Sustained renal interstitial macrophage infiltration following chronic angiotensin II infusions

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Ozawa Y, Kobori H, Suzaki Y, Navar LG. Sustained renal interstitial macrophage infiltration following chronic angiotensin II infusions. Am J Physiol Renal Physiol 292: F330–F339, 2007. First published June 27, 2006; doi:10.1152/ajprenal.00059.2006.—Chronic angiotensin (ANG) II infusions into rats lead to augmented intrarenal levels of ANG II and inflammatory factors, impaired renal function, and progressive hypertension. Residual effects persist after cessation of ANG II infusions, as manifested by a hypertensive response to high-salt intake. This study was performed to determine the residual cytokines and chemokines following the cessation of ANG II infusion. Male Sprague-Dawley rats, maintained on a normal diet, received either a sham operation or continuous ANG II infusion (120 ng/min) subcutaneously via minipumps. The ANG II-infused rats were further subdivided into three subgroups. Minipumps were removed on day 12 with subsequent harvesting of kidneys at 0, 3, and 6 days after cessation of ANG II infusion. After 12 days of ANG II infusion, systolic blood pressure, interstitial fibrosis, and preglomerular hypertrophy were still present. Importantly, interstitial fibrosis and preglomerular hypertrophy were still present. Six days after cessation of ANG II infusion. Importantly, augmented mRNAs of interstitial matrix metalloproteinase (MMP)-1 (1.55 ± 0.15 vs. 1.00 ± 0.13, relative ratio) and transforming growth factor (TGF)-β1 (1.52 ± 0.16 vs. 1.00 ± 0.08) persisted 6 days after the withdrawal of ANG II infusion (1.60 ± 0.20 for MCP-1 and 1.43 ± 0.17 for TGF-β1). Thus, the ANG II-induced activation of MCP-1 and TGF-β1 is sustained and may account for the persistent effect of chronic ANG II infusions on interstitial macrophage infiltration, suggesting a possible mechanism for the development of salt sensitivity in ANG II-dependent hypertension.

MATERIALS AND METHODS

Animal experimental protocols. All procedures and protocols used in this study were approved by the Institutional Animal Care and Committee of Tulane University. The rats were placed in individual metabolic cages and had free access to water and regular rat chow throughout the study. Male Sprague-Dawley rats (175–200 g, N = 47) were selected randomly to receive either a sham operation (N = 11) or ANG II infusion (N = 36) at 120 ng/min subcutaneously via minipumps. The ANG II-infused rats were randomly assigned to one of the following three subgroups: ANG II + group (N = 14), which received ANG II infusion for 12 days followed by tissue harvesting; ANG II +3 group (N = 10), which received ANG II infusion for 12 days followed by removal of minipumps and tissue harvesting 3 days later; and ANG II +6 group (N = 12), which received ANG II infusion for 12 days followed by removal of minipumps and tissue harvesting 6 days later. In addition, the sham group was killed at day 18 to serve as controls.

Systolic BP, measured by the tail-cuff method, was evaluated every 3 days. Twenty-four-hour urine samples were collected the day before the tissue harvesting, and the protein concentration in urine samples

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was measured as previously described (23–25, 27, 29). The kidneys were harvested to evaluate ANG II content, electromobility shift assay (EMSA), quantitative real-time PCR (QRT-PCR), histological analysis, and immunohistochemical analysis.

Measurements of kidney ANG II. One-half of one kidney was immediately homogenized in ice-cold 100% methanol and centrifuged. The supernatant was processed for measurement of kidney ANG II by solid-phase extraction and radioimmunoassay. Kidney samples were extracted on C18 reverse-phase bond elute columns (Varian), and radioimmunoassay was performed as previously described (23–25, 27, 29).

EMSA. EMSA was performed as previously described (26). Nuclear protein was extracted from one-half of one kidney by using a commercially available kit (Panomics). EMSA for NF-κB was done with a commercially available kit (Panomics) (17). Briefly, 10 μg of nuclear extracts, which were prepared from the kidney cortex, were incubated in the presence of poly(dI-dC) at room temperature. Then the biotinylated labeled was added and incubated for another 30 min. The mixture was run on a 6% agarose gel and blotted on a positively charged membrane (Pall). Horseradish peroxidase-conjugated streptavidin was added and detected.

