Endotoxin-induced Renal Inflammatory Response

ONCOSTATIN M AS A MAJOR MEDIATOR OF SUPPRESSED RENIN EXPRESSION

Received for publication, April 4, 2000
Published, JBC Papers in Press, May 9, 2000, DOI 10.1074/jbc.M002830200

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The systemic response to endotoxin is characterized by hypotension and severe reductions in blood pressure, leading to cardiovascular collapse that can accompany septicemia. The renin/angiotensin system would normally be expected to respond to hypotensive challenge; however, inflammation appears to modify this response. This study identifies a strong acute phase response of the kidney that is characterized by enhanced expression of serum amyloid A, haptoglobin and tissue inhibitor for metalloproteinase-1 and a reduced expression of renin. Equivalent regulatory effects were observed for the immortalized As4.1 kidney cell line that models certain features of juxtaglomerular cells. Oncostatin M, a known endotoxin-responsive proinflammatory cytokine, proved to be an effective inhibitor of renin gene expression. Suppression by oncostatin M involves activated STAT5 and requires an inhibitory element in the renin promoter that functions separately from cell type-specific enhancer elements. The renal acute phase reaction, unlike the liver acute phase reaction, is more strongly dependent on locally produced inflammatory factors.

The tissue damage, infection, or endotoxin induces a local inflammatory reaction by the recruitment of an interacting network of effector cells and intercellular mediators (1). The various cells respond through panels of inflammatory factors, which on one side augment the local response and, on the other side, enter circulation causing systemic propagation of the acute phase reaction. The liver response, in particular, stands out through the massive changes in acute phase plasma proteins. The local inflammatory response invariably affects endothelial cells, which in part through NO-mediated mechanisms effect relaxation of adjacent smooth muscle (2, 3). Vasodilation in turn assists in plasma effusion and extravasation of chemotactically mobilized leukocytes. The accompanying drop in blood pressure should serve generally as a signal for compensatory reaction to restore normal vascular tone by mobilization of the renin/angiotensin system. Although inflammatory conditions have consistently been noted to elevate angiotensinogen mRNA levels in liver (4) and angiotensinogen levels in plasma in parallel with the induction of acute phase proteins (APP) (5, 6), plasma renin concentration and plasma renin activity have been noted to exhibit complex and variable kinetic alterations (7, 8), and increases in renin expression have not been reported. In this study we identify a kidney response to endotoxin and systemic inflammatory mediators that involves an enhanced expression of plasma proteins generally associated with the liver acute phase reaction and a reduced expression of renin. The suppression of renin is contrasted by an induction of renal serum amyloid A (SAA), haptoglobin, and TIMP-1.

EXPERIMENTAL PROCEDURES

Reporter Gene Constructs and Expression Vectors—The CAT reporter gene constructs were: the STAT3- and STAT5-sensitive p8xHRRE-CAT (9) and pTIMP-1-CAT (containing TIMP-1 promoter region −62 to +47; Ref. 10); marine Ren−IC promoter-CAT constructs, pRen-1(−3.2 kb)-CAT, pRen-1(−2.6 kb)-CAT, pRen-1(−2.1 kb)-CAT, pRen-1(−4.1 to −2.6 kb)-CAT, pRen-1(−3.1 kb)-CAT, pRen-1(−2.6 kb)-CAT, pRen-1(−2.1 kb)-CAT, pRen-1(−3.1 kb)-CAT, and pRen-1(−2.6 kb)-CAT (the predicted cAMP response element site at −2699 to −2690 mutated from TGACATCA to TtctATCA; and pRen-1(−3.1 kb)-CAT, pRen-1(−2.6 kb)-CAT, and pRen-1(−2.1 kb)-CAT generated by standard cloning techniques). The following expression vectors were used: rat STAT5B, dominant negative mutant STAT5BΔ40C, STAT3 (12), and dominant negative mutant STAT3Δ55C (13); the dominant negative A-CREB (14) and A-Fos (15) in pRC/CMV (InVitroGen); and pEGFP-N1 (Upstate Biotechnology, Inc.).

