Increased Angiotensin II–Induced Hypertension and Inflammatory Cytokines in Mice Lacking Angiotensin-Converting Enzyme N Domain Activity


Abstract—Angiotensin-converting enzyme (ACE) is composed of the N- and C-terminal catalytic domains. To study the role of the ACE domains in the inflammatory response, N-knockout (KO) and C-KO mice, models lacking 1 of the 2 ACE domains, were analyzed during angiotensin II–induced hypertension. At 2 weeks, N-KO mice have systolic blood pressures that averaged 173±4.6 mm Hg, which is more than 25 mm Hg higher than the blood pressures observed in wild-type or C-KO mice (146±3.2 and 147±4.2 mm Hg). After 3 weeks, blood pressure differences between N-KO, C-KO, and wild-type were even more pronounced. Macrophages from N-KO mice have increased expression of tumor necrosis factor α after stimulation with either lipopolysaccharide (about 4-fold) or angiotensin II (about 2-fold), as compared with C-KO or wild-type mice. Inhibition of the enzyme prolyl oligopeptidase, responsible for the formation of acetyl-SerAspLysPro and other peptides, eliminated the blood pressure difference and the difference in tumor necrosis factor α expression between angiotensin II–treated N-KO and wild-type mice. However, this appears independent of acetyl-SerAspLysPro. These data establish significant differences in the inflammatory response as a function of ACE N- or C-domain catalytic activity. They also indicate a novel role of prolyl oligopeptidase in the cytokine regulation and in the blood pressure response to experimental hypertension. (Hypertension. 2012;59:283-290.) ● Online Data Supplement

Key Words: angiotensin-converting enzyme ■ hypertension ■ angiotensin II ■ prolyl oligopeptidase ■ acetyl SDKP

Angiotensin-converting enzyme (ACE) is a zinc-dependent carboxy dipeptidase that is responsible for the conversion of angiotensin I to angiotensin II. Although ACE is a single polypeptide chain, it is composed of 2 homologous and independent catalytic domains, often termed the N- and C-terminal domains. Each domain binds a zinc molecule that is required for catalysis. Despite genetic divergence, the 2 catalytic domains retain significant structural homology, particularly in areas critical for catalysis. However, in vitro analysis has demonstrated that the two catalytic sites differ in affinity and effectiveness for cleaving individual peptide substrates. For example, the C-terminal catalytic site has a higher K_m for angiotensin I than the N-terminal domain. In contrast, the 4 amino acid ACE substrate acetyl-SerAspLysPro (AcSDKP) is cleaved almost exclusively by the N-terminal catalytic domain. It is released from its precursor, the activatase thymosin β_4, by the action of prolyl oligopeptidase (POP), a serine peptidase. AcSDKP is elevated in N-knockout (KO) mice and in patients on ACE inhibitors. However, AcSDKP is only one of several peptides produced or degraded by POP; other POP substrates include angiotensin I, angiotensin II, bradykinin, bradykinin-potentiating protein, substance P, oxytocin, and vasopressin.

There is growing recognition that inflammation plays an important role in the development of hypertension. Recent reports have demonstrated that immune cells significantly modulate blood pressure changes and target organ damage, particularly in angiotensin II–induced hypertension. Moreover, mice deficient in certain proinflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin (IL)-6, have a blunted hypertensive response to chronic angiotensin II infusion. To understand the functional role of the N- and C-domains of ACE in the cytokine and the inflammatory response, N-knockout (KO) and C-KO mice, models lacking 1 of the 2 ACE domains, were analyzed during angiotensin II–induced hypertension. At 2 weeks, N-KO mice have systolic blood pressures that averaged 173±4.6 mm Hg, which is more than 25 mm Hg higher than the blood pressures observed in wild-type or C-KO mice (146±3.2 and 147±4.2 mm Hg). After 3 weeks, blood pressure differences between N-KO, C-KO, and wild-type were even more pronounced. Macrophages from N-KO mice have increased expression of tumor necrosis factor α after stimulation with either lipopolysaccharide (about 4-fold) or angiotensin II (about 2-fold), as compared with C-KO or wild-type mice. Inhibition of the enzyme prolyl oligopeptidase, responsible for the formation of acetyl-SerAspLysPro and other peptides, eliminated the blood pressure difference and the difference in tumor necrosis factor α expression between angiotensin II–treated N-KO and wild-type mice. However, this appears independent of acetyl-SerAspLysPro. These data establish significant differences in the inflammatory response as a function of ACE N- or C-domain catalytic activity. They also indicate a novel role of prolyl oligopeptidase in the cytokine regulation and in the blood pressure response to experimental hypertension. (Hypertension. 2012;59:283-290.) ● Online Data Supplement