QRT-PCR. One-half of one kidney of each rat was placed into RNAlater (Ambion) and kept at −80°C. Total RNA was extracted using a commercially available kit (Qiagen) and treated with DNase I treatment (Invitrogen) to remove contaminated genomic DNA. QRT-PCR was performed as previously described (28, 40, 41). For the evaluation of NF-κB expression, mRNA levels of RelA (p65), a part of NF-κB complex, were measured. For the evaluation of extracellular matrix accumulation, MMP-9 and tissue plasminogen activator (tPA) were measured. For the evaluation of cellular proliferation, MCP-1 and transforming growth factor (TGF)-β1 and transforming growth factor (TGF)-β1 were measured. For the evaluation of cellular proliferation, a competitive slide to the slide stained by α-smooth muscle isofrm of actin was stained for PCNA, and the preglomerular arteriolar cells expressing PCNA were counted using the Image-Pro Plus software. The data were expressed as the number of positive cells per preglomerular arteriolar vessel wall. For all measurements, 20 randomly selected preglomerular arteriolar vessel walls were examined as described previously (38).

CD68 was used to identify the macrophages and monocytes in the renal interstitial tissues of each rat. The number of CD68-positive cells in 20 fields of tubular interstitium at high magnification was counted using the Image-Pro Plus software, and the average for each rat was taken as previously described (21, 28).

Statistical analyses. Data are expressed as means ± SE and analyzed by using one-way analysis of variance, followed by the post hoc “Hsu’s multiple comparison with the best” test. *P < 0.05 was considered statistically significant.

RESULTS

The temporal profiles of systolic BP from each group are depicted in Fig. 1A. Systolic BP was similar among the groups before the treatments. ANG II infusion progressively increased systolic BP, reaching average levels of 208 ± 7, 213 ± 6, and 199 ± 8 mmHg in the three ANG II-infused groups compared with 136 ± 3 mmHg in the control group. Following cessation of ANG II infusion, systolic BP returned toward the normal range at 3 days (138 ± 6 mmHg) and 6 days (139 ± 4 mmHg). Urinary excretion rate of total protein in each group is shown in Fig. 1B. In association with systolic BP, urinary protein excretion rate was significantly increased after 12 days of ANG II infusion compared with the sham-operated rats (43 ± 6 vs. 11 ± 2 mg/day). Urinary protein excretion returned to levels not significantly different from those in the sham group at 3 (16 ± 2 mg/day) and 6 days (14 ± 2 mg/day) following cessation of ANG II infusion.

Table 1. The sequence information of the primers and the probes for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Probe</th>
</tr>
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<tbody>
<tr>
<td>GAPDH</td>
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<td>CTGCCCTCAACCTGCTTGA</td>
<td>CTGCCGAAACCCCTCGAGATGAG</td>
</tr>
<tr>
<td>Angiotensinogen</td>
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<td>AGGACCTACCAGGTCCCG</td>
<td>TTCTTTGACACTGCTTGATCGC</td>
</tr>
<tr>
<td>RelA</td>
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</tr>
<tr>
<td>tPA</td>
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<td>CTGGACTGCTTCGTTGTC</td>
<td>TCTTTGACACTGCTTGATCGC</td>
</tr>
<tr>
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<td>CCAATATCGGAACTGCTGAG</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>TAATCGATGGGATCTTGTG</td>
<td>AAGAATCTGTCCTTGTG</td>
<td>CCGATATCGGAACTGCTGAG</td>
</tr>
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MMP, metalloproteinase; tPA, tissue plasminogen activator; MCP, monocyte chemotactic protein; TGF-β1, transforming growth factor-β1.
Angiotensinogen mRNA levels in renal cortex and kidney ANG II contents in each group are shown in Fig. 2, A and B, respectively. Similar to systolic BP and proteinuria, angiotensinogen mRNA levels and kidney ANG II contents were concomitantly and significantly increased by 12 days of ANG II infusion compared with the sham-operated rats by 43 and 66%, respectively. The angiotensinogen mRNA levels and kidney ANG II contents returned to levels not different from those in sham rats within 3 days after ANG II infusions were stopped.