Cell Transfection and Analysis—As4.1 cells (American Type Culture Collection CRL 2193) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and antibiotic. Transfections were carried out with FuGene6 (4 µg of DNA and 6 µl of FuGene6) according to the manufacturer’s recommendation (Roche Molecular Biochemicals). All transfections included 0.25 µg of pEGFP-N1 as marker, 3 µg of CAT reporter construct, and 0–0.75 µg of expression vector for transcription factors. Transfected cultures were passaged, and subcultures were treated in serum free Dulbecco’s modified Eagle’s medium containing 0–100 ng/ml of recombinant human IL-6 (Genetics Institute, LIF (Immunex Corp), epidermal growth factor (Collaborative Research), growth hormone (Genentech), mouse OSM (produced by transiently transfected COS cells; Ref. 16), 10 ng/ml IL-1β (Immunex Corp.), TNFα (Genentech), and 10 µg/ml LPS (serotype 0127: B8; Sigma). A digital image of each GFP positive culture was taken under an inverted fluorescence microscope (Nikon) with a SPOT camera, and

* This work was supported in part by National Institutes of Health Grants DK33886 (to H. B.) and HL48459 (to K. W. G.), by funds from the Arthritis Society of Canada (to C. D. R.), and Roswell Park Cancer Institute Support Grant CA16056. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: APP, acute phase protein; CREB, cAMP response element-binding protein; DEE, distal enhancer element; GFF, green fluorescent protein; IL, interleukin; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; NRE, negative regulatory element; OSM, oncostatin M; SAA, serum amyloid A; STAT, signal transducer and activator of transcription; TIMP-1, tissue inhibitor for metalloproteinase-1; CAT, chloromphenicol acetyltransferase; TNF, tumor necrosis factor; kb, kilobase(s).
fluorescence above background for a constant view area was quantitated in the NIH Image program version 1.62. The integrated net pixel values served as a measure for transfection efficiency. CAT activity in serially diluted cell extract was determined, normalized to the GFP signal for each culture, and expressed as relative CAT activity. Aliquots of cell extract were separated on 10% SDS-polyacrylamide gels. Proteins were electrotransferred onto protean membranes (Schleicher & Schuell). Membranes were reacted with primary antibodies to phosphotyrosine (pY20, Transduction Laboratories; 4G10, Upstate Biotechnologies), STAT3 and STAT5 (Santa Cruz Biotechnology), phosphorylation-specific anti-STAT3, STAT5, and ERK1/2 (New England Biolabs), followed by secondary horseradish peroxidase-conjugated antibodies (Capel) and visualized by ECL (Amersham Pharmacia Biotech). SAA isoforms synthesized and secreted by As4.1 cells were identified by immunoblotting using isofrom-specific antibodies against mouse SAA proteins (generously provided by Dr. E. P. Benditt, University of Washington, Seattle, WA).

Inflammatory Reactions in Mice and RNA Analysis—Adult (10–12 weeks old) male and female C57BL/6 mice received either two subdermal injections of 25 μl of turpentine on both flanks of the hind legs (sterile tissue injury model) or a single intraperitoneal injection of 50 μg of LPS in 100 μl of phosphate-buffered saline/25 g of body weight (endotoxemia model). Each experimental series included 2–5 replicate animals/treatment. Treatment with phosphate-buffered saline alone served as control. To activate an OSM-specific systemic reaction, mice received an intraperitoneal injection of 0.5 ml of phosphate-buffered saline containing 2 × 10^8 plaque-forming units of the adenovirus with deletion of the early regions 1 and 3 region (17) and insertion of the cDNA for murine OSM under the control of the murine cytomegalovirus promoter/enhancer, AdmOSM (18), or of the control virus, AdlI70-3. RNAs were extracted from liver and kidney by the Trizol method (Life Technologies, Inc.). Aliquots of 5–20 μg of total RNA were separated on a formaldehyde containing agarose gel, transferred to nitrocellulose, and reacted with 32P-labeled cDNAs to mouse renin, haptoglobin, SAA-3 or SAA-1, α1-protease inhibitor, or TIMP-1. The hybridization signals were quantitated by PhosphorImager scanning and analysis with the ImageQuant program (Molecular Dynamics). The digital image of ethidium bromide stained 28 S rRNA band served as loading marker and was quantitated by analysis with the NIH Image program 1.62. The hybridization signal was normalized to the 28 S rRNA signal. In each experimental series, the mean value of the control animals was defined as 100%, and the values of each animal were then expressed relative to that control mean values.