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response secondary to the induction of experimental hypertension, we chronically infused angiotensin II into 2 lines of genetically engineered mice harboring point mutations that specifically inactivate either the N- or the C-terminal catalytic domain of ACE; these mice are termed N-KO and C-KO, respectively. In these mouse models, the genetic mutations have no effect on the tissue distribution or levels of ACE expression. Under basal conditions, N-KO and C-KO mice have normal and equivalent blood pressures and plasma levels of angiotensin II. Thus, the N-KO and C-KO mice permit investigation of ACE domain-specific functions independent of secondary effects of basal blood pressure changes, a typical bias introduced with either ACE inhibitors or the genetic elimination of all ACE expression.

Methods

The systolic blood pressure of N-KO, C-KO, and WT mice was repeatedly measured by tail-cuff plethysmography for 2 or 3 weeks during the infusion of 980 ng/kg per minute of angiotensin II by osmotic minipump. For the blood pressure analysis, at least 21 animals per group were studied. For in vivo experiments in which POP was inhibited, N-KO and wild-type (WT) mice received 2 weeks of angiotensin II (980 ng/kg per minute) and received daily intraperitoneal (IP) injections of 10 mg/kg of S-17092. Blood pressure was measured as described above. At least 9 mice per group were studied.

For in vitro production of cytokines, peritoneal macrophages from N-KO, C-KO, and WT mice were collected after thioglycollate injection. After purification, cells were cultured overnight with 1 mg/mL lipopolysaccharide (LPS). Supernatant concentrations of TNFα, IL-12, and IL-10 were measured by ELISA. To measure in vivo production of TNFα, N-KO, C-KO, and WT mice were injected IP with 45 mg/kg of LPS, and sera levels of TNFα were measured by ELISA. For all figures showing cytokine production, the number of mice studied is indicated by individual data points.

To examine the role of AcSDKP in blood pressure control, WT or N-KO mice received 980 ng/kg per minute of angiotensin II for 2 weeks. Groups of 10 WT mice received 0, 0.5, 1.5, or 3.0 mg/kg per day AcSDKP beginning 1 week before the start of angiotensin II infusion and continuing throughout the experiment. Blood pressure was repeatedly measured as described above. In a separate experiment, all N-KO mice received daily IP injections of 10 mg/kg of S-17092 in addition to angiotensin II. Five N-KO mice also received 1.5 mg/kg per day of AcSDKP by osmotic minipumps. Blood pressure was repeatedly measured. A full description of the Materials, Methods, and Statistics is found in the online Data Supplement (available at http://hyper.ahajournals.org).

Results

Under basal conditions, the systolic blood pressures of the N-KO, C-KO, and WT mice averaged 111 ± 2 mm Hg, 105 ± 2 mm Hg, and 112 ± 1 mm Hg. The mice were implanted with osmotic minipumps that delivered 980 ng/kg per minute of angiotensin II for 2 weeks. The blood pressure was determined 3, 5, 7, 10, 12, and 14 days after the start of angiotensin II infusion (Figure 1). At day 5, the blood pressures of both N-KO and C-KO mice were significantly higher than WT mice. The blood pressures of the C-KO mice reached a peak at day 7 (164 ± 5 mm Hg) and then decreased so that by day 14, the pressures of these mice were virtually identical to WT. In contrast, the blood pressures of the N-KO mice remained significantly above the WT animals from day 5 onward and at day 14 averaged 173 ± 5 mm Hg (P < 0.001 for N-KO versus WT or C-KO). Thus, after a 2-week infusion of angiotensin II, N-KO mice had systolic blood pressures that averaged 26 and 27 mm Hg greater than C-KO or WT mice.

