Novel anti-inflammatory mechanisms of N-Acetyl-Ser-Asp-Lys-Pro in hypertension-induced target organ damage

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Ac-SDKP decreased secretion of TNF-α by 10.2 ± 0.33. We also examined in mice with ANG II-induced hypertension the effects of Ac-SDKP on cardiac macrophage infiltration and activation (galectin-3 expression) and fibrosis.

METHODS

BMSC Isolation and Culture

This protocol was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee. BMSC were isolated from mouse femurs and tibias as reported previously (16). Hind legs from two mice were cleaned thoroughly, and marrow was flushed from the femora and tibias using ice-cold PBS. The collected bone marrow cells were pooled, washed in ice-cold PBS, and resuspended in macrophage growth medium (IMDM; HEPES buffered) with 5% (vol/vol) FBS...
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solution, and the fluorescence emitted by CyQuant GR dye was warmed buffer. The detached cells were incubated in lysis buffer-dye extractant was added. Cells were incubated for 12 h, and those attached increasing doses of Ac-SDKP (0.01, 0.1, 10, and 100 nM) were added in the presence or absence of a chemoattractant (3×10^6/H9262).

Macrophage Differentiation

BMSC were treated with either Ac-SDKP or PBS and cultured for 7 days in MGM. Ac-SDKP was added daily to overcome degradation by ACE and maintain Ac-SDKP concentration, according to previous reports by others (4, 6, 11, 36). In addition, ACEi was not used in this study since captopril, an ACEi, has been shown to directly inhibit proliferation of hematopoietic stem and progenitor cells in murine bone marrow cultures (8). On day 8, cells were harvested with PBS supplemented with 10 mM EDTA and 4 mg/ml lidocaine. Typically, marrow from the femurs and tibias of one mouse yielded 2×10^7 to 6×10^7 macrophages after 7 days in culture. Macrophages were stained with FITC-conjugated F4/80 as the primary antibody and anti-mouse IgG2a-FITC as the isotype control. Approximately 3,000 cells were analyzed, and only viable cells were gated. Surface expression of F4/80 by mature macrophages was quantified by flow cytometry using the FL1 channel, and data were expressed as mean fluorescence intensity (MFI).

Macrophage Migration Assays

Macrophage migration studies were performed in a modified Boyden chamber. We used a Chemicon QCM 96-well migration assay with an 8-μm pore size, which is appropriate for monocytes and macrophages. The upper chamber was filled with a macrophage suspension (10×10^6 cells per well) derived from mouse spleens, and 150 μl of serum-free medium were added to the lower chamber in the presence or absence of a chemotactant (3 μg/galectin-3) or M-CSF (100 ng/ml). For the inhibition studies with Ac-SDKP, gradually increasing doses of Ac-SDKP (0.01, 0.1, 10, and 100 nM) were added and the samples were preincubated for 30 min before the chemoattractant was added. Cells were incubated for 12 h, and those attached to the bottom of the porous membrane were detached using a prewarmed buffer. The detached cells were incubated in lysis buffer-dye solution, and the fluorescence emitted by CyQuant GR dye was quantified by a fluorescence plate reader using a 480/520 nm filter set. We found that Ac-SDKP was most effective at 10 nM, and thus we used this dose in all subsequent experiments.

Macrophage Activation In Vivo and In Vitro

TNF-α expression by activated macrophages in vitro. Macrophages obtained from differentiation of BMSC were used for this study according to a method described previously (1). The mature macrophages were stimulated by a purified endotoxin (10 μg/ml LPS) with and without Ac-SDKP (1 and 10 nM) for 48 h. TNF-α in the cell supernatant was quantified by ELISA according to the manufacturer’s instructions (R&D Systems). The viable cells present in each culture well were stained with crystal violet, and absorbance was measured according to manufacturer’s instructions. The TNF-α level in the supernatant was normalized to the absorbance recorded from the corresponding culture wells.

Infiltration by activated macrophages in vivo. Male C57Bl6/J mice (12- to 14-wk-old) were divided into three groups: 1) vehicle (n = 5), 2) ANG II (n = 7), and 3) ANG II + Ac-SDKP (n = 7). Mice were anesthetized with sodium pentobarbital (50 mg/kg ip). A small incision was made between the shoulder blades, and a pocket was created subcutaneously, just large enough to hold an osmotic minipump (Alzet 2004). The pump was implanted to administer ANG II (750 μg·kg⁻¹·day⁻¹) and/or Ac-SDKP (800 μg·kg⁻¹·day⁻¹; synthesized by W. Neugebauer, University of Sherbrooke, Canada) or saline plus 0.01 N acetic acid. Mice were treated for 8 wk. Systolic blood pressure was measured by tail cuff twice a week. At the end of the experiment, animals were anesthetized with 50 mg/kg sodium pentobarbital. The heart was stopped at diastole with an intraventricular injection of 15% KCl and rapidly excised. The LV (including the septum) was weighed and sectioned transversely from apex to base. Picrosirius red staining for detection of collagen in the heart. Sections measuring 6 μm were deparaffinized, rehydrated, and stained with picrosirius red using a modification of the method of Sweat et al. (39). Briefly, sections were postfixed in Bouin’s fluid, followed by iron hematoxylin stain to show the nuclei. