Published in final edited form as:
Peptides. 2007 November ; 28(11): 2164–2170.

Mice heterozygous for adrenomedullin exhibit a more extreme inflammatory response to endotoxin-induced septic shock

Ryan Dackor[a,b] and **Kathleen Caron**[a,b,*]

a *Department of Cell & Molecular Physiology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599 USA*

b *Genetics Department, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599 USA*

Abstract

Adrenomedullin (AM) is a highly conserved peptide that can act as a potent vasodilator, anti-microbial factor and anti-inflammatory factor. Several studies have implicated diverse roles for AM in regulating the inflammatory and hemodynamic responses to septic shock. Moreover, during sepsis the receptors that mediate AM signaling [*calcitonin receptor-like receptor* (*calcrl*) and *receptor activity modifying proteins* (*RAMP*) 2 and 3] undergo dynamic and robust changes in their expression. Although numerous studies have used animal models to study the role of administered or increased AM in septic animals, genetic studies to determine the consequences of reduced AM during septic shock have not yet been performed. Here, we used a murine model of lipopolysaccharide (LPS)-induced septic shock to assess the inflammatory response in mice heterozygous for the *AM* gene. Following LPS challenge, $AM^{+/-}$ mice had higher expression of *TNF-α* and *IL-1β* than LPS-treated wild-type (WT) controls. Consequently, serum TNF-α was also significantly elevated in LPS-treated $AM^{+/-}$ mice compared to WT LPS-treated mice. We also observed higher serum levels of liver enzymes, suggesting more advanced end-organ damage in mice with genetically reduced AM. Finally, we found that *RAMP2* and *calcrl* expression levels were markedly reduced in LPS-treated mice, whereas *RAMP3* expression was significantly elevated. Importantly, these changes in receptor gene expression were conserved in $AM^{+/-}$ mice, demonstrating that AM peptide itself does not impact directly on the expression of the genes encoding its receptors. We therefore conclude that during septic shock the dynamic modulation of AM and its receptors primarily functions to dampen the inflammatory response.

Keywords

adrenomedullin; septic shock; inflammation; genetic model; mice

1. Introduction

Adrenomedullin (AM) is a peptide encoded by a highly conserved gene that may have evolved from an antimicrobial peptide in early eukaryotic organisms into a potent vasodilator in higher mammalian species [37]. AM causes relaxation of vascular smooth muscle cells (VSMCs)

*Corresponding Author: Department of Cell & Molecular Physiology, CB # 7545, 6330 MBRB 103 Mason Farm Rd., The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599 USA, Phone: (919) 966-5215, FAX: (919) 966-5230, e-mail: Kathleen caron@med.unc.edu.

[7], reduces endothelial cell permeability [10] and is a biologically-relevant antimicrobial peptide involved in the innate immune response [1]. The 52-amino acid peptide is produced and secreted by many mammalian tissues and is most highly expressed by VSMCs [30] and endothelial cells [29]. Stimuli for AM synthesis and secretion include angiotensin II, endothelin-1, hypoxia, oxidative stress and inflammatory cytokines such as TNF-α and IL-1β [6]. Thus, the biological functions of AM in mammals are numerous, diverse and likely inter-related.

Plasma levels of AM are significantly elevated in humans with a wide variety of physiological and pathological conditions, including cardiovascular disease, normal pregnancy and septic shock [8]. In patients with septic shock, AM peptide levels are 25 to 30 folds higher than in normal individuals [11,21]. Since AM is a potent vasodilator [15], it is reasonable to assume that increased plasma AM contributes to the extreme hypotension observed in the early stages of septic shock. However, our recent studies using genetically engineered mice that lack one copy of the *AM* gene demonstrate that reduction of endogenous AM to 50% of wild-type (WT) levels has no effect on the acute hypotension that occurs in an LPS-induced murine model of septic shock [2]. These results suggest that AM may play other primary roles during septic shock.

AM possesses anti-inflammatory [9], bactericidal [1], and positive inotropic [12] properties, which are all beneficial responses to sepsis. When treated with endotoxin, mice over-expressing AM in their vasculature experience less severe hemodynamic and inflammatory responses, less liver damage and lower mortality rates compared to WT endotoxin-treated controls [28]. AM has also been shown to reduce TNF-α expression and release in macrophage cell lines and rat Kupffer cells [33]. More recently, administration of AM to rats with α-toxin-induced sepsis reduced vascular hyperpermeability and resulted in dramatically improved survival rates [31]. Finally, dynamic and robust changes in the expression of AM and its receptors occur in the lungs in response to septic shock [4,22]. Taken together, these results suggest that the beneficial roles of AM during septic shock may primarily be to minimize organ damage by influencing the immune response and/or vascular permeability, rather than by regulating blood pressure. Yet, experiments to genetically confirm the primary function of AM during septic shock have not yet been performed.

The AM peptide contains a 6-residue ring structure and amidated C-terminus which, due to conserved sequence homology and structural motifs, places it in the calcitonin family of peptides, including calcitonin, calcitonin gene related peptide (CGRP), amylin and intermedin [37]. Peptides of this family also share a unique mechanism of G-protein coupled receptor signaling by a novel class of single transmembrane proteins called receptor activity modifying proteins (RAMPs). RAMPs were first identified through their association with the calcitonin receptor-like receptor (CLR) and can interact with many other class II GPCRs to determine receptor ligand binding specificity [27]. In the case of CLR, association with RAMP1 produces a CGRP receptor, while association with RAMP2 or RAMP3 produces a receptor specific for AM. In this way, the spatial and temporal expression of RAMP proteins determines the tissue responsiveness to either CGRP or AM.