To investigate if the pressure differences between N-KO mice, C-KO and WT mice were sustained, smaller numbers of mice were infused with angiotensin II for 21 days. On days 19 and 21, the N-KO mice averaged 200 ± 5 and 190 ± 4 mm Hg (n = 5), whereas WT averaged 147 ± 5 and 147 ± 4 mm Hg (n = 4, P ≤ 0.001). C-KO mice averaged 142 ± 8 and 127 ± 10 mm Hg (n = 3, P < 0.001). Thus, the sustained elevation of blood pressure in the N-KO group was maintained in the 3rd week of angiotensin II infusion.

To understand the difference in blood pressure response between the N-KO, C-KO, and WT mice, we measured blood and kidney angiotensin peptides under basal conditions and after 14 days of angiotensin II infusion (Figure 2). Under basal conditions, the 3 groups had similar levels of angiotensin II in the blood; kidney levels were also equivalent among the 3 groups. Infusion of angiotensin II significantly elevated blood angiotensin II in all mice, but among the groups there were no significant differences. At 14 days, kidney angiotensin II levels were also markedly higher than basal levels. However, whereas levels in C-KO and WT mice were virtually identical, renal levels of
Angiotensin II in the N-KO mice were 1.5-fold those of the other two groups (n=14 per group; P<0.015).

Previously, we reported that basal blood angiotensin I levels were elevated in C-KO mice. This is also true for renal angiotensin I levels and reflects the elevated renin found in this model (Figure 2).

Angiotensin 1–7 is reported as an N-terminal–specific substrate of ACE. Thus, we wondered if the level of this peptide would be elevated in the N-KO mice, similar to the elevated levels of the N terminal substrate AcSDKP. However, under basal conditions, no significant differences in blood or renal angiotensin 1–7 were found in WT, N-KO, or C-KO mice (Supplemental Figure 1). With the infusion of angiotensin II, there was a rise in angiotensin 1–7 levels, with the slope of the rise being greatest for the N-KO group. However, by 2-way ANOVA, taking into account time and genotype, there was no group significance. Finally, at 14 days, there were no significant differences by 1-way ANOVA. Bradykinin showed no significant differences between the groups (Supplemental Figure 1).

To understand the role of inflammation in the blood pressure response of the N-KO mice, we first studied a simple model of inflammatory cytokine release in response to LPS. In this experiment, there was no angiotensin II infusion. Four days after IP injection of thioglycollate, peritoneal macrophages from N-KO, C-KO, and WT mice were collected and cultured overnight with LPS (a model activator of macrophages). TNFα levels in the supernatants were then measured (Figure 3A). To our surprise, there was a marked difference in cytokine profile; macrophages derived from N-KO mice produced far more TNFα than either C-KO or WT mice (N-KO versus C-KO or WT, P<0.001). Study of the time course of TNFα protein expression showed that as early as 4 hours after LPS addition, there were differences between N-KO and WT cells; by 8 hours, the differences were pronounced (Supplemental Figure 2).

To investigate whether differences in TNFα expression also exist in vivo, groups of N-KO and WT mice were injected IP with LPS. Maximum blood levels of TNFα were found 90 minutes after the LPS injection (Figure 3B). At this point, TNFα levels in the N-KO mice were nearly double those of the WT mice (P<0.005). In contrast, the same experiment performed with C-KO mice showed a rise of TNFα virtually identical to WT. Thus, both in vitro and in vivo data show that in the absence of ACE N-terminal catalytic activity, TNFα expression was increased.

Thioglycollate-elicited macrophages were also examined for LPS-stimulated expression of other cytokines. IL-12, a proinflammatory cytokine, showed a pattern...
similar to TNFα, namely after culture for 18 hours with LPS, N-KO derived macrophages made 3-fold the cytokine levels of WT cells (P<0.001 for N-KO vs either C-KO or WT). Cells from N-KO mice produced far more TNFα than the equivalent cells from the other groups. In contrast, expression of the anti-inflammatory cytokine IL-10 by N-KO cells was only about half that produced by either WT or C-KO macrophages (Supplemental Figure 3B). In summary, the inflammatory response of macrophages from N-KO mice was substantially different from that of either WT or C-KO derived cells; in response to LPS, N-KO cells produced cytokines typical of a strong proinflammatory response. In contrast, C-KO–derived cells resembled WT.

