furthermore, the importance of inhibitory mechanisms in control of activin A gene expression is also indicated by the observation that activin A mRNA in the liver actually decreased at 4–5 h after LPS treatment.

The regulation of follistatin was addressed only indirectly in the present study. It has already been established that follistatin is stimulated by LPS and inflammatory cytokines, including TNF-α, IL-1β, and activin A itself, over a much longer time frame than is activin A (13, 15, 29, 40). Accordingly, a significant increase in follistatin mRNA in the liver was first observed at ~5 h in the present study, at the same time as serum follistatin levels increased. However, the data were equivocal, given that neither cycloheximide nor actinomycin D had any inhibitory effect on follistatin levels in the serum following LPS, suggesting that stored protein, rather than new protein synthesis, was largely responsible.

Although actinomycin D alone had no affect on the acute release of activin A or follistatin, it did cause a prolonged release of both activin A and TNF-α from 3 h after LPS until the end of the experiment. This may be attributed to the fact that, in addition to being an mRNA transcription inhibitor, actinomycin D is also able to stabilize preexisting, long-lived
mRNA species, such as activin A mRNA, thereby enhancing protein production from these mRNA (5, 32). Previous studies have reported that activin A is produced by a number of different bone marrow cells, even under normal conditions, including osteoclasts (11), chondrocytes (44), “monocytoïd” cells (39), and stromal or “fibroblastoid” cells (35, 43). Furthermore, blood-derived neutrophils are a source of activin A in humans (4). On the basis of the immunostaining in the present study, it appears that about half of the cells in murine bone marrow express activin A under normal conditions and that labeling is particularly evident in the various neutrophil precursors, megakaryocytes, osteoclasts, and the endothelial/fibroblastic cells that line the bone, vasculature, and cell colonies. One hour after an LPS challenge, activin A-containing cells and neutrophil precursors in the bone marrow were greatly reduced in number, and activin A-containing neutrophils appeared throughout the lung. Preferential translocation of neutrophils from the bone marrow to the lungs is a characteristic feature of LPS-induced inflammation and is responsible for lung damage in such models (1, 3, 31, 33). Neutrophils previously have been implicated as a source of activin A in lung inflammation following allergen challenge in humans (14). At least part of the increase in activin A in the blood and in tissues where neutrophils become resident following LPS, most notably the lungs, may be attributed to activin A from these bone marrow cells, in particular. This observation is consistent with accumulating evidence that activin A plays a central role in inflammatory lung damage in various models (2, 9, 18). This does not mean that neutrophils alone are responsible for the increase in activin A following LPS treatment. Release of activin A from other bone marrow cells, such as the stromal cells, also appears to occur. Moreover, it can be expected that cells in other tissues, such as epithelial cells in the lungs and hepatocytes and hepatic stellate cells in the liver (27), for example, may also contribute to this increase.

These data provide novel quantitative information about the sites of production of activin A and follistatin in the mouse. The data indicate that the liver may be the major site of activin A production (i.e., synthesis as opposed to content) under normal conditions in the adult male mouse, in contrast to previous studies in the rat and mouse in which liver expression was reported to be relatively low (19, 34). Another notable observation was that follistatin protein concentrations are normally elevated in tissues of the male reproductive tract, most notably in the vas deferens and prostate. The vas deferens also appears to occur. More-


