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Constitutive Repression of Interleukin-1 α in Endothelial Cells

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Abstract

Activation of complement stimulates inflammation and provides an initial vigorous defense against infection. Insertion of the membrane attack complex in cell membranes of vascular endothelial cells induces changes in cell differentiation that promote coagulation, thrombosis, inflammation, and immunity. These changes are mediated by production of interleukin (IL)-1 α by endothelial cells, which acts locally on endothelial cells to contain infection and promote healing of the affected site. In healthy tissues, however, promoting coagulation and inflammation would be dysphysiologic. Accordingly, endothelial cell activation by the membrane attack complex depends on both transcriptional regulation of IL-1 α and availability of that cytokine to broadly modify endothelial cell physiology. Here, we report that the IL-1 α gene contains a suppressor sequence that cooperates with histone modification to regulate production of IL-1 α by endothelial cells. The suppressor sequence binds C/EBP (CCAAT enhancer-binding protein) family DNA-binding proteins isolated from the nucleus of quiescent endothelial cells. These results suggest constitutive suppression of IL-1 α maintains quiescence of endothelium and that terminal complement complexes remove that suppression, allowing IL-1 α transcription and, ultimately, activation of endothelium to proceed.

Keywords

calcineurin; complement; endothelium; histone deacetylase; inflammation; interleukin-1

Quiescent endothelial cells actively maintain the form and function of blood vessels. For example, quiescent endothelial cells promote angiogenesis by synthesizing growth factors such as vascular endothelial growth factor and platelet-derived growth factor¹ and control angiogenesis by producing inhibitors of cellular growth and migration, such as heparan sulfate² and nitric oxide³ (reviewed elsewhere^{4,5}). Endothelial cells preserve blood fluidity and prevent thrombosis in conditions of health by presenting a nonadhesive and anticoagulant surface^{6,7} and relaxing vascular smooth muscle.⁸ This antiinflammatory, anticoagulant, prosuffusing posture maintains blood and tissue homeostasis.

Endothelial cells also sense injury and infection. Thus, endothelial cells express receptors for endotoxin,⁹ thrombin,¹⁰ and inflammatory cytokines such as interleukin (IL)-1.^{11–13} Stimulation of these receptors by corresponding agonists causes changes in endothelial cells that promote inflammation,^{14–16} coagulation,^{14,17,18} and diminished blood flow.¹⁹ This response has been called endothelial cell activation.^{20,21}

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Ideally, endothelial cells should coordinate responses to local and systemic inflammatory mediators for which they bear receptors. For example, endothelial cells respond to endotoxin via Toll-like receptor 4²² and to cytokines by activating latent cytoplasmic nuclear factor (NF)- κ B transcription factors, thus initiating a global nuclear factor NF- κ B-driven proinflammatory gene transcription program. In this way, receptor-mediated activation of endothelial cells begins an inflammatory paradigm that includes transcription of more than 100 genes involved in acute and chronic inflammation,²³ including inflammatory mediators such as adhesion molecules and chemokines,^{14–16} procoagulant molecules such as plasminogen activator inhibitor-1,¹⁸ vasoconstrictors such as thromboxane A₂,¹⁹ and production of IL-1 α .^{13,20,22,24–26}

Activation of complement on endothelial cells can change endothelial cell physiology in ways similar to receptor-mediated activation.¹⁷ Activation of complement may occur spontaneously²⁷ or in the context of disease or tissue trauma.^{28–30} The fragments of C3 and C5 (C3a and C5a) activate G protein-coupled receptors (C3aR, C5aR) and promote fusion of Weibel-Palade bodies with cell membranes.³¹ Externalization of Weibel-Palade bodies promotes adhesion of leukocytes but does not activate endothelial cells, as commonly understood.³² Insertion of C5b678 complexes in endothelial cell membranes promotes formation of polymeric C9, the membrane attack complex, which can activate endothelial cells fully. However, by itself, the membrane attack complex does not stimulate the broad range of changes described above but, rather, stimulates only the production of IL-1 α , which may act on endothelium in an autocrine manner, evoking the broader manifestations of receptor-mediated activation including production of procoagulant, vasoactive, and inflammatory mediators typical of activated endothelium.^{17,33} Consistent with this concept, inhibiting the function of IL-1 α prevents activation of endothelial cells by the membrane attack complex.³⁴ On the other hand, if IL-1 α is carried away by free-flowing blood, the broader range of changes may not occur.³³

How the membrane attack complex selectively activates IL-1 α expression in endothelial cells is incompletely understood. The membrane attack complex acts via calcineurin to activate NK- κ B-dependent transcription of the IL-1 α gene, whereas cytokines and other agonists function independently of calcineurin.³⁵ Still unclear is how transcription of IL-1 α is differentially controlled. Because acetylation of histones controls transcription of other inflammatory cytokines,^{36–38} we asked whether histone acetylation may regulate transcription of IL-1 α . We report here that the IL-1 α gene contains a suppressor sequence that cooperates with histone modification to regulate production of IL-1 α and thus governs endothelial cell response to complement.