The above data examined N-KO mice for TNFα expression after LPS. To study the effects of angiotensin II, N-KO and WT mice were infused with the peptide for 14 days. When the animals were killed, peritoneal cells were collected, treated with brefeldin A to inhibit protein secretion, then stained for surface expression of F4/80 (a macrophage marker), fixed, permeabilized, and stained for cytoplasmic TNFα, which was analyzed by flow cytometry. Representative data are shown in Figure 4A, where the top panel shows data from an N-KO mouse and a WT is shown on the bottom. The percentage of F4/80+/TNFαhigh cells versus total F4/80+ cells is shown in the box. Combining data from at least 9 mice per group, N-KO mice averaged 40.3±4.7% F4/80+/TNFαhigh, whereas WT averaged 19.8±2.8% (P<0.002) (Figure 4B). Cumulatively, our data establish a compelling case for differences in cytokine expression; to our knowledge, no other group

**Figure 3.** Macrophage tumor necrosis factor-α (TNFα) production in response to lipopolysaccharide (LPS). A, Peritoneal macrophages from N-knockout (KO), C-KO, and wild-type (WT) mice were collected after thioglycollate injection. After purification, the macrophages were cultured with LPS for 18 hours and TNFα was measured. Each dot represents data from 1 mouse. Group means and SEM are also shown (P<0.001 for N-KO vs either C-KO or WT). Cells from N-KO mice produced far more TNFα than the equivalent cells from the other groups. B, N-KO, C-KO, and WT mice received a single IP injection of LPS. After 60, 90, and 120 minutes, plasma concentrations of TNFα were determined. Each dot is data from a single mouse. Group means and SEM are also shown. At 90 minutes, levels of TNFα are significantly higher in N-KO mice compared with the 2 other groups (P<0.005).

**Figure 4.** Macrophage production of tumor necrosis factor-α (TNFα) in response to angiotensin II. After mice received angiotensin II for 14 days, peritoneal cells were collected by lavage. The cells were then stained with fluorescent-tagged antibody to F4/80 and TNFα. Fluorescence intensity was measured by FACS. A, Typical results from an N-KO (top) and WT mouse (bottom). The percentage of F4/80+/TNFαhigh cells versus total F4/80+ cells is shown in the box. B, This percentage is shown for 9 WT and 10 N-KO mice. The group mean and SEM are also indicated (P<0.002).
has ever identified differences in the inflammatory response as a function of ACE N- or C-domain catalytic activity.

Part of the reason why N-KO mice have higher blood pressures in angiotensin II–induced hypertension may be the differences in the inflammatory response discussed above. Further, we wondered if these differences might be a consequence of the known increased AcSDKP in the N-KO model, as AcSDKP can influence the inflammatory response. To test this, we blocked formation of AcSDKP in N-KO and WT mice by injection of the POP inhibitor S-17092 for 4 days before the collection of thioglycollate-induced peritoneal macrophages.17,18 Macrophages were then cultured overnight with LPS, and TNFα levels in the supernatants were measured (Figure 5A). These data showed that POP inhibition reduced N-KO macrophage production of TNFα to levels equivalent to those of cells from WT mice. Further, the effect of S-17092 was limited to the N-KO mice. Thus, these data indicate that POP activity affects in vitro macrophage production of TNFα.

To test the effects of POP in vivo, and specifically whether this contributes to the increased blood pressure response to angiotensin II, groups of N-KO and WT mice were treated with the POP inhibitor S-17092 by daily IP injection beginning 3 days before implantation of the minipumps delivering angiotensin II (Figure 5B). Blood pressure was then measured during the 14 day angiotensin II infusion. S-17092 had little effect on WT mice. However, N-KO mice treated with S-17092 increased blood pressure in response to angiotensin II significantly less than N-KO mice without S-17092 (P < 0.001 on day 14). Or, put differently, the 25 mm Hg average difference in blood pressure between N-KO and WT mice treated with angiotensin II was completely eliminated by blocking POP.