RESULTS

Two separate mouse models of in vivo inflammatory reactions were examined for effects on kidney. One model, the LPS-initiated intraperitoneal inflammation model, couples a systemic distribution of LPS and activation of endotoxin-responsive cells at different organ sites (19, 20) to produce inflammatory cytokines, including IL-1b, TNFα, IL-6, LIF, and OSM (21–23). The second model, the turpentine-induced sterile tissue injury model, causes a confined but strong local inflammation and initiates a systemic acute phase reaction by means of humoral mediators of which IL-6 is the most significant component (24).

Within 24 h following LPS administration, the characteristic induction of the representative APPs, haptoglobin and SAA, was observed for liver (Fig. 1A, bottom panel). Analysis of kidney RNA showed an appreciable induction of the same two plasma proteins, although the peak values observed were a fraction of those found for liver. The kidney RNA analysis also revealed a 2–3-fold LPS-mediated reduction of renin mRNA (Fig. 1, A, top panel, and B, left panel), which appeared to be inversely correlated with the prominent induction of TIMP-1 and SAA.

A quantitatively distinct pattern of regulation was evident in animals undergoing turpentine-elicted tissue injury. It was more effective in inducing APP genes in the liver and of SAA and haptoglobin in the kidney but produced only a minor induction of TIMP-1 (Fig. 1A) and somewhat lower suppression of renin than LPS treatment (Fig. 1B, left panel). The more prominent effects of turpentine induction on liver and kidney APP genes was attributed to systemically distributed cytokines, of which IL-6 is probably a major component (24, 25). The LPS-specific activity was attributed to both systemically distributed (26) and locally elicited production of inflammatory mediators that act on cells in the same tissue context. The particularly effective induction of TIMP-1 and reduction of renin may represent the effects of LPS-dependent renal mediators.

To profile the effects of specific factors on renal gene regulation, we turned to the As4.1 mouse kidney cell line that exhibits features characteristic of juxtaglomerular cells, such as prominent renin expression (27). These cells were treated with cytokines predicted to be induced by LPS, namely OSM, IL-6, LIF, IL-1β, and TNFα (Fig. 1, C and D). Time course analysis indicated maximal effects on gene induction at 24 h of treatment (data not shown). OSM yielded the most prominent suppression of renin (Fig. 1B, right panel); smaller but significant reductions were also obtained with IL-6, IL-1β, and TNFα.

**FIG. 1.** Gene regulation in kidney (A and B) and As4.1 cells (C–E). A, male C57BL/6 mice, in duplicates as indicated at the bottom, were treated for 24 h with LPS or turpentine. 20 μg of kidney and 5 μg of liver RNA were analyzed by Northern blot hybridization for the mRNAs listed at the right. B, compilation of the relative levels of renin mRNA expression determined in individual mice from five independent series (left panel) and in four separate experiments with of As4.1 cells (right panel). Note that the culture of untreated control As4.1 cells in each experimental series is defined as 100%. C–E, in four separate experimental series, As4.1 cells were treated for 24 h with serum-free medium containing the factors listed at the bottom. RNA (20 μg) were analyzed by Northern blot hybridization.
transfected As4.1 cells (Fig. 3A). As4.1 cells supported a minor induction of the OSM-responsive, STAT5-sensitive test construct p(6xHRRE)-CAT, whereas the 4.1-kb renin-CAT construct yielded a prominent basal expression that was reduced by OSM and other effectors. The relative rank order of activities in the transfection reporter assay were comparable with those detected at the level of renin mRNA abundance (Fig. 3A versus Fig. 1, C and D). The profile of regulation for the transfected TIMP-1 promoter contrasted sharply with that observed for the renin promoter, and the robust induction of the TIMP-1-CAT construct by OSM was in accordance with regulation of the endogenous TIMP-1 gene. A strong contributing activity of OSM-activated STAT proteins was suggested.

The prominent cell type-specific expression of the 4.1-kb renin-CAT construct has been described to depend on two critical elements: a promoter proximal element at position – 60 and a distal enhancer element (DEE) at position –2690 (11). A set of promoter truncations and mutations was tested to localize the functional element(s) responsible for OSM suppression (Fig. 3B). With deletion of the DEE, essentially all expression was abolished. Removal of the intervening sequence between DEE and position –117 reduced the basal expression of the promoter less than 2-fold but eliminated suppression by OSM. The mutation of a cAMP response element modulator/CREB-binding element within the DEE reduced basal expression but did not reintroduce either positive or negative responsiveness to OSM. By retaining more 5’ flanking sequence upstream of –117, the region from –694 to –365 was found to restore full sensitivity to OSM inhibition of promoter activity. This region contains at –650 a negative regulatory element (NRE) that has been suggested to exert an inhibitory activity on constitutive expression of the renin gene (29).