During inflammation and septic shock, there are robust and dynamic changes in the expression of the *RAMP* and *calcrl* genes that are responsible for mediating AM signaling. For example, TNF-α significantly reduced the expression of *calcrl* (the gene encoding CLR), *RAMP1* and *RAMP2* in cultured smooth muscle cells of human coronary artery in a time and dose-dependent manner [20]. Moreover, Ono et al. have also shown that *calcrl* and *RAMP2* expression was significantly decreased in lungs of LPS-induced septic mice, while *RAMP3* expression levels were elevated nearly 40-fold [22]. In a related fashion, the amount of AM binding protein (AMBP) is significantly reduced during the hypodynamic phase of sepsis, which may account

for the reduced responsiveness to elevated plasma AM during the late phase of sepsis [32,34, 36,37]. These results suggest that the modulation of AM signaling during septic shock is complex (involving both receptor modulation and active peptide bioavailability) and finely tuned in order to maintain homeostatic balance in response to severe physiological insults. However, whether AM signaling itself is involved in these dynamic receptor responses remains unclear.

Our previous studies with genetically engineered mouse models have shown that mice lacking both copies of the *AM* gene or the *calcrl* gene die at mid-gestation from extreme hydrops fetalis and cardiovascular defects [3,5]. Adult female mice heterozygous for *AM* display profound reproductive defects [16] and are protected from hypertension-induced cardiovascular end organ damage [2]. Otherwise, adult male and female *AM* heterozygous mice are born at the expected Mendelian ratios, survive to adulthood and have normal blood pressures under basal and stressed conditions with no obvious phenotypic defects.

To determine if genetic reduction of endogenous AM affects the septic response in mice, we challenged $AM^{+/-}$ mice in an LPS-induced model of septic shock. Since AM is consistently reported as an anti-inflammatory peptide, we were particularly interested in determining whether genetic reduction of endogenous AM in vivo could alter the inflammatory response in septic animals. We also used our genetic model to determine if the dynamic gene expression changes observed in the AM receptor signaling genes during septic shock are dependent on the expression levels of AM peptide.

2. Experimental Methods

2.1. Experimental Animals and LPS Treatment

The generation and phenotype of mice with a targeted deletion of the *AM* gene have been previously described [3]. Experiments were carried out with 8–10 week old male mice bred under a controlled environment and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill. Animals used in these experiments were produced by $AM^{+/-}$ intercrosses and were maintained on an isogenic 129S6/SvEv genetic background. To induce septic shock in WT and $AM^{+/-}$ mice, 60 mg/kg LPS (*Escherichia coli* O55:B5; Sigma, St. Louis, MO) was injected I.P. (unless otherwise stated).

2.2. Gene expression analysis

Gene expression was analyzed by quantitative reverse transcription-PCR with the Mx3000P Real-Time PCR machine from Stratagene. Taqman primer and probe sequences for *calcrl* detection have recently been published. Primers for *RAMP2* were 5′-CAGAATCAATCTCATCCCACTGA-3′ and 5′-GTCCATGCAACTCTTGTACTCATAC-3′. The probe for *RAMP2* detection was 5′-FAM-TGGAAGACTACGAAACACATGTCCTACCTTG-TAMRA-3′. Primers for *RAMP3* were 5′-GGTCATTAGGAGCCACGTGT-3′ and 5′-GGGCTAAACAAGCCACAGCT-3′. The probe for *RAMP3* detection was FAM-5′-CAGCCCACACTGGACACAGAATCGTG-TAMRA-3′. For *L1* detection, we used a pre-designed, Assays on Demand primer/probe set (Applied Biosystems). Primers for *TNF-α* were 5′-CTGTCTACTGAACTTCGGGGTGAT-3′ and 5′-GGTCTGGGCCATAGAACTGATG-3′. The probe for *TNF-α* detection was 5′-FAM-ATGAGAAGTTCCCAAATGGCCTCCCTC-TAMRA-3′. Primer and probe sequences for *AM* and *IL-1β* detection have been previously published [3] and [14]. A GAPDH primer/probe set was purchased from Applied Biosystems (part # 4308313) and used as an internal control for all samples. RNA was isolated from lungs with Trizol reagent (Invitrogen), DNase treated, and purified with an RNeasy Mini Kit (Qiagen). 200 ng of lung RNA was used per reaction and each sample was run in triplicate. The ΔΔ Ct method was used to determine the relative

levels of gene expression [17]. For these studies, the lung was chosen since it represents a primary site of inflammation following LPS exposure.

2.3. Measurement of serum TNF-α levels

For basal in vivo serum TNF-α concentrations, tail-vein bleeds were performed prior to LPS challenge. Mice were then administered LPS for one hour and serum samples were again obtained by tail-vein bleed. TNF-α concentrations were quantified using anti-TNF-α antibody by ELISA technique (product # EMTNFA2; Pierce, Rockford, IL). Recombinant TNF-α was used as a standard control.

2.4. ALT and AST measurement

Blood chemical analysis for serum alanine (ALT) and aspartate (AST) aminotransferase activities was performed in the Animal Clinical Laboratory Core Facility of the University of North Carolina at Chapel Hill using a Chemical Analyzer VT250 (Ortho-Clinical Diagnostics Comp. Rochester, NY).

2.5. Statistical analysis

Statistical analysis was performed using a Student's *t* test with unequal variance. A p value equal to or less than 0.05 was considered statistically significant.

3. Results

3.1 AM gene expression is induced significantly less in $AM^{+/-}$ mice treated with LPS

Using quantitative RT-PCR, *AM* gene expression was measured in lungs of WT and $AM^{+/-}$ mice treated with or without LPS for 2 hours. In untreated mice, the basal level of *AM* gene expression in $AM^{+/-}$ mice was 53% of WT mice, consistent with our previous demonstration that the gene targeting strategy effectively reduced *AM* gene expression by approximately half wildtype levels[3]. *AM* gene expression was dramatically elevated in both WT and $AM^{+/-}$ mice following LPS administration. However, the level of *AM* expression achieved in $AM^{+/-}$ mice following LPS treatment was only 37% of the level achieved by WT LPS-treated animals (Fig. 1).

3.2 Elevated TNF-α and IL-1β expression in lungs of $AM^{+/-}$ septic mice

Since the lungs are a primary site of inflammation during septic shock, we used quantitative RT-PCR to determine the expression of the pro-inflammatory cytokines *TNF-α*, *IL-1β*, and *IL-6* in lungs of WT and $AM^{+/-}$ mice treated with or without LPS for 45 minutes. Both WT and $AM^{+/-}$ animals showed a robust and rapid increase in *TNF-α* expression following LPS administration. However, induction of *TNF-α* expression was significantly higher in lungs of $AM^{+/-}$ mice than in lungs of WT control mice (Fig. 2). Similarly, *IL-1β* expression was drastically elevated in both WT and $AM^{+/-}$ mice upon LPS challenge, and this increase was significantly higher in $AM^{+/-}$ mice than in WT mice (Fig. 2). Although the expression of *IL-6* was higher in $AM^{+/-}$ mice than in WT controls, this data did not reach statistical significance (data not shown). These results demonstrate that the lungs of mice with only one copy of the *AM* gene ($AM^{+/-}$) are more susceptible to the inflammatory response of endotoxemic shock than WT mice with both copies of the endogenous *AM* gene.

3.3 Elevated serum TNF-α levels in $AM^{+/-}$ septic mice

Serum TNF-α was quantified using ELISA before and during LPS challenge in WT and $AM^{+/-}$ mice. Basal TNF-α levels prior to LPS treatment were similar in both genotypes (30 pg/ml +/− 5.59 for $AM^{+/-}$ versus 19.41 pg/ml +/− 4.69 for WT, p=0.174, Fig. 3). Both WT and $AM^{+/-}$ mice had a marked elevation in TNF-α 1 hour after LPS administration. However,

$AM^{+/-}$ mice showed a more robust response and secreted more than two-fold higher TNF-α than WT mice within the first hour of LPS challenge (598.83 pg/ml +/− 59 for $AM^{+/-}$ versus 268.18 pg/ml +/− 41 for WT, p=0.0004, Fig. 3). These results are consistent with our experiments measuring TNF-α gene expression and provide further evidence that $AM^{+/-}$ mice are more susceptible to LPS-induced inflammation than WT controls.

3.4. Increased ALT and AST activity in $AM^{+/-}$ septic mice

Liver damage is a hallmark characteristic of endotoxin-induced septic shock and can be assessed by liver enzyme activity. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are thus accurate indicators of liver cell damage and inflammation [25], as well as heart damage [26]. In untreated mice of both genotypes, serum ALT levels were similar (11 U/L +/− 2.3 for $AM^{+/-}$ versus 9.73 U/L +/− 1.06 for WT, p=0.63). Following a 24-hour, 100 mg/kg LPS challenge, $AM^{+/-}$ mice showed significantly higher ALT activity than WT controls (528.1 U/L +/− 158 for $AM^{+/-}$ versus 69.4 U/L +/− 15.6 for WT, p=0.02). Similarly, basal serum AST activity was not significantly different between WT and $AM^{+/-}$ mice (105.7 U/L +/− 7.5 for $AM^{+/-}$ versus 111.8 U/L +/− 6.1 for WT, p=0.56). However, after 24 hours of LPS challenge, $AM^{+/-}$ mice exhibited significantly higher AST activity (878 U/L +/− 181 for $AM^{+/-}$ versus 283.1 U/L +/− 45.5 for WT, p=0.01) (Fig. 4). Perhaps due to the isogenic 129S6/SvEv strain used in these studies, histological analysis and myeloperoxidase assays for neutrophil infiltration revealed no significant differences between LPS-challenged livers of WT and $AM^{+/-}$ mice. Nevertheless, these data clearly demonstrate that reduction of *AM* gene expression by 50% in $AM^{+/-}$ mice leads to more severe end-organ damage after septic shock than in mice with 2 copies of the *AM* gene.

3.5 Dynamic regulation of AM receptor genes in $AM^{+/-}$ septic mice

To determine whether the expression of the *AM* gene affects homeostatic alterations in the expression of genes encoding for AM receptors (*calcrl*, *RAMP2*, and *RAMP3*) during sepsis we used quantitative RT-PCR of total lung RNA from septic WT and $AM^{+/-}$ mice. We found that *RAMP2* and *calcrl* expression levels were markedly reduced 97.3% and 98.1%, respectively, while *RAMP3* expression was elevated 23.8 fold in WT mice treated with LPS. Importantly, similar changes in gene expression were observed in $AM^{+/-}$ mice; *RAMP2* expression was reduced 92.8%, *calcrl* was reduced 95% and *RAMP3* was elevated 24.9 fold (Fig. 5). In addition, we also measured expression levels of another putative AM receptor, called L1, in WT and $AM^{+/-}$ septic mice, and found the expression level of this gene to be reduced in LPS-treated mice of both genotypes (75.7% reduction in WT mice and 68% reduction in $AM^{+/-}$ mice, data not shown). Taken together, our results support the concept that robust and dynamic changes in the expression of genes responsible for mediating AM signaling occur during septic shock. Moreover, since the magnitude of these changes was not different between WT and $AM^{+/-}$ mice, we conclude that the level of AM peptide is not primarily involved in mediating responsiveness to AM signaling at the level of receptor expression.

4. Discussion

Many in vitro studies have suggested an important regulatory role for AM in sepsis and other inflammatory diseases [13,18,19,30,33,35]. Moreover, genetic over-expression of AM in the mouse vasculature or therapeutic administration of AM peptide in rats or mice had beneficial effects on reducing the inflammatory and hemodynamic insults elicited by septic shock [28]. However, whether genetic reduction of AM can cause a more severe response to sepsis has not yet been determined. The present study was designed to test the effects of a genetic reduction in AM in a murine model of LPS-induced septic shock.

We found that the administration of LPS induced a more severe inflammatory response in $AM^{+/-}$ mice than in WT LPS-treated mice. Regulation of the inflammatory response by AM occured at both transcriptional and translational levels. The expression of two pro-inflammatory cytokines, *TNF-α* and *IL-1β*, was significantly higher in $AM^{+/-}$ septic mice than in WT septic mice. In addition, serum TNF-α levels were more than 2-fold higher in $AM^{+/-}$ septic mice than in WT septic mice. These observations are consistent with previous reports showing that AM reduces the production of TNF-α in macrophages and Kupffer cells [33].