Although AcSDKP is produced by POP, it is only one of several peptide end-products of this peptidase. To directly investigate the effect of AcSDKP on blood pressure, we infused this peptide by minipump in WT mice. The mice were treated with 1 of 3 doses of AcSDKP (0.5, 1.5 or 3.0 mg/kg per day) beginning 1 week before the start of angiotensin II infusion, and AcSDKP infusion was continued through the 14 day angiotensin II infusion period. Blood pressure was measured at several time points throughout the experiment. At no time did the mice receiving AcSDKP show a significant difference in blood pressure from that observed in WT mice treated with angiotensin II in the absence of AcSDKP (Supplemental Figure 4).

The lack of blood pressure effects observed with AcSDKP infusion may be due to the large amount of ACE in WT mice and the ability of this enzyme (which has N-terminal activity) to degrade AcSDKP. To address this, we studied N-KO mice treated with angiotensin II in which POP activity was blocked by daily S-17092 injection but in which AcSDKP levels were increased by minipump infusion of 1.5 mg/kg per day of peptide, a dose consistent with previous studies.19 These mice were compared with equivalent N-KO mice treated with angiotensin II and S-17092 but without AcSDKP supplementation (Figure 6). No significant elevation of blood pressure was found with AcSDKP infusion. Further, FACS analysis of TNFα production by peritoneal macrophages showed no effect with AcSDKP infusion, as compared with mice treated with angiotensin II and S-17092 but without AcSDKP supplementation (Supplemental Figure 5). Thus, these data do not indicate a role of AcSDKP in the elevated blood pressure or inflammation of N-KO mice treated with angiotensin II.
Discussion

ACE inhibitors are widely used to treat hypertension and heart disease. The goal of such therapy is blocking all ACE activity and these inhibitors appropriately block both ACE domains. However, the clinical emphasis on the role of ACE in cardiovascular disease often obscures the implications of an enzyme that has maintained 2 independent catalytic sites throughout millions of years of evolution. These domains can be individually studied in the N-KO and C-KO mice; perhaps the most important conclusion from these mice is that they establish beyond doubt that the 2 ACE catalytic domains are different. In vivo, it is the ACE C-terminal domain that normally produces the substantial majority of angiotensin II. In the C-KO mouse, typical concentrations of blood angiotensin II are only maintained through marked compensatory elevations of renin and angiotensin I levels. In contrast, N-KO mice have blood renin and angiotensin I levels that are essentially equivalent to those of WT animals.

Further evidence for the asymmetry of ACE domain activity is the behavior of N-KO and C-KO mice when each strain was infused for 2 weeks with angiotensin II. C-KO mice demonstrated a very marked elevation of blood pressure during the first week of peptide infusion. This peaked at 7 days and then diminished slowly during the second week to levels equivalent to those of WT mice. Although C-KO mice have a basal elevation of angiotensin I and renin, we do not know whether this contributes to the exaggerated blood pressure 7 days after the start of angiotensin II infusion.

In contrast to the C-KO model, the N-KO mouse shows an initial elevation of blood pressure that is greater than that of WT and that continues to increase and to maintain the pressure differential with WT mice for at least 21 days. At the end of the infusion period, N-KO mice have blood pressures that averaged approximately 25 mm Hg higher than those seen in C-KO or WT mice. Our studies present 2 possible reasons for this difference in blood pressure response to angiotensin II. One possibility reflects the asymmetrical accumulation of angiotensin II within the kidneys of the N-KO mice, as compared with WT or C-KO animals. The increased renal angiotensin II in the N-KO model may partly explain the elevated blood pressure, since previous work has demonstrated that elevated renal levels of angiotensin II are critical for the hypertensive response to angiotensin II. Both previous study of N-KO mice, and the data presented in the present report, show no significant difference in blood levels of angiotensin II in N-KO versus WT mice. Thus, some other mechanism must be responsible for enhanced renal accumulation of angiotensin II in a low renin environment. One possibility, suggested by the literature, is differences in intratubular generation of angiotensin II.

In addition to the contribution of renal angiotensin II, we believe an additional influence is differences in the inflammatory milieu present in N-KO versus C-KO or WT mice. N-KO mice show both in vitro and in vivo evidence for elevated production of TNFα and other cytokines generally associated with an increased inflammatory response. This was seen in response to in vitro stimulation with LPS and also in peritoneal macrophages collected after the 2-week infusion of angiotensin II. There is now substantial evidence that a significant part of blood pressure elevation seen in response to angiotensin II infusion is due to immune activation. Thus, the enhanced macrophage pro-inflammatory cytokine production in N-KO mice exposed to angiotensin II is a second physiological explanation for why these mice have an exaggerated blood pressure response.

One obvious difference between N-KO and WT mice is that N-KO mice have chronically elevated AcSDKP levels. AcSDKP is 1 of 4 peptides reported to be specific substrates of the ACE N-domain (AcSDKP, angiotensin 1–7, LH-RH, amyloid β1–42). AcSDKP is produced by the enzyme POP, which can be inhibited by the pharmaceutical S-17092, a very specific POP inhibitor. We treated N-KO mice with S-17092, using conditions previously shown to reduce plasma AcSDKP levels to WT levels. With POP inhibition, N-KO macrophage production of TNFα in response to LPS was restored to WT levels. Further, POP inhibition reduced the angiotensin II–mediated elevation of blood pressure in N-KO mice to the levels seen in WT mice. However, we were unable to establish that AcSDKP was the cause of the N-KO phenotype. Specifically, when N-KO mice were treated with

Figure 6. Effects of acetyl-SerAspLysPro (AcSDKP) on blood pressure and inflammation. N-knockout (KO) mice were all treated with angiotensin (Ang) II and S-1702. One group also received 1.5 mg/kg per day AcSDKP by minipump. n=5 per group. By Student t test, P=0.05 for all days.

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angiotensin II, S-17092, and AcSDKP, no increase in the blood pressure response was observed as compared with mice treated with angiotensin II and S-17092 but without AcSDKP. Further, AcSDKP supplementation did not affect peritoneal macrophage production of TNFα. Previous published work showed that the short term administration of AcSDKP was associated with a reduced fibrotic response and not with any type of proinflammatory response.26 Thus, our data and previous published findings are consistent in indicating that the elevated AcSDKP levels in N-KO mice are not the primary cause of the N-KO phenotype. Of the other known ACE N-terminal–specific substrates, none appear to be good candidates to explain the N-KO data. Angiotensin 1–7 is generally considered a vasodilator. LH-RH and β amylod are not suspected of influencing blood pressure effects.

POP is a 710-residue serine endopeptidase that is widely distributed in tissues.18 The renal cortex is particularly rich in this enzyme. Although POP was discovered in human uterus through its ability to degrade oxytocin, the enzyme is thought to be active on several peptides, including angiotensin I and angiotensin II (releasing angiotensin 1–7), bradykinin, and vasopressin. Several studies have implicated POP in central nervous system function, including learning and memory, and this has led to the development of several specific and high-affinity inhibitors.6 For example, S-17092 was shown to have a Ki for POP of 1.3 nmol/L in the rat and to not inhibit several other peptidases, including aminopeptidase M, dipeptidylpeptidase IV, neprilysin, endopeptidases 3.4.24.15 and 3.4.24.16, calpains, and ACE. Although there has been some speculation about a role of POP in blood pressure control, there has been little experimentation addressing this topic.27 POP knockout mice have been created, but their blood pressure was not reported.28 Our data are notable for the difference in effect between N-KO and WT mice treated with a POP inhibitor; the blood pressure–lowering effect is far more pronounced in the N-KO. To us, this implies an important balance between the actions of POP and ACE. One well-recognized product of POP is AcSDKP, which is destroyed by ACE. However, this does not appear to be the cause of the N-KO phenotype. We conclude that other POP products must also interact with the ACE N-terminus.