Materials and Methods

Materials

Human serum was used as a source of complement.^{17,34} Recombinant tumor necrosis factor (TNF) α and antibodies against IL-1 α were from R&D Systems (Minneapolis, Minn). FK506 and trichostatin A were from Calbiochem (La Jolla, Calif). Antibodies to CCAAT enhancer-binding proteins (C/EBPs) were from Santa Cruz Biotechnology (Santa Cruz, Calif).

Endothelial Cell Culture and Analysis of IL-1 α mRNA

Porcine aortic endothelial cells (passages 4 to 8) were cultured as described.³⁴ Human aortic endothelial cells were from Cambrex Bio-Science Walkersville Inc (Santa Rosa, Calif) and cultured as suggested by the supplier. Total RNA from confluent cultures of endothelial cells was isolated by the guanidinium thiocyanate method. RT-PCR was performed as described previously.³⁴ The identities of the PCR products were confirmed by sequencing

(data not shown). To compare relative levels of various mRNA to GAPDH mRNA, a semiquantitative RT-PCR strategy was used.¹⁷ Sequences of oligonucleotides for PCR were as follows:

- Human IL-1 α : CCAGGCGTAGGTCTGGAGTCTCACTTGTCT and TGTTGCGGCAGGAAGGCTTAGGTATTATTC
- Human GAPDH: CATGCCATCACTGCCACCCAGAAGACTGTG and GAAATGAGCTTGACAAAGTGGTCGTTGAGG
- Porcine IL-1 α : CGGGAAGATTCTGAAGAAGAGACGGTTGAG and TGGGCGGCTGATTTGAAGTAGTCCATATTG
- Porcine GAPDH: CCGCGTCCCTGAGACACGATGGTGAAGGTC and TTCAAGTGAGCCCCAGCCTTCTCCATGGTC

The amplified products were fractionated on a 1.2% agarose–Tris/boric acid/EDTA (TBE) gel and visualized by staining with ethidium bromide. The resulting bands were quantitated using the Gel Doc 2000 and Quantity One software (Bio-Rad, Hercules, Calif).

Construction of IL-1 α Luciferase Reporter Plasmids, Transfection, and Luciferase Assay

The porcine IL-1 α promoter region was cloned from liver genomic library prepared in Lambda FIX II vector (Stratagene, La Jolla, Calif). The 5' noncoding region of IL-1 α encompassing a 3.25-kb DNA fragment was sequenced. The human IL-1 α promoter containing 156 bp upstream of the IL-1 α transcription start site and the repressor region was isolated from genomic DNA from human aortic endothelial cells. The DNA region was amplified using AGTTTTAGCCAGTATCGAGTTGAATGAACATAGAA and AAGCCTGAGTCAGTCTTCTTCGCCTTTTGTAATTG or AGTTTTAGCCAGTATCGAGTTGAATGAACATAGAA and AACTAGTTTCCTATGAGGGCATGGGTGAATACAACACTGA oligonucleotides (GenBank accession no. X03833.1). The PCR product was isolated and sequenced.

Human and porcine IL-1 α promoter regions, point mutants, and deletions were ligated to pGL3 basic luciferase reporter vector (Promega). Transient transfections of endothelial cells on 24-well plates at 40% to 50% confluence were performed using 0.5 μ g of reporter plasmid and 0.1 μ g of *Renilla* luciferase control reporter plasmid (pRL-TK; Promega, Madison, Wis) and Superfect transfection reagent (Qiagen, Valencia, Calif). Luciferase activity was assayed using the Dual-Luciferase Reporter Assay system (Promega) and a TD-20/20 Luminometer (Turner Designs, Sunnyvale, Calif).

Electrophoretic Mobility-Shift Assays

Proteins were extracted from cell nuclei of confluent cultures of porcine or human endothelial cells as described.³⁹ Nuclear protein content was measured using Bio-Rad Protein Assay (Bio-Rad).