A representative EMSA of NF-κB in each group is shown in Fig. 3A. NF-κB activity (Fig. 3B) was significantly enhanced by 80% after 12 days of ANG II infusion compared with the sham-operated rats. After cessation of ANG II infusion, NF-κB activity returned to normal levels within 6 days, with the level at 3 days being at an intermediate stage. Along with NF-κB activity, mRNA levels of RelA were also significantly increased by 60% after 12 days of ANG II infusion compared with the sham-operated rats. RelA mRNA levels returned to the normal range within 3 days following cessation of ANG II infusion (Fig. 3C).

Representative Masson’s trichrome-stained sections from the sham group (Fig. 4A), ANG II+0 group (Fig. 4B), ANG II+3 group (Fig. 4C), and ANG II+6 group (Fig. 4D), respectively, are depicted in each panel. The collagen-positive areas are shown in blue. The average percentages of the collagen-positive regions are shown in Fig. 4E. Chronic ANG II infusions caused significant increases in interstitial fibrosis compared with the sham-operated rats (0.16 ± 0.02 vs. 0.07 ± 0.01%). Interstitial fibrosis was still present at 3 days after cessation of ANG II infusion (0.17 ± 0.02%), but returned to near control levels by 6 days after (0.08 ± 0.01%). After 12 days of ANG II infusion, mRNA levels of MMP-9 (Fig. 4F) and tPA (Fig. 4G) were significantly increased by 117 and 42%, respectively, compared with the sham-operated rats. Following cessation of ANG II infusion, mRNA levels of MMP-9 and tPA also returned to the normal range within 3 days.

Fig. 2. Angiotensinogen (AGT) mRNA levels in renal cortex (A) and kidney ANG II contents (B) in each group. After 12 days of ANG II infusion, AGT mRNA levels and kidney ANG II contents were significantly increased compared with that of the sham-operated rats. Cessation of ANG II infusion normalized AGT mRNA levels and kidney ANG II contents within 3 days. *P < 0.05 compared with the sham group.

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Representative immunohistochemical slides for α-smooth muscle isoform of actin from sham group (Fig. 5A), ANG II +0 group (Fig. 5B), ANG II +3 group (Fig. 5C), and ANG II +6 group (Fig. 5D) are depicted in each panel. Figure 5E represents a negative control slide where the primary antibody was omitted and was replaced by phosphate-buffered saline.

DISCUSSION

In this study, we investigated the effects of cessation of ANG II infusion on various intrarenal indexes of proliferation, fibrosis, and inflammation. After discontinuing the ANG II infusion, the intrarenal values for ANG II and angiotensinogen returned to control levels, but several indications of a sustained inflammatory process persisted. At 3 days following cessation of ANG II infusion, interstitial fibrosis and preglomerular hypertrophy were still present. The persistence of vascular wall hypertrophy after transient ANG II infusion is consistent with the previous report by Franco et al. (13). In addition, the number of CD68-positive cells remained elevated on day 6 after cessation of ANG II infusion (4.41 ± 0.1 vs. 3.56 ± 0.18 μm). Evidence of preglomerular arteriolar wall thickening persisted at 3 days after cessation of ANG II infusion (4.41 ± 0.19 μm), but returned to near control levels by 6 days after (3.78 ± 0.10 μm). To address the cellular proliferative activity in preglomerular arteriolar walls, a consecutive slide was stained for PCNA, and the preglomerular arteriolar cells expressing PCNA were counted. The average numbers of positively stained cells were not altered among the groups, either by 12 days of ANG II infusion or in the groups where ANG II infusions were discontinued (1.1 ± 0.1 for sham group, 1.1 ± 0.1 for ANG II + 0 group, 1.0 ± 0.1 for ANG II + 3 group, and 1.1 ± 0.1 positive cells/preglomerular arteriolar for ANG II + 6 group, Fig. 5G).