In addition to increased cytokine production, there was also a significant elevation in ALT and AST enzyme activity in $AM^{+/-}$ septic mice, when compared to WT septic mice. These results are in agreement with a report by Shindo et al. showing that mice overexpressing AM in their vasculature were resistant to LPS-induced liver damage [28].

Notably, we failed to observe any differences in end-organ histology (liver, kidney), pulmonary edema formation or overall survival of $AM^{+/-}$ septic mice compared to control septic mice at all doses and times examined. We therefore conclude that a modest genetic reduction in *AM* to 50% of WT levels does not significantly impact on the pathological morbidity and mortality of septic shock. Nevertheless, it is likely that the robust induction of *AM* expression and the modulation of its receptors plays an important role in mediating the septic response.

Recent studies by others revealed a dynamic change in the expression of genes involved in AM signaling during sepsis. Nagoshi et al. found that TNF-α significantly reduced the expression of *calcrl*, *RAMP1* and *RAMP2* in cultured smooth muscle cells of human coronary artery in a time and dose-dependent manner [20]. Furthermore, Ono et al. examined the expression of *calcrl* and *RAMP1*, *2*, and *3* in several tissues from LPS-induced septic mice. *calcrl* and *RAMP2* expression were significantly decreased in lungs of septic mice, whereas *RAMP3* message levels were increased approximately 40 folds in lungs after 12 hours of LPS challenge [22]. Using a polymicrobial model of sepsis, Ornan et al also showed that *RAMP3* expression is elevated in lungs during the early hyperdynamic stage of sepsis, but not in the later hypodynamic phase [24]. Similar to these studies, we also found that LPS induced a significant change in the expression patterns of genes involved in AM signaling, characterized by a "switch" from *RAMP2* to *RAMP3* expression in the lungs of septic animals. Importantly, we further established that genetic reduction of *AM* does not impact on the magnitude of these changes, since the response to LPS was similar in $AM^{+/-}$ and WT mice. Although some studies [23,24] have suggested that modulation of *RAMP2* and *RAMP3* may act to alter the clearance of AM peptide, our data show that the absolute level of AM peptide does not directly impact on their expression.

We conclude that during septic shock the dynamic modulation of AM and its receptors primarily functions to dampen the inflammatory response. Therefore, our in vivo studies using mice with genetically reduced levels of AM support the use of AM therapy to help counteract the detrimental effects of inflammation during septic shock in humans.

Acknowledgements

The authors would like to thank current and past members of the Caron Laboratory for helpful advice and discussions. This work was supported in part with funds from the Burroughs Wellcome Fund and a National Institutes of Health grant HD046970 to KMC.

References

1. Allaker RP, Zihni C, Kapas S. An investigation into the antimicrobial effects of adrenomedullin on members of the skin, oral, respiratory tract and gut microflora. FEMS Immunol Med Microbiol 1999;23:289–293. [PubMed: 10225288]

2. Caron K, Hagaman J, Nishikimi T, Kim HS, Smithies O. Adrenomedullin gene expression differences in mice do not affect blood pressure but modulate hypertension-induced pathology in males. Proc Natl Acad Sci U S A 2007;104:3420–3425. [PubMed: 17360661]

3. Caron KM, Smithies O. Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. Proc Natl Acad Sci U S A 2001;98:615–619. [PubMed: 11149956]

4. Cheung BM, Hwang IS, Li CY, O WS, Tsang KW, Leung RY, Kumana CR, Tang F. Increased adrenomedullin expression in lungs in endotoxaemia. J Endocrinol 2004;181:339–345. [PubMed: 15128282]

5. Dackor RT, Fritz-Six K, Dunworth WP, Gibbons CL, Smithies O, Caron KM. Hydrops fetalis, cardiovascular defects, and embryonic lethality in mice lacking the calcitonin receptor-like receptor gene. Mol Cell Biol 2006;26:2511–2518. [PubMed: 16537897]

6. Eto T, Kato J, Kitamura K. Regulation of production and secretion of adrenomedullin in the cardiovascular system. Regul Pept 2003;112:61–69. [PubMed: 12667626]

7. Fung E, Fiscus RR. Adrenomedullin induces direct (endothelium-independent) vasorelaxations and cyclic adenosine monophosphate elevations that are synergistically enhanced by brain natriuretic peptide in isolated rings of rat thoracic aorta. J Cardiovasc Pharmacol 2003;41:849–855. [PubMed: 12775961]

8. Gibbons C, Dackor R, Dunworth W, Fritz-Six K, Caron KM. Receptor Activity-Modifying Proteins: RAMPing up Adrenomedullin Signaling. Mol Endocrinol 2007;21:783–796. [PubMed: 17053041]

9. Gonzalez-Rey E, Chorny A, O'Valle F, Delgado M. Adrenomedullin protects from experimental arthritis by down-regulating inflammation and Th1 response and inducing regulatory T cells. Am J Pathol 2007;170:263–271. [PubMed: 17200199]

10. Hippenstiel S, Witzenrath M, Schmeck B, Hocke A, Krisp M, Krull M, Seybold J, Seeger W, Rascher W, Schutte H, Suttorp N. Adrenomedullin reduces endothelial hyperpermeability. Circ Res 2002;91:618–625. [PubMed: 12364390]

11. Hirata Y, Mitaka C, Sato K, Nagura T, Tsunoda Y, Amaha K, Marumo F. Increased circulating adrenomedullin, a novel vasodilatory peptide, in sepsis. J Clin Endocrinol Metab 1996;81:1449–1453. [PubMed: 8636349]

12. Ihara T, Ikeda U, Tate Y, Ishibashi S, Shimada K. Positive inotropic effects of adrenomedullin on rat papillary muscle. Eur J Pharmacol 2000;390:167–172. [PubMed: 10708720]

13. Isumi Y, Kubo A, Katafuchi T, Kangawa K, Minamino N. Adrenomedullin suppresses interleukin-1beta-induced tumor necrosis factor-alpha production in Swiss 3T3 cells. FEBS Lett 1999;463:110–114. [PubMed: 10601648]

14. Kagari T, Doi H, Shimozato T. The importance of IL-1 beta and TNF-alpha, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. J Immunol 2002;169:1459–1466. [PubMed: 12133972]

15. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–560. [PubMed: 8387282]

16. Li M, Yee D, Magnuson TR, Smithies O, Caron KM. Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. J Clin Invest 2006;116:2653–2662. [PubMed: 16981008]

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 2001;25:402–408. [PubMed: 11846609]

18. Mittra S, Bourreau JP. Gs and Gi coupling of adrenomedullin in adult rat ventricular myocytes. Am J Physiol Heart Circ Physiol 2006;290:H1842–1847. [PubMed: 16327020]

19. Mittra S, Hyvelin JM, Shan Q, Tang F, Bourreau JP. Role of cyclooxygenase in ventricular effects of adrenomedullin: is adrenomedullin a double-edged sword in sepsis? Am J Physiol Heart Circ Physiol 2004;286:H1034–1042. [PubMed: 14766677]

20. Nagoshi Y, Kuwasako K, Cao YN, Imamura T, Kitamura K, Eto T. Tumor necrosis factor-alpha downregulates adrenomedullin receptors in human coronary artery smooth muscle cells. Peptides 2004;25:1115–1121. [PubMed: 15245870]

21. Nishio K, Akai Y, Murao Y, Doi N, Ueda S, Tabuse H, Miyamoto S, Dohi K, Minamino N, Shoji H, Kitamura K, Kangawa K, Matsuo H. Increased plasma concentrations of adrenomedullin correlate

with relaxation of vascular tone in patients with septic shock. Crit Care Med 1997;25:953–957. [PubMed: 9201046]

22. Ono Y, Okano I, Kojima M, Okada K, Kangawa K. Decreased gene expression of adrenomedullin receptor in mouse lungs during sepsis. Biochem Biophys Res Commun 2000;271:197–202. [PubMed: 10777702]
23. Ornan DA, Chaudry IH, Wang P. Pulmonary clearance of adrenomedullin is reduced during the late stage of sepsis. Biochim Biophys Acta 1999;1427:315–321. [PubMed: 10216248]
24. Ornan DA, Chaudry IH, Wang P. Saturation of adrenomedullin receptors plays an important role in reducing pulmonary clearance of adrenomedullin during the late stage of sepsis. Biochim Biophys Acta 2002;1586:299–306. [PubMed: 11997081]
25. Renner EL, Dallenbach A. Increased liver enzymes: what should be done? Ther Umsch 1992;49:281–286. [PubMed: 1352416]
26. Senthil S, Sridevi M, Pugalendi KV. Cardioprotective effect of oleanolic acid on isoproterenol-induced myocardial ischemia in rats. Toxicol Pathol 2007;35:418–423. [PubMed: 17455091]
27. Sexton PM, Morfis M, Tilakaratne N, Hay DL, Udawela M, Christopoulos G, Christopoulos A. Complexing receptor pharmacology: modulation of family B G protein-coupled receptor function by RAMPs. Ann N Y Acad Sci 2006;1070:90–104. [PubMed: 16888151]
28. Shindo T, Kurihara H, Maemura K, Kurihara Y, Kuwaki T, Izumida T, Minamino N, Ju KH, Morita H, Oh-hashi Y, Kumada M, Kangawa K, Nagai R, Yazaki Y. Hypotension and resistance to lipopolysaccharide-induced shock in transgenic mice overexpressing adrenomedullin in their vasculature. Circulation 2000;101:2309–2316. [PubMed: 10811600]
29. Sugo S, Minamino N, Kangawa K, Miyamoto K, Kitamura K, Sakata J, Eto T, Matsuo H. Endothelial cells actively synthesize and secrete adrenomedullin. Biochem Biophys Res Commun 1994;201:1160–1166. [PubMed: 8024557]
30. Sugo S, Minamino N, Shoji H, Kangawa K, Kitamura K, Eto T, Matsuo H. Production and secretion of adrenomedullin from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. Biochem Biophys Res Commun 1994;203:719–726. [PubMed: 8074727]
31. Temmesfeld-Wollbruck B, Brell B, David I, Dorenberg M, Adolphs J, Schmeck B, Suttorp N, Hippenstiel S. Adrenomedullin reduces vascular hyperpermeability and improves survival in rat septic shock. Intensive Care Med 2007;33:703–710. [PubMed: 17318497]
32. Wang P, Yoo P, Zhou M, Cioffi WG, Ba ZF, Chaudry IH. Reduction in vascular responsiveness to adrenomedullin during sepsis. J Surg Res 1999;85:59–65. [PubMed: 10383838]
33. Wu R, Zhou M, Wang P. Adrenomedullin and adrenomedullin binding protein-1 downregulate TNF-alpha in macrophage cell line and rat Kupffer cells. Regul Pept 2003;112:19–26. [PubMed: 12667621]
34. Yang S, Zhou M, Chaudry IH, Wang P. Novel approach to prevent the transition from the hyperdynamic phase to the hypodynamic phase of sepsis: role of adrenomedullin and adrenomedullin binding protein-1. Ann Surg 2002;236:625–633. [PubMed: 12409669]
35. Zaks-Zilberman M, Salkowski CA, Elsasser T, Cuttitta F, Vogel SN. Induction of adrenomedullin mRNA and protein by lipopolysaccharide and paclitaxel (Taxol) in murine macrophages. Infect Immun 1998;66:4669–4675. [PubMed: 9746563]
36. Zhou M, Ba ZF, Chaudry IH, Wang P. Adrenomedullin binding protein-1 modulates vascular responsiveness to adrenomedullin in late sepsis. Am J Physiol Regul Integr Comp Physiol 2002;283:R553–560. [PubMed: 12184987]
37. Zudaire E, Portal-Nunez S, Cuttitta F. The central role of adrenomedullin in host defense. J Leukoc Biol 2006;80:237–244. [PubMed: 16769769]