All oligonucleotides were from the Mayo Clinic Molecular Biology Core Facility. Probes were prepared by end-labeling with [γ ³²P]ATP (Amersham Bioscience) and T4 polynucleotide kinase (New England Biolabs Inc) and purified using G-25 Sephadex Quik Spin Column (Roche Diagnostics, Indianapolis, Ind).

In a typical electrophoretic mobility-shift assay (EMSA) reaction, nuclear extract (4 to 5 μ g) and labeled probes (0.25 pmol per reaction) were incubated on ice for 15 minutes, as described.³⁹ For antibody supershift experiments, 1 μ L of various antibodies was included. Protein–DNA complexes were resolved on 4% nondenaturing polyacrylamide gels. The gels were dried and subjected to autoradiography.

Results

Complement Activates IL-1 α Gene Transcription

We previously found that the membrane attack complex of complement, and other pore-forming proteins, activate endothelial cells by calcium- and calcineurin-dependent production and secretion of IL-1 α .^{17,34,35} Generation of the membrane attack complex of complement is specifically required because serum depleted of complement component C8, or serum that has been heated to inactivate complement, does not activate endothelial cells.¹⁷ Thus, as Figure 1A shows, activation of complement on cultured porcine aortic endothelial cells stimulates expression of IL-1 α mRNA. Cells treated with the calcineurin inhibitor FK506 express significantly less IL-1 α mRNA in response to complement than cells not treated with the drug, suggesting calcineurin-dependent signals stimulate IL-1 α transcription.³⁵

Histone Acetylation Regulates IL-1 α Gene Expression

Epigenetic modification of chromosome structure regulates transcription of the IL-1 α gene in CD4⁺ T cells⁴⁰ and promotes activation of inflammatory genes such as IL-6 and TNF α in human blood monocytes³⁶ and mouse microglia.³⁷ Because epigenetic modifications that regulate gene expression also operate in endothelium,⁴¹ we asked whether acetylation of histones regulates transcription of IL-1 α in endothelial cells. We investigated porcine endothelial cells because we determined previously that these cells, like human endothelial cells, respond to complement and other pore-forming proteins by regulating the availability of IL-1 α .^{17,34,35,42,43} We cultured porcine aortic endothelial cells with trichostatin A, an inhibitor of histone deacetylase, and then tested for expression of IL-1 α by RT-PCR. Endothelial cells cultured with trichostatin A had increased expression of IL-1 α mRNA within 2 hours of adding the inhibitor (Figure 1B) and reached levels 40-fold higher than untreated cells in 20 hours. IL-1 α protein was secreted by the endothelial cells treated with trichostatin A, as detected by immunoblot analysis of cell culture supernatants using anti-porcine IL-1 α -specific antibodies (Figure 1C).

Identification of a Novel IL-1 α Gene Suppressor Element

Cytokines, chemokines, and complement stimulate IL-1 α transcription by activating nuclear transport of NF- κ B.^{23,35} Because inhibition of histone deacetylase stimulated IL-1 α production without addition of an NF- κ B agonist, we reasoned that changes in chromatin structure imparted by histone acetylation may diminish the activity of a constitutive suppressor of IL-1 α gene transcription. To determine whether a *cis*-acting element suppresses the IL-1 α gene, we cloned the porcine IL-1 α gene promoter, along with the contiguous 5' and 3' regions, into luciferase reporter plasmids and tested for suppressor function. Reporter plasmids containing the firefly luciferase gene regulated by various regions of the IL-1 α gene promoter were transfected into porcine aortic endothelial cells, and the production of luciferase was assayed 36 hours later. Figure 2A shows a schematic representation of the promoter region and the first 2 exons of the porcine IL-1 α gene.^{44,45} (See Figure I in the online data supplement [<http://circres.ahajournals.org>] for the complete nucleotide sequence of this region of the porcine IL-1 α gene [NM 214029].) The 5' end of this sequence, immediately preceding exon 1, contains the IL-1 α minimal gene promoter, and this region strongly induces expression of the IL-1 α luciferase reporter in porcine aortic endothelial cells (Figure 2B). However, luciferase production was abolished when the promoter was combined with 700 bp of the contiguous 3' sequence (Figure 2B), suggesting the latter includes a suppressor.

To determine which region(s) within the 700-bp sequence downstream of the promoter region suppresses IL-1 α transcription, we generated nested deletion mutants of the region in

luciferase reporter plasmids and tested them for suppression of reporter production. Thus, porcine aortic endothelial cells were transfected with the mutated reporter plasmids and tested for suppression of IL-1 α gene promoter activity. This analysis revealed that the sequence responsible for suppression of the IL-1 α gene promoter resides within a region located 169 bp 3' of the IL-1 α transcriptional start site (Figure 2A and 2B). Because this region of the IL-1 α gene repressed gene expression and is located “downstream” of the gene promoter and transcriptional start site, and within the first intron of the gene, we call it “repressor downstream” or RepD.

To identify a specific nucleotide sequence within RepD that acts as a suppressor, we mutated specific sequences within suppressor region and tested the mutants for suppression of the IL-1 α gene promoter. A 60-bp sequence suppressed luciferase reporter gene expression in porcine endothelial cells (Figure 3). Mutations or deletions within regions we call RepD-A or RepD-B abolished much RepD repressor function and mutations or deletions in both regions fully disabled the repressor (Figure 3). RepD specifically controls the IL-1 α promoter, as it has no repressor activity when placed 5' or 3' of the SV40 promoter (data not shown).

A RepD Sequence in the Human IL-1 α Gene

We have previously shown that pore-forming proteins induce production of IL-1 α in both swine and human endothelial cells.³⁴ Accordingly, we asked whether RepD also regulates transcription of human IL-1 α . We first tested whether IL-1 α expression by human aortic endothelial cells was regulated by epigenetic modification by treating the cells with an inhibitor of histone deacetylase. Like porcine cells, human aortic endothelial cells treated with trichostatin A had increased expression of IL-1 α mRNA detected by RT-PCR (Figure 1B). To test whether the human IL-1 α gene is regulated by RepD, we cloned the human IL-1 α gene promoter along with the contiguous 5' and 3' sequences into luciferase reporter vectors and tested for suppressor function by transfection of human aortic endothelial cells. The human IL-1 α gene contained a RepD-A sequence (Figure 3) approximately 270 bp 3' of the gene promoter region (Figure 4A; see supplemental Figure II for the complete nucleotide sequence of this region of the human IL-1 α gene). The human IL-1 α RepD-A contains a 12-bp sequence identical to the porcine RepD-A, and luciferase production in human aortic endothelial cells transfected with reporter plasmids containing human IL-1 α promoter and RepD-A region was suppressed by \approx 50% (Figure 4B). Suppression by porcine RepD was \approx 90% (Figure 3). Greater suppression of the reporter gene with porcine RepD may reflect the presence of the RepD-B sequence that is lacking in human RepD. This difference in reporter gene suppression between species should not be taken to predict the degree of suppression in vivo, which likely also depends on cytokine balance, complement levels, flora, etc.

RepD-Binding Proteins

As a first step toward identifying the protein(s) that regulate the IL-1 α gene in endothelial cells, we extracted proteins from the nuclei of quiescent porcine aortic endothelial cells, in which transcription of IL-1 α is suppressed, and performed EMSAs using the nuclear proteins and ³²P-labeled oligonucleotide probes derived from RepD. Nuclear proteins from the endothelial cells formed 2 separate complexes with the full RepD sequence probe (Figure 5). To distinguish the 2 complexes formed with RepD, we tested oligonucleotides representing the 5' half (RepD-A) or the 3' half (RepD-B) of RepD as cold competitors in EMSAs. The oligonucleotide derived from RepD-A inhibited formation of the slower-migrating complex but not the faster-migrating complex formed between porcine endothelial cell nuclear protein(s) and the RepD probe (Figure 5). In contrast, oligonucleotides containing poly-thymidine or a poly-adenosine mutations that disrupt RepD repressor

activity (Figure 3) did not compete with the labeled RepD probe, suggesting the protein(s) that forms the slow migrating complex binds specifically to sequences within RepD-A. The oligonucleotide derived from RepD-B partially inhibited formation of the faster-migrating complex but not the slower-migrating complex. As would be expected, RepD oligonucleotides with a poly-adenosine mutation of RepD-B prevented competition (Figure 5). The slower-migrating complex bound to RepD-A and the faster-migrating complex to RepD-B because a slower-migrating complex formed with the radiolabeled RepD-A probe and a faster-migrating complex formed on the radiolabeled RepD-B probe (Figure 5), consistent with the 2 complexes that formed on the full-length RepD probe. These results suggest that 2 distinct protein complexes synergistically regulate IL-1 α gene transcription by binding to RepD.

RepD Binds C/EBP From Endothelial Cells

The RepD sequence contains a site potentially recognized by C/EBP. The promoter regions of TNF α , IL-8, and G-CSF genes also have such sequences.⁴⁶ To investigate whether the DNA-binding complexes from endothelial cells contain C/EBP, we performed EMSAs using ³²P-labeled oligonucleotide probes with native RepD-A sequences or RepD-A mutated within the putative C/EBP-binding region (Figure 6). RepD-A oligonucleotides with changes within the C/EBP-binding sequence did not form complexes with nuclear proteins extracted from porcine aortic endothelial cells (Figure 6A), suggesting protein(s) with the C/EBP DNA-binding specificity may regulate IL-1 α via RepD.

To determine whether C/EBP family members may regulate IL-1 α transcription, we performed electrophoretic mobility “supershift” analysis using antibodies to C/EBP family members or to other transcription factors. Antibodies directed against C/EBP β retarded mobility of the RepD-A complex to a small but reproducible extent (Figure 6B), whereas antibodies specific for C/EBP α (Figure 6B) or NFATc (data not shown) did not. None of the antibodies reacted with the component(s) of the RepD-B–protein complex (Figure 6B). These results suggest that suppression of IL-1 α gene is mediated by RepD interaction with C/EBP β , although other nuclear proteins may play a role by binding to the RepD-B sequence.

Discussion

Activation of complement is a seminal event in inflammation and immunity.³³ Complement is the first system to be activated by infectious microorganisms, and it initiates an immediate and potent defense. Besides directly killing micro-organisms, the membrane attack complex promotes procoagulant posture of endothelial cells^{14,17} and recruits platelets to vessel walls, both of which circumscribe sites of infection and produce a barrier that isolates the pathogen.³³ Although complement clearly defends the host, it may also damage healthy endothelium and tissues.⁴⁷ For this reason, presumably, endothelial cells resist injury from complement and rapidly repair such injury that may occur.^{28,33} This resistance reflects both control of production and responsiveness to IL-1 α .³⁴ Here, we report that a transcriptional repressor controls IL-1 α production by quiescent endothelial cells.

The physiological changes in endothelial cells induced by the membrane attack complex are predominantly mediated by production of IL-1 α and autocrine or paracrine action of that cytokine.³³ Because the membrane attack complex is produced constitutively,³³ the quiescent condition of endothelial cells depends on control of IL-1 α transcription. We show here that such control is exerted, at least in part, by repression of IL-1 α transcription enforced by epigenetic modification of DNA⁴⁰ and/or histones (Figure 1) and sequence-specific binding of repressor proteins. Toward the latter, we report a DNA sequence within the IL-1 α gene that we call RepD and that functions as a transcriptional repressor. RepD

differs from a repressor-binding site reported by McDowell et al⁴⁸ located between –448 and –435 nucleotides with respect to the human IL-1 α transcriptional start site. Although McDowell et al did not report which physiological stimuli disable the upstream repressor, we found that histone deacetylase inhibitors activate IL-1 α transcription independently of other agonists. Thus, cellular signaling pathways that regulate histone deacetylase may regulate IL-1 α by disabling the function of a repressor(s). Given our previous finding that the membrane attack complex activates calcineurin-dependent IL-1 α transcription in endothelial cells by targeting NF- κ B to the IL-1 α gene,³⁵ and the report by Zhang et al⁴⁹ that calcineurin promotes export of histone deacetylase(s) from the nucleus,⁴⁹ leaving unopposed histone acetylases that acetylate histones in chromatin and thereby “open” certain genes for activation by NF- κ B⁵⁰ or other transcriptional activators, we speculate the membrane attack complex may selectively target transcription of the IL-1 α gene by calcineurin-dependent removal of histone deacetylase activity from the nucleus.

Suppression of IL-1 α transcription in endothelial cells by RepD and derepression by calcineurin-dependent signals and histone deacetylase may provide a switch by which IL-1 α , via action on endothelium, participates in host defense in one context and protects endothelium from harm in another. After the initial encounter with a microorganism, complement activation increases production of membrane attack complexes and promotes small vessel permeability by disrupting endothelial integrity.²⁸ These changes promote generation of thrombin, activation of platelets, and formation of fibrin clots and platelet plugs, contributing to reduced blood flow and sequestration of infectious agents. When blood flow is reduced, IL-1 α accumulates and stimulates production of chemokines, influx of leukocytes, acute inflammation,^{14,16} and subsequent initiation of adaptive immune responses.^{51,52} However, in normal tissues, the proinflammatory action of IL-1 α would be detrimental and is prevented by multilayered mechanisms. Thus, under physiological conditions, transcription of the IL-1 α gene is constitutively suppressed, as we show here, and endothelial cells remain inured to small amounts of complement spontaneously activated even in healthy tissues. Moreover, even if transcriptional suppression fails, small amounts of IL-1 α produced are dissipated in the rapid blood flow. Blood vessels adjacent to injured or infected sites may experience increased exposure to IL-1 α , but endothelial cell responses to IL-1 α are antagonized by a secreted IL-1 α receptor antagonist,⁵³ synthesis of a nonsignaling IL-1 receptor,⁵⁴ constitutive suppression of IL-1 α transcription, and feedback inhibition of IL-1 α synthesis by IL-1 receptor signaling [S.S. and J.L.P., unpublished results, 2006]. Thus, constitutive suppression of IL-1 α gene transcription and removal of that suppression by histone modifications allow proinflammatory production of IL-1 α to predominate in appropriate circumstances and potentially enable complement, via highly regulated production of IL-1 α , to serve as a master regulator of endothelial function in health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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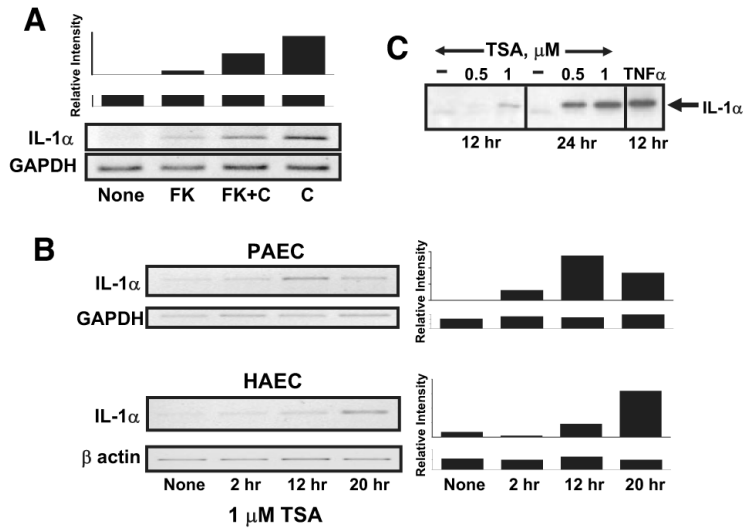


Figure 1. IL-1 α expression is activated by complement and regulated by histone deacetylase in endothelial cells. Expression of IL-1 α was evaluated in confluent cultures of porcine (PAEC) or human (HAEC) aortic endothelial cells. A, Complement-stimulated IL-1 α expression requires calcineurin. Porcine aortic endothelial cells were left untreated (none) or were treated with 100 nmol/L FK506 (FK) for 2 hours to inhibit calcineurin. The cells were then exposed to 12% human serum for 4 hours to activate complement (indicated as C). Total cellular RNA was prepared and analyzed for IL-1 α mRNA using quantitative RT-PCR. The samples were also analyzed for GAPDH as a control for equal mRNA loading and quality. The representative gels shown at the bottom are representative of 3 separate experiments. The densitometry scan bar graph at the top reflect IL-1 α /GAPDH mRNA levels. B, IL-1 α expression is regulated by histone deacetylase. Confluent cultures of porcine or human aortic endothelial cells were treated with 1 μ mol/L trichostatin A (TSA) for various periods of time, as shown, to inhibit histone deacetylase. Total cellular RNA was prepared, and expression of IL-1 α mRNA or control GAPDH or β -actin mRNA was evaluated by quantitative RT-PCR as described above. C, Porcine aortic endothelial cells were treated with 0.5 or 1.0 μ mol/L trichostatin A or with the drug vehicle alone for 12 or 24 hours. A positive control sample was stimulated with 10 nmol/L TNF α . Secretion of IL-1 α into cell culture supernatants was analyzed from equal volumes of supernatant by immunoblot using anti-porcine IL-1 α antibodies. The results show that complement or an inhibitor of histone deacetylase induces IL-1 α gene expression in endothelial cells.