Perspectives

The lack of ACE N-terminal activity is associated with an exaggerated blood pressure response to angiotensin II, and an increased proinflammatory immune response to lipopolysaccharide or angiotensin II, as compared with WT or C-KO mice. These data underline important physiologic differences in the function of the 2 catalytic domains of ACE, a novel finding. All commonly used ACE inhibitors block catalytic activity of both ACE domains; once we understand the actions of the ACE N- and C-terminal domains, there may be clinical implications of ACE site-specific inhibition, which may ultimately translate into new treatment strategies using site-specific ACE inhibitors.

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Disclosures

None.

References


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INCREASED ANGIOTENSIN II INDUCED HYPERTENSION AND INFLAMMATORY CYTOKINES IN MICE LACKING ANGIOTENSIN CONVERTING ENZYME N DOMAIN ACTIVITY

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Detailed Methods

Materials. LPS (Escherichia coli 055:B5) was from Sigma-Aldrich (St. Louis, MO). The prolyl oligopeptidase inhibitor S-17092 was a generous gift from Servier (Suresnes, France). Anti-mouse IL-10 functional grade purified antibody was from eBioscience (San Diego, CA). Murine tumor necrosis factor α (TNFα), IL-10 and IL-12/p40 ELISA kits were purchased from eBioscience (San Diego, CA) and used according to manufacturer's instructions.

Mice. The generation of mice expressing site-inactivated ACE was previously described in detail.1,2 Briefly, N-KO mice express a full length ACE protein where the N-terminal catalytic site was inactivated by mutating the two zinc-binding histidines (395H and 399H) to lysines. In the C-KO strain, 993H and 997H, which are responsible for the zinc binding and catalytic activity of the C-terminal enzymatic site, were mutated to lysine. During the back breeding of mice to C57BL/6, animals were selected for mating so that all mice possess only a single renin gene (Ren-1c). 8 to 12-week old N-KO, C-KO or wild-type littermates were used. Animal breeding and experimental procedures were approved by Cedars-Sinai Medical Center Institutional Animal Care and Use Committee.

Peptide measurement. Mice were anesthetized and blood was collected from the inferior vena cava directly into a syringe containing 5 ml of 4 mol/l guanidine thiocyanate using a 25-gauge needle. The left kidney was then rapidly removed and homogenized in 5 ml of 4 mol/l guanidine thiocyanate. The blood and tissue homogenates were frozen and stored at -80°C until shipped on dry ice to St. Vincent's Institute of Medical Research, where peptide measurements were performed. Peptides were measured using HPLC-based radioimmunoassays, as previously described.3

Blood pressure. Systolic blood pressures were measured as previously described.4 Briefly, blood pressure was measured in conscious mice using a Visitech Systems BP2000-automated tail-cuff system. Mice were trained for several days before data acquisition. On each day that blood pressure was determined, 20 measurements were collected and averaged to calculate the pressure.

Angiotensin II administration. Angiotensin II (Bachem AG) at the dose of 980 ng/kg/min was infused via osmotic mini-pumps (Alzet model 1002). The mini-pumps were implanted subcutaneously between the scapulas following manufacturer’s instructions. Angiotensin II was infused for either 14 or 21 day. At the end of the experiment, the mice were sacrificed by CO2 inhalation and cervical dislocation.

Flow cytometry and cytokine determination. To measure cytokine expression, peritoneal cells were collected and cultured for 5 hrs with 5 µg/ml brefeldin A. Cells were surface-stained with FITC-conjugated anti-F4/80 (BioLegend) followed by intracellular staining of APC-conjugated anti-TNFα (eBioscience) with fixation and permeabilization buffer (eBioscience). The stained samples were analyzed on a Beckman Coulter CyAn ADP and data were analyzed with FlowJo software.