To identify signaling molecules that potentially connect the OSMR to the renin gene regulatory element, we assessed the relative contribution of the particularly strong activated ERK and STAT pathways (Fig. 2A). Inhibition of MEK-1 by U0126 during OSM treatment did not affect OSM inhibition (Fig. 4A). Dominant negative A-Fos was unable to modify promoter activity. In contrast, the dominant negative A-CREB essentially abolished all promoter activity, likely through neutralization of the DEE-interacting cAMP response element modulator/CREB proteins.

Overexpression of the dominant negative STAT3Δ55C was inconsequential; however, the dominant negative STAT5BΔ40C severely reduced basal expression, as well as expression in OSM-treated cells (Fig. 4A). The inhibition by STAT5BΔ40C in the absence of OSM treatment probably reflected the elevated DNA binding activity of STAT5 proteins as a result of overexpression (30). The same inhibitors and dominant negative transcription factors were used to characterize the regulation of the TIMP-1-CAT reporter gene construct. A
fundamentally different, inverse pattern of action was observed. In this case, U0126 inhibition of MEK lowered the OSM-dependent OSM induction. Similarly, A-Fos, the dominant forms for STAT3 and STAT5 reduced TIMP-1-CAT expression (Fig. 4B).

To determine whether the probable mode of STAT5BΔ40C inhibition of renin promoter activity was mediated via the NRE-containing region, different length promoter proximal 5'-flanking regions linked to the DEE were challenged by OSM treatment in the presence of overexpressed STAT5BΔ40C (Fig. 4C). Significant suppression was evident for the −696 promoter construct, which included the NRE. The suppression of basal and OSM-induced inhibition by truncated STAT5 suggests that interaction of STAT5 either directly with the DNA site or indirectly through an NRE-associated component is sufficient to obtain inhibition. That STAT5B, with or without the C-terminal transactivation domain, could act as an inhibitor was confirmed by demonstrating that suppression of renin promoter activity by overexpressed wild type STAT5B occurred to the same extent as observed for truncated STAT5B (Fig. 4C).

The combined results from the tissue culture analyses strongly support a prominent renin suppressing activity of OSM that in part could account for the effects observed in kidneys of LPS-treated mice (Fig. 1, A and B, left panel). To directly assess an inhibitory activity of OSM in vivo, we next determined renin mRNA levels in the kidneys of mice infected with an adenoviral vector expressing murine OSM (Fig. 5). Whereas the animals infected with control virus did not show appreciable changes in APP expression, the AdmOSM-treated animals demonstrated within 48 h an enhanced level of liver APP mRNA and serum APP levels that were characteristic for OSM and a prominent reduction of renin mRNA in the kidney. Taken together, these data support the notion that endotoxin action in kidney, presumably by resident macrophages, may...

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**FIG. 3.** Regulation of transfected Ren-CAT and TIMP-1-CAT constructs. As4.1 cells were transfected with the CAT reporter constructs as indicated at the top in A and on the left in B. Subcultures of the transfected cells were treated for 24 h with the factors listed at the bottom. Specific CAT activities are determined. In B, the values represent the means ± S.D. (n = 3–5).

**FIG. 4.** Effects of transcription factors on Ren-1 promoter activity. Cells were transfected with the CAT-reporter gene constructs together with the expression vectors for dominant negative transcription factors listed at the top. Subcultures were treated for 24 h with the factors listed at the bottom. The relative CAT activities represent the mean values ± S.D. (n = 3).

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FIG. 5. In vivo action of OSM. Mice received control adenovirus (Ad-Control) or mOSM-expressing adenovirus (Ad-mOSM). Kidney and liver RNAs were analyzed 48 h later by Northern hybridization for the mRNAs indicated at the right.

involves the local production of OSM, which, together with other inflammatory cytokines and endotoxin, suppresses renin expression in juxtaglomerular cells in vitro by mechanisms comparable with that observed for As4.1 cells in vitro.

DISCUSSION

The inflammation-specific reduction of renin expression identifies this gene product as a potential negative acute phase reactant. Although secretion of renin, a regulated process in juxtaglomerular cells, may not precisely track the kinetics of the acute phase-induced reduction observed for renin mRNA levels during the chronic stage of inflammatory reaction, a decrease in the RCP is expected that will assume physiologically relevant consequences for blood pressure homeostasis.