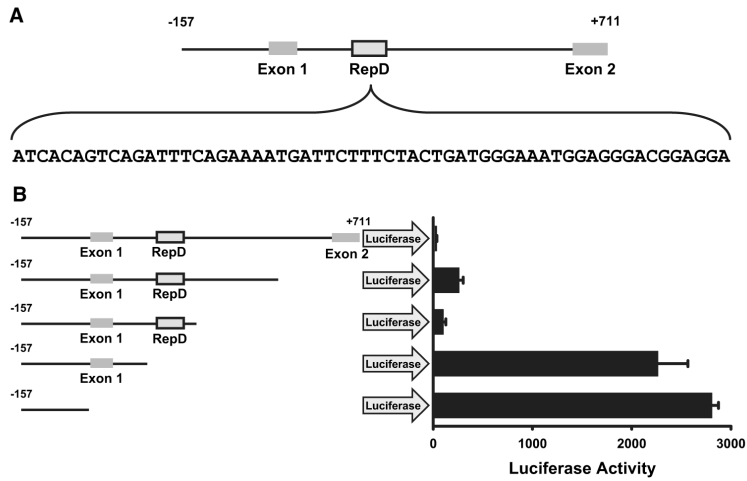


Figure 2. Identification of a repressor element in the porcine and human *IL-1 α* gene. A, Schematic representation of the porcine *IL-1 α* gene promoter and first 2 exons. The nucleotide sequence of a repressor element (boxed) is shown. Because the repressor element is located “downstream” or 3’ to the *IL-1 α* gene promoter, between exon 1 and exon 2, we named it repressor downstream, or RepD (see below). B, The *IL-1 α* gene promoter or the promoter plus 700 bp of contiguous 3’ sequence (downstream) encoding the first 2 exons of *IL-1 α* was cloned into a luciferase reporter plasmid, and the reporter plasmids were transfected into porcine aortic endothelial cells. Production of the luciferase reporter measured 36 hours later revealed that the 700-bp sequence abolished luciferase reporter gene production. The bar graph reflects the mean \pm SEM of triplicate samples and is representative of 3 experiments. Deletions within the 700-bp repressor region sequence showed that the 163-bp region located \approx 100 bp 3’ of the *IL-1 α* gene promoter contained the repressor.

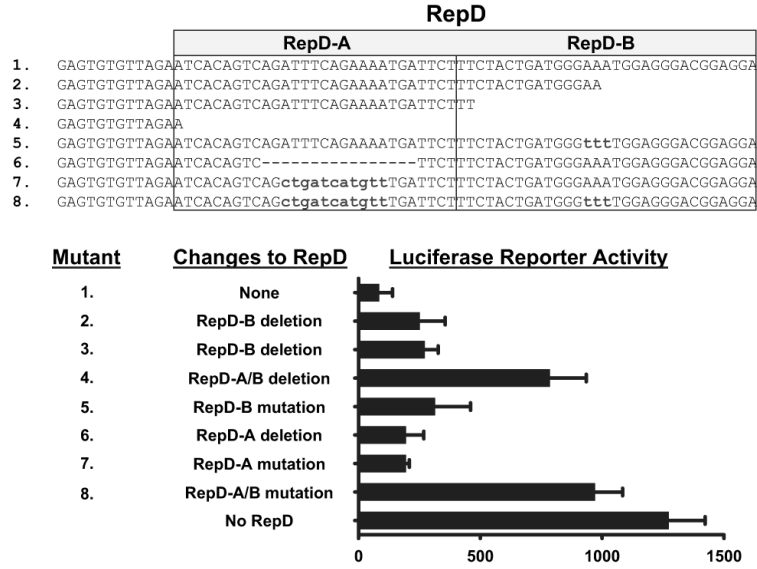


Figure 3. Resolution and characterization of the IL-1 α gene repressor RepD sequence. Luciferase reporter plasmids containing the IL-1 α gene promoter and RepD or nucleotide deletions or substitutions within the 60-bp RepD sequence reveal that RepD consists of 2 functional domains (shown as RepD-A and RepD-B). The bar graph reflects the mean \pm SEM for triplicate samples and is representative of 3 experiments.