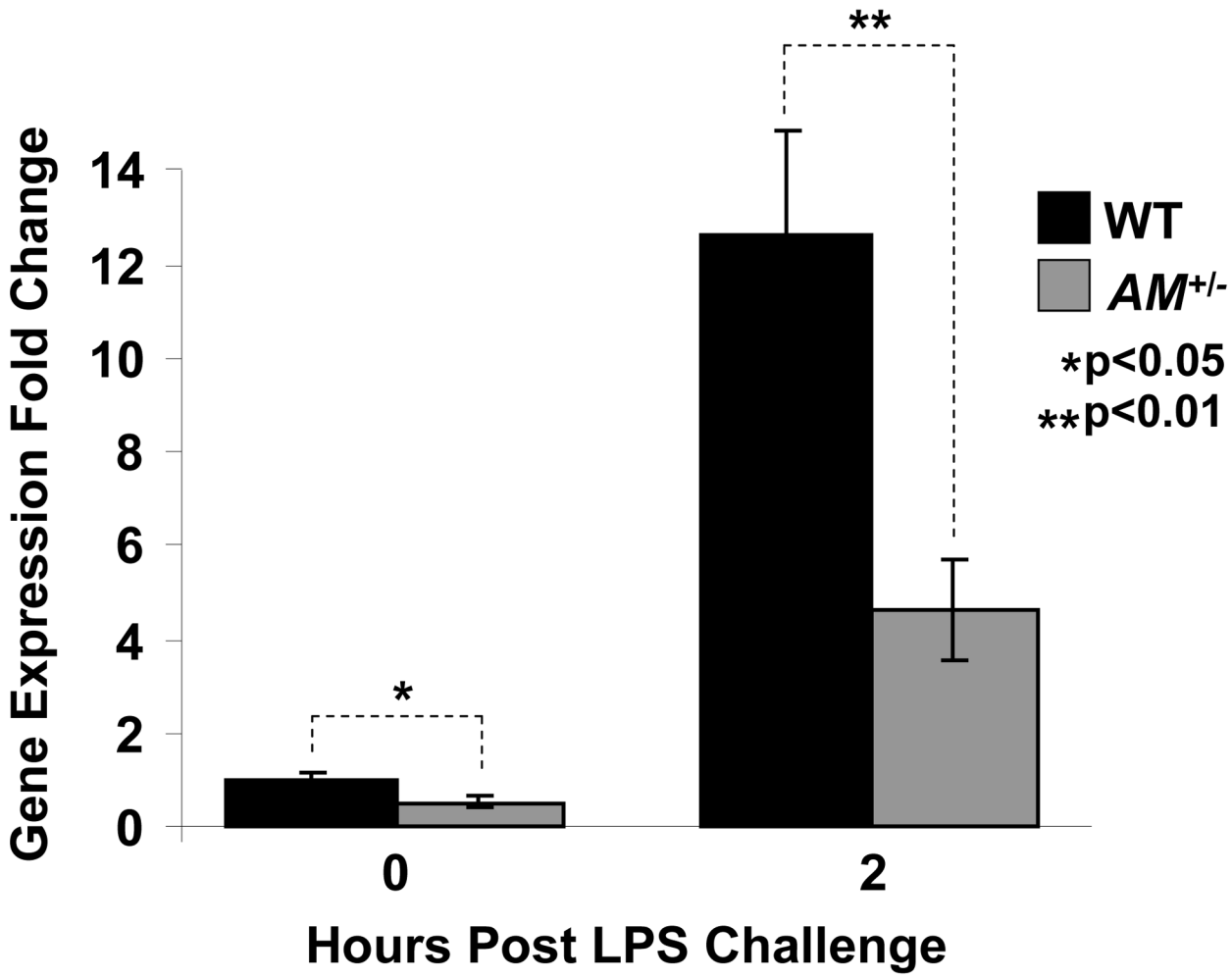

Figure 1.
AM gene expression levels before and after LPS challenge. Total lung RNA was prepared from WT and $AM^{+/-}$ mice treated with or without LPS for 2 hours. *AM* gene expression was measured by quantitative RT-PCR. At least 7 animals were used in each group.

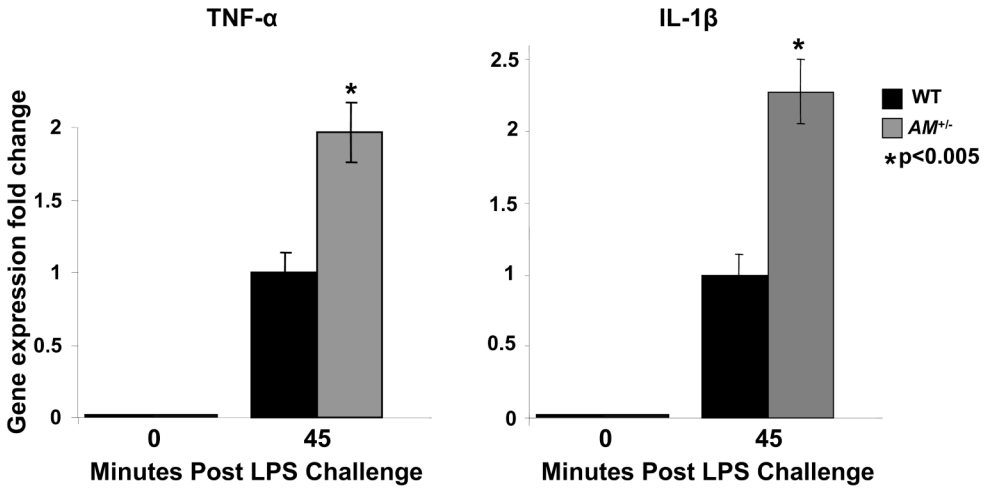

Figure 2.