Representative immunohistochemical slides for CD68, a surface marker of macrophage/monocyte from the sham group (Fig. 6A), ANG II + 0 group (Fig. 6B), ANG II + 3 group (Fig. 6C), and ANG II + 6 group (Fig. 6D) are illustrated in each panel. Figure 6E represents a negative control slide, where the primary antibody was omitted and was replaced by phosphate-buffered saline. CD68-positive cells are shown in brown. The average numbers of positively stained cells are shown in Fig. 6F. Chronic ANG II infusions significantly increased CD68-positive cells from 16 ± 1 to 26 ± 2 positive cells/mm², and these remained elevated in the groups where ANG II infusion was discontinued for 3 (26 ± 2 positive cells/mm²) and 6 days (24 ± 2 positive cells/mm²), suggesting the persistence of interstitial inflammation compared with the sham-operated rats. Similarly, mRNA levels of MCP-1 (Fig. 6G) and TGF-β1 (Fig. 6H) were significantly increased after 12 days of ANG II infusion compared with those seen in sham-operated rats (1.55 ± 0.15 vs. 1.00 ± 0.13 for MCP-1 and 1.52 ± 0.16 vs. 1.00 ± 0.08 for TGF-β1). The augmented mRNA levels of MCP-1 and TGF-β1 persisted at 3 days (1.91 ± 0.23 for MCP-1 and 1.38 ± 0.23 for TGF-β1) and remained unabated at 6 days (1.60 ± 0.20 for MCP-1 and 1.43 ± 0.17 for TGF-β1) following the withdrawal of ANG II infusion.
Moreover, in our present study, the augmented mRNA levels of MCP-1 and TGF-β persisted 6 days after the withdrawal of ANG II infusion. This sustained activation of MCP-1 and TGF-β indicates possible pathways to account for the development of salt sensitivity in ANG II-dependent hypertension.

It is interesting to note that kidney angiotensinogen expression and kidney ANG II contents were restored to the control levels within 3 days after cessation of ANG II infusion. Although it has been previously demonstrated that the production of renal angiotensinogen is upregulated in ANG II-dependent hypertension, contributing to the sustained production of renal ANG II (23–25, 27, 29), the renal angiotensinogen and ANG II activation was not maintained without a source of elevated exogenous ANG II infusion.

It is also interesting to note that the ANG II-induced renal inflammation continued even while the systolic BP and urinary protein excretion returned to the normal levels. These results suggest that the renal inflammation does not contribute to the development of proteinuria, at least, at this stage in this experimental model. The rapid attenuation of proteinuria after the cessation of ANG II infusion indicates that the primary mechanism mediating proteinuria during the early days of ANG II infusion is hemodynamic and not yet structural.

ANG II-induced preglomerular arteriolar wall thickening can occur as a consequence of proliferation, hypertrophy, or a combination of both (8). To address the cellular proliferative activity in preglomerular arteriolar walls, the preglomerular arteriolar cells expressing PCNA were counted. The average numbers of positively stained cells were not different among the groups, while the average thickness of preglomerular arteriolar wall was increased by ANG II infusion. These data indicate that ANG II-induced preglomerular arteriolar wall thickening during the early response to ANG II infusion primarily represents preglomerular arteriolar hypertrophy rather than proliferation.

From our results, cessation of ANG II infusion immediately reversed the RelA mRNA expression and the NF-κB activity in a manner similar to that seen with ANG II and angiotensinogen.
Although it has been reported that the angiotensinogen expression is elevated independently from the elevation of BP in ANG II-dependent hypertension (27, 29), the signaling pathway from ANG II to an enhanced angiotensinogen production remains unclear. One transcriptional factor, NF-κB, has been reported to stimulate the angiotensinogen expression in cultured hepatocytes (6). Furthermore, the inflammatory response to unilateral ureteral obstruction (UUO) due to activation of ANG II receptors appears to be mediated via NF-κB pathways (11). Thus our results are consistent with previous data showing a linkage of NF-κB to angiotensinogen and ANG II. These data suggest that NF-κB activates angiotensinogen expression and that inflammation would then be accelerated by the stimulation of intrarenal ANG II production, independent from the exogenous ANG II. Using transgenic mice that have different numbers of angiotensinogen gene copies, Fern et al. (12) demonstrated that the angiotensinogen expression is related to the renal interstitial fibrosis resulting from obstructive nephropathy. Also, the accelerated kidney angiotensinogen expression was accompanied by activated NF-κB in recurrent focal segmental glomerular sclerosis following renal transplantation (47). An involvement of activated NF-κB in the develop-