Peritoneal macrophage extraction. Thioglycollate-elicited peritoneal exudates were collected via peritoneal lavage 4 days after a 2 ml intraperitoneal injection of 3% thioglycollate broth. 1x10^6/ml cells were cultured at 37°C, 5% CO2 in RPMI 1640 medium enriched with 10% fetal calf serum, 50 µM 2-ME, 0.5 mM sodium pyruvate, 10 mM HEPES buffer, 50 units/ml penicillin, 50 µg/ml streptomycin, and 2mM L-glutamine. For purification of macrophages, peritoneal exudates were allowed to adhere for 2 h, after which
non-adherent cells were removed with phosphate buffered saline (PBS) to achieve a
>95% purity of macrophages. For flow cytometry assay, peritoneal exudates were cultured in Costar low adherence culture plates. Then, the macrophages were cultured as above with or without lipopolysaccharide (LPS, 1 mg/ml) for 18 h. The concentration of TNFα, IL-10, and IL-12/p40 in the supernatant was determined by ELISA. In Fig. 6a, daily intraperitoneal S-17092 (40 mg/kg) was given was given for 4 days.

**POP inhibition with S-17092.** The specific POP inhibitor S-17092 was administered daily to mice IP at a dose of 10 mg/kg in normal saline from a freshly prepared solution. For *in vitro* experiments, injection of S-17092 began 4 days before the collection of thioglycollate-induced peritoneal macrophages. The macrophages were carefully counted to verify that the yield of macrophages was not affected by S-17092. For *in vivo* inhibition of POP, S-17092 administration began four days before angiotensin II mini-pump implantation and continued daily until the mice were sacrificed.

**In vivo LPS challenge.** Mice were injected intraperitoneally with 45 mg/kg of LPS in sterile PBS, and blood was collected 60 min, 90 min, and 120 min after injection. TNFα concentration in sera was measured by ELISA.

**Statistics.** All results are expressed as mean ± SEM, with p<0.05 considered statistically significant. Unless otherwise stated, one-way ANOVA analysis with a Tukey correction was used to analyze significance between groups. For analysis of angiotensin 1-7 levels, data was analyzed using two-way ANOVA taking into account the interaction between genotype and time. Where indicated, we use a two tailed Student's t-test.

**Online Supplement References**

S1. Angiotensin 1-7 and bradykinin peptide levels.

On day 0, no significant differences in blood or renal angiotensin 1-7 were found in WT, N-KO or C-KO mice. With the infusion of angiotensin II, there was a rise in angiotensin 1-7 levels, with the slope of the rise being greatest for the N-KO group. However, by 2-way ANOVA, taking into account the interaction of time and genotype, there were no significant differences between the groups. Also, on day 14, there were no significant differences by 1-way ANOVA. There was no significant difference in blood or kidney bradykinin levels between the 3 groups of mice. For both angiotensin 1-7 and bradykinin, the number of mice studied on day 0 was 12, 6 and 12 for N-KO, C-KO and WT, while for day 14 the number of mice studied was 17, 12 and 15 for N-KO, C-KO and WT. Blood levels of peptide are fmol/ml, while kidney levels are fmol/g.
S2. Time course of TNFα expression.

Peritoneal macrophages from N-KO, C-KO and WT mice were collected after thioglycollate injection and cultured with LPS. At the indicated times after LPS addition, the concentration of TNFα was measured by ELISA (n=3 per point).
S3. Peritoneal macrophages from N-KO, C-KO and WT mice were collected after thioglycollate injection. After purification, the macrophages were cultured with LPS for 18 hrs. The concentration of IL-12 (left) or IL-10 (right) was then determined. Cells from N-KO mice make significantly more IL-12 than from the other groups (p<0.02). N-KO cells make less of the inhibitory IL-10. While p values by Student’s t test were significant (N-KO vs WT: 0.03; N-KO vs C-KO: 0.05), these values were not significant by one way ANOVA with the Tukey correction (N-KO vs WT: 0.07; N-KO vs C-KO: 0.14).
S4. Effect of AcSDKP on blood pressure in WT mice treated with angiotensin II

WT mice were treated with angiotensin II (♦), angiotensin II + 0.5 mg/kg/day AcSDKP (■), angiotensin II + 1.5 mg/kg/day AcSDKP (▲), and angiotensin II + 3.0 mg/kg/day AcSDKP (●) via minipump. Blood pressure was measured on days 5, 7, 10, and 14 after implantation. The figure shows the group means and SEM. No significant differences were observed. n=10 per group.