Gene expression levels of *TNF-α* and *IL-1β* following LPS challenge. Total lung RNA was prepared from WT and $AM^{+/-}$ mice treated with or without LPS for 45 minutes. Expression of these genes was measured by quantitative RT-PCR. Four mice were used in each group.

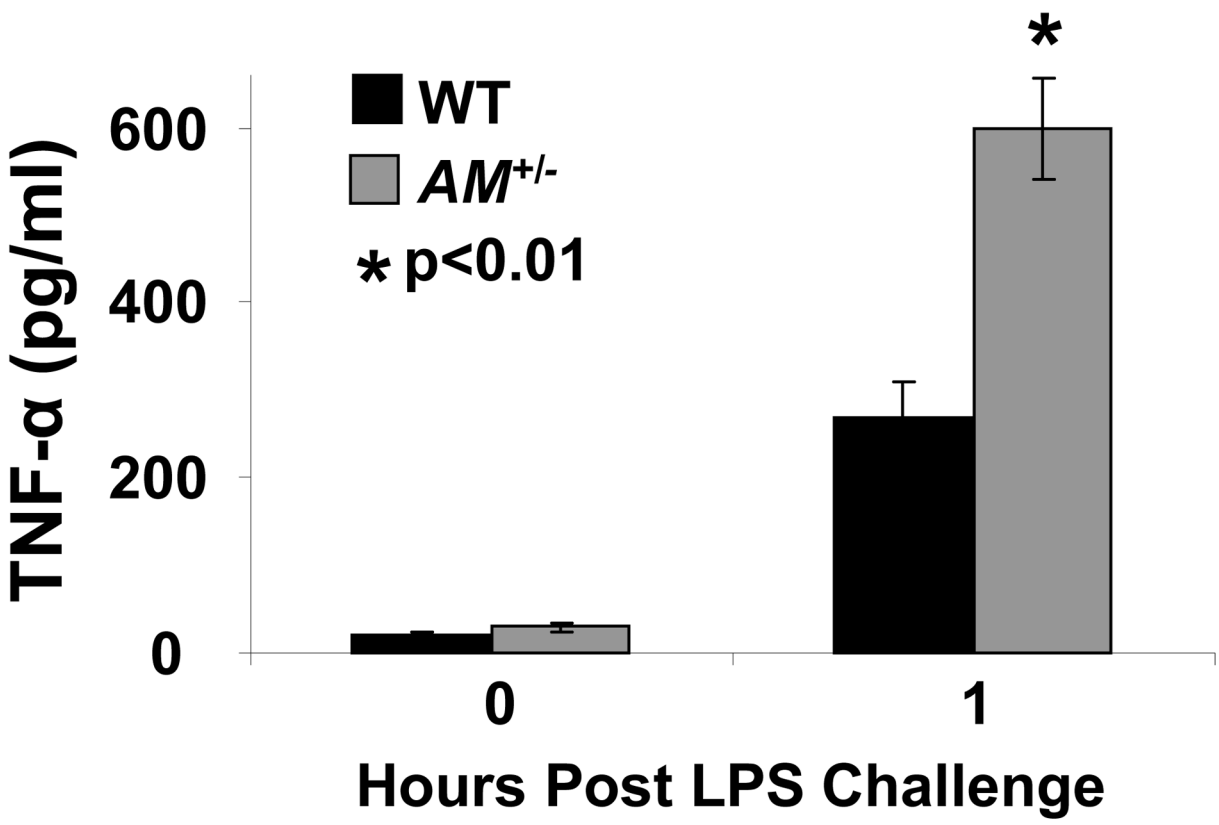

Figure 3.
Serum TNF-α levels following LPS challenge. Serum from WT and $AM^{+/-}$ mice was collected by tail-vein bleed at the indicated time points following treatment with 100 mg/kg LPS. TNF-α was analyzed by ELISA. n=8 for WT mice and n=9 for $AM^{+/-}$ mice.