Fig. 5. A–F: representative immunohistochemical slides (A–E) for α-smooth muscle isoform of actin from each group (magnification: ×40). Chronic ANG II infusions (B) significantly increased preglomerular arteriolar (AA) wall thickness compared with the sham-operated rats (A). Evidence of preglomerular hypertrophy was still present at 3 days after cessation of ANG II infusion (C), but returned to near control levels by 6 days after (D). E: a negative control slide where the primary antibody was omitted and was replaced with phosphate-buffered saline. The detailed procedure of the semi-automatic image analysis of preglomerular hypertrophy with the robotic system was described previously (28). G: to address the cellular proliferative activity in AA walls, the numbers of the AA cells expressing proliferating cell nuclear antigen (PCNA) were counted. The average numbers of positively stained cells were not altered among the groups. *P < 0.05 compared with the sham group. N = 11, 14, 10, and 12 for sham, ANG II+0, ANG II+3, and ANG II+6, respectively.
Development of renal injury was also reported in ANG II-dependent gene-targeted rats (33). Thus the consistent changes in NF-κB activity, angiotensinogen mRNA levels, and renal ANG II contents suggest a mechanistic linkage.

In the present study, mRNA levels of both MMP-9 and tPA were restored rapidly to the control levels, although the increased mRNA levels of MCP-1 and TGF-β1 were maintained. MMP-9 is involved in remodeling in several tissues (2, 3, 62); McMillan et al. demonstrated that MMP-9 expression was increased in cultured glomerular epithelial cells of membranous nephropathy model (32). In the UUO animal, which is a renal fibrosis model, Yang et al. (60) demonstrated that tPA activated MMP-9, but did not alter the TGF-β1 level. Renal fibrosis was less in UUO in tPA-deficient mice than in control mice (60). ANG II also increased MCP-1 levels, which may be responsible for aggregation of inflammatory cells into the renal interstitium and increased the TGF-β1 levels. In the present study, these inflammatory markers remained elevated 3 and 6 days after ANG II infusion was discontinued, suggesting a mechanistic link to the salt sensitivity previously reported (13, 18, 31, 44). This mechanism may participate in the development of ANG II nephropathy (48, 59) and should be an independent pathway from that of MMP-9 and tPA. Thus our results suggest that the pathways through MCP-1 and TGF-β1

![Fig. 6. A–F: representative immunohistochemical slides (A–E) for CD68, a surface marker of macrophage/monocyte from each group (magnification: ×40). Chronic ANG II infusions (B) significantly increased interstitial inflammation compared with the sham-operated rats (A). Evidence of interstitial inflammation was still present at 3 days (C) and 6 days (D) after cessation of ANG II infusion. E: negative control slide where the primary antibody was omitted and was replaced with phosphate-buffered saline. The detailed procedure of the semiautomatic image analysis of interstitial inflammation with the robotic system was described previously (28). After 12 days of ANG II infusion, mRNA levels of monocyte chemotactic protein (MCP)-1 (G) and transforming growth factor (TGF)-β1 (H) were significantly enhanced compared with that of the sham-operated rats. The augmented mRNA levels of MCP-1 and TGF-β1 were persistent after 6 days of the withdrawal from continuous ANG II infusion. *P < 0.05 compared with the sham group. N = 11, 14, 10, and 12 for sham, ANG II+0, ANG II+3, and ANG II+6, respectively.

![Graphs showing mRNA levels of MMP-9 and TGF-β1](http://ajprenal.physiology.org/)
are important mechanisms for developing salt-sensitive hypertension after chronic ANG II infusion.

There are inconsistent findings regarding whether TGF-β1 is an inflammatory effector or an anti-inflammatory effector. In one study, TGF-β1 transgenic mice did not develop interstitial inflammation with macrophage infiltration following UUO (57). However, emerging evidence in experimental and human studies suggests that TGF-β1 is a major contributor to renal fibrosis, because TGF-β1 directly induces both increased synthesis and reduced degradation of matrix proteins, leading to a net accumulation of pathological matrix (5, 14, 46, 63). Therefore, TGF-β1 is considered an important mechanism of the observed phenomenon in the present model.