At issue in this study is the cell type-specific regulation of APP production and the relative role of specific inflammatory cytokines. Although the hepatic induction of APP genes and the synthesis of their cognate products clearly represents a dominant process systemically, the availability or accumulation of the individual plasma proteins in physiologically significant levels at distant sites of inflammation may require on the order of several hours to occur. An alternative mechanism to gain protective APPs at sites of inflammation has been suggested to occur through local production. Extrahepatic APP synthesis has been noted in organs such as lung and intestine that are especially exposed to insults (31). Our data indicate that kidney possesses a capability to carry out a similar process that involves positively and negatively affected genes. The response of As4.1 cells suggests that the positive APPs comprise a cellular response for juxtaglomerular cells in situ that is similar to that identified for interstitial myofibroblasts and mediated by the same signal transduction systems as the inhibition of renin (32).

The primary regulators of positive as well as negative APP have emerged as IL-1- and IL-6-type cytokines. Increased transcription of various APP genes is attributable to the action of specific sets of transcription factors that generally include CCAAT/enhancer-binding protein β and nuclear factor κB, stimulated primarily by IL-1 cytokines, and STAT3 and AP-1 components, stimulated by IL-6 cytokines. The mode of regulation of negative APPs is less well understood but appears to involve in part the same transcription factors as those acting on positive APP genes (33).

The contrasting regulatory action of OSM on renin versus TIMP-1 gene expression in As4.1 cells revealed a novel mechanism. Although transcriptional activity of the renin gene appears to be moderated by a number of proinflammatory cytokines and dexamethasone (Fig. 1), the signaling through OSMR emerged as particularly effective. The identification of STAT5 as an inhibitor of renin gene expression explains in part the OSM effect, as OSMR signaling, unlike that of the other IL-6 cytokine receptors, involves STAT5 as a major signal-transducing component. The observation that other effectors, such as LPS, IL-1β, TNFα, and dexamethasone, and even to some extent IL-6, all of which are not associated with prominent STAT5 activation, can also reduce renin expression implies that STAT5 is not the exclusive suppressor of renin but that additional pathways act on renin gene expression. The identity of those intracellular mediators and their genetic targets within the renin gene remain to be established.

STAT5 has been recognized to be a preferred substrate for activation by several hematopoietin receptors and generally been associated with stimulated transcription (34). The inhibition of renin by STAT5 involves a mode of action that appears to be distinct from its stimulatory functions. Moreover, although there is a clear requirement for specific promoter elements, the suppression effect is manifested irrespective of the presence of the transactivating domain on STAT5. Precedent for such an inhibitory activity has been reported recently for STAT5 in regulation of the IRF-1 promoter by prolactin (35).

We have been unable to identify a STAT5-binding element in the renin promoter or to detect a direct physical interaction of activated STAT5B with the region containing the NRE. Conceivably, STAT5 may bind to DNA recognition sequences, but that interaction is undetectable by the in vitro assay conditions employed, or STAT5 may be acting indirectly through association with other transcription factors (squelching). In either case, we would expect that STAT5 exerts its transcriptional suppression by interfering with the strong transcriptional regulators of the renin gene.

Our results demonstrate the regulatory capability of the kidney in response to an acute inflammatory process. Preliminary results with animals treated repeatedly with LPS for several days indicate a persistent expression pattern of enhanced APPs and reduced renin. Yet to be determined is the influence of inflammatory stimuli, which are expected to be present during chronic renal inflammatory diseases. Long-term intrarenal tissue damage, coupled with inflammatory cell activities, would inevitably introduce new conditions that will affect the regulatory phenotype of the kidney. This will include not only an adaptive regulation of the renin/angiotensin system but also a profound tissue repair reaction that results in a change in tissue composition, as best exemplified by interstitial fibrosis (32, 36).

Acknowledgments—We thank ImmuneX Corp. and the Genetics Institute for providing recombinant cytokines, Dr. F. L. Graham for control adenovirus, Dr. W. Liao for mouse SAA-1 cDNA, Dr. P. Soloway for TIMP-1 cDNA, Dr. Benditt for SAA antisera, and Colleen Kane-Haas, Erin Kinzie, and colleagues at the laboratory animal facility for technical assistance.

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