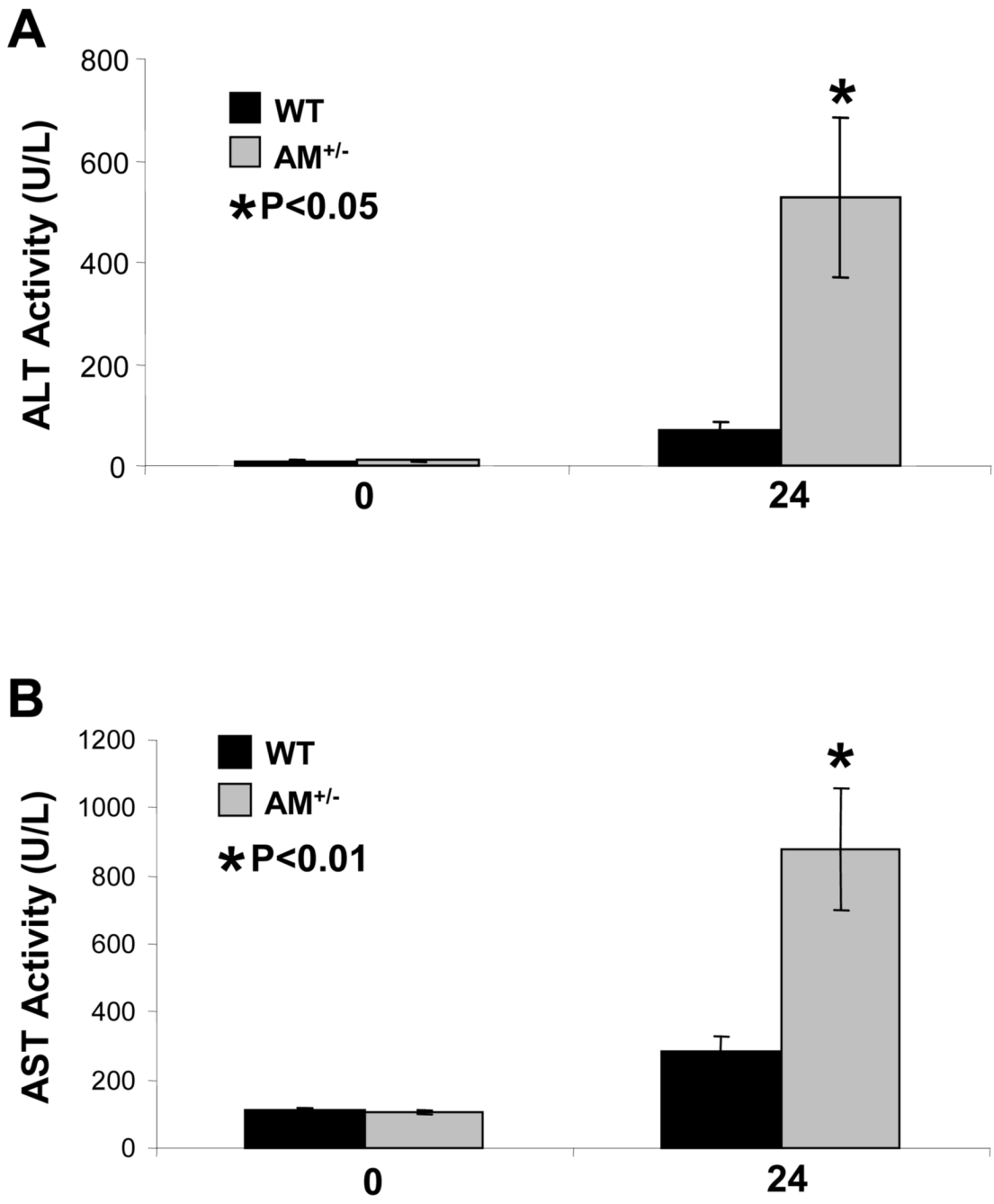

Figure 4.
Serum (*A*) ALT and (*B*) AST levels following LPS challenge. Serum from WT and $AM^{+/-}$ mice treated with or without LPS for 24 hours was collected. At least 6 mice were used in each group.

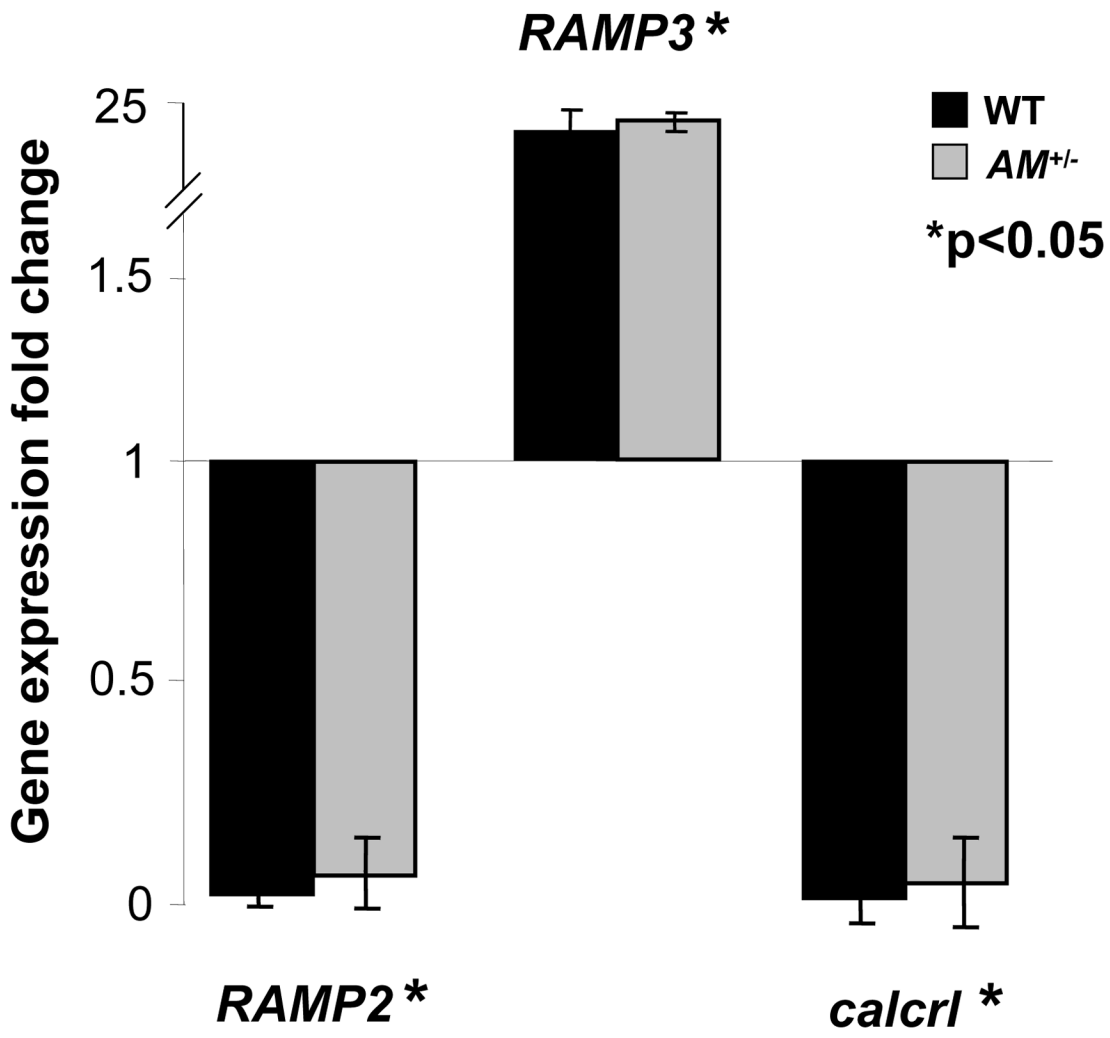

Figure 5.

AM receptor gene expression in WT and $AM^{+/-}$ mice following LPS challenge. Total lung RNA was prepared from WT and $AM^{+/-}$ mice treated with or without LPS for 12 hours. Expression of *RAMP2*, *RAMP3* and *calcrl* were measured by quantitative RT-PCR. Four mice were used in each group.