There are also inconsistent findings regarding the linkage between TGF-β1 and NF-kB. Some experimental studies suggest that the changes of TGF-β1 levels are parallel to the changes of NF-kB levels (35, 52, 61). However, in another study, TGF-β1 was shown to suppress NF-kB activation in mice following UUO (57). Moreover, it was reported that TGF-β1 induction occurred without activation of the NF-kB pathway in mesangial cells (9). One possible explanation to account for these inconsistent findings is that the activated period may be different between TGF-β1 and NF-kB. Additional studies will be required to address this issue.

It is not clear in this study whether the observed effects were directly related to ANG II or BP-dependent indirect effects of ANG II. Our laboratory has addressed this important issue in another recent study (28). Spontaneously hypertensive rats (SHR) showed enhanced intrarenal ANG II and hypertension as well as renal injury at 14 wk of age compared with SHR at 7 wk of age. Two groups of SHR received either an ANG II type 1 receptor blocker (ARB; olmesartan, 5 mg/day) or triple therapy (HRH; hydralazine, 7.5 mg/day; reserpine, 0.15 mg/day; hydrochlorothiazide, 3 mg/day) during weeks 7–14. ARB treatment and HRH treatment equivalently prevented hypertension. While ARB treatment prevented the augmented intrarenal ANG II and the development of renal injury, HRH treatment failed to prevent the augmented intrarenal ANG II or the development of renal injury. These results indicate that the development of renal injury of SHR at 14 wk of age was related directly to intrarenal ANG II and was independent from BP. We recognize that this is an important issue; however, the emphasis of the present study was on the evaluation of the residual effects which might be responsible for salt-sensitive hypertension.

Recent evidence suggests that the renal proinflammatory response, such as immune cell accumulation in the kidneys, may play an important role in mediating sodium retention and, thereby, in the development of hypertension (43). In a model of salt-sensitive hypertension, a high-salt diet significantly increased total circulating leukocyte counts in the Dahl salt-sensitive rats but not in the Dahl salt-resistant rats (49). A recent report indicates that a high-salt diet increases tubulointerstitial infiltration of inflammatory cells in a model of salt-sensitive hypertensive rats (50). Several studies have also shown that reducing renal inflammatory cell infiltration prevents the development of salt-sensitive hypertension (1, 44). These data also support our findings in this study.

In summary, this study demonstrates that chronic ANG II infusions to normal rats significantly increased systolic BP (Fig. 1A), urinary protein excretion rate (Fig. 1B), angiotensinogen mRNA levels (Fig. 2A), kidney ANG II content (Fig. 2B), NF-kB activity (Fig. 3, A and B), RelA mRNA levels (Fig. 3C), interstitial fibrosis (Fig. 4, A–E), mRNA levels of MMP-9 (Fig. 4F) and tPA (Fig. 4G), preglomerular hypertrophy (Fig. 5, A–F), macrophage infiltration (Fig. 6, A–F), and mRNA levels of MCP-1 (Fig. 6D) and TGF-β1 (Fig. 6H). Cessation of ANG II infusion normalized systolic BP, urinary protein excretion rate, mRNA levels of angiotensinogen, RelA, MMP-9 and tPA, kidney ANG II content, and NF-kB activity within 3 days. However, interstitial fibrosis and preglomerular hypertrophy were still present at 3 days, and the increases in macrophage infiltration persisted even at 6 days after cessation of ANG II infusion. Importantly, the augmented mRNA levels of MCP-1 and TGF-β1 also remained significantly elevated 6 days after cessation of ANG II infusion. These data indicate that ANG II-induced activation of MCP-1 and TGF-β1 is sustained even after the ANG II is discontinued and may account for the persistent effect of ANG II infusion on enhanced macrophage infiltration, suggesting a possible mechanism responsible for the continued injury and the development of salt sensitivity in ANG II-dependent hypertension.

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