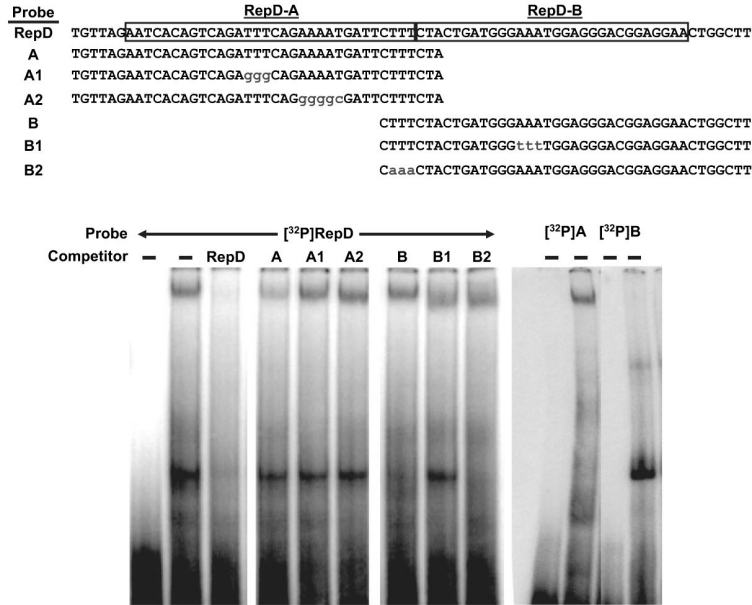


Figure 5. Protein factors in endothelial cell nuclei bind RepD sequences. Nuclear proteins were extracted from porcine aortic endothelial cells and incubated with ³²P-labeled oligonucleotide probes derived from the RepD sequence as shown (top). A control reaction contained labeled probe without protein (first lane). The protein–DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and detected on autoradiography film. The experiment revealed that 2 protein–DNA complexes form simultaneously on the radio-labeled RepD sequence. The slower-migrating complex is diminished when cold competitor oligonucleotide probes from intact RepD or from RepD-A are included but not when cold RepD-A sequences include the specific mutations shown. The faster-migrating complex is diminished by cold RepD or RepD-B oligonucleotides but not when RepD-B contains the mutation shown. Protein–DNA complexes formed with ³²P-labeled oligonucleotide probes derived from RepD-A or RepD-B confirm that the slower-migrating complex forms specifically with the RepD-A sequence and that the faster-migrating complex forms specifically with the RepD-B sequence (right). Data are representative of 3 experiments.

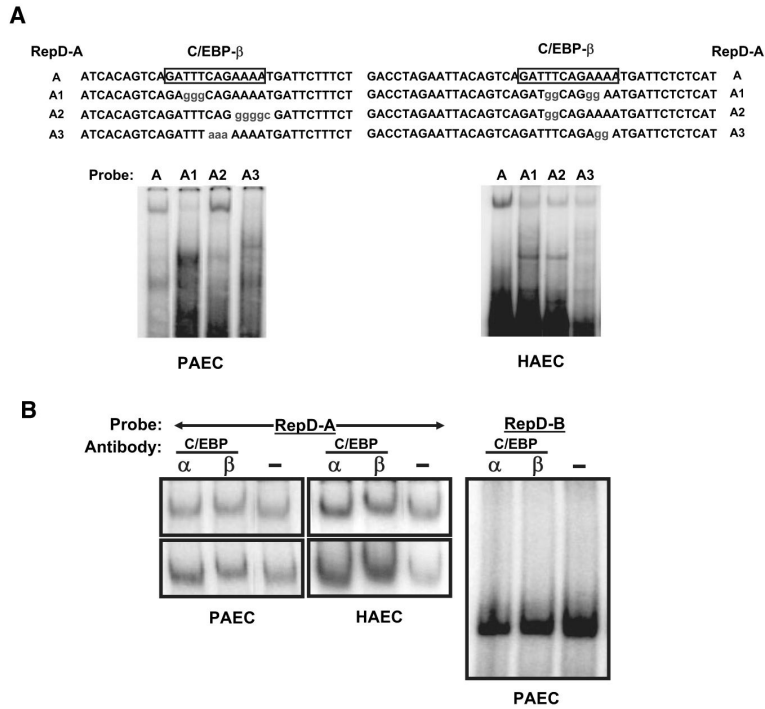


Figure 6. The IL-1α gene repressor RepD binds to a C/EBP. Nuclear proteins were extracted from porcine (PAEC) or human (HAEC) aortic endothelial cells and incubated with ³²P-labeled oligonucleotide probes derived from the RepD-A sequence, as shown in A. The protein–DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and detected by autoradiography of dried gels. A, Nuclear proteins from porcine or human aortic endothelial cells form complexes with RepD-A oligonucleotides but not with RepD-A oligonucleotides containing mutations in the CEBP-β-binding sequence. B, Antibodies to C/EBP-β (lanes marked β) react with protein–RepD-A oligonucleotide complexes and retard migration of the migration through gels. Antibodies against other proteins including C/EBP-α (lanes marked α) or NFATc (data not shown) were without effect. Shown at left side are several examples of experiments using porcine or human porcine aortic endothelial cells probed with the RepD-A probe and anti C/EBP or control (-) antibodies. Antibodies against C/EBP-α or C/EBP-β do not react with the faster-moving complex that forms with RepD-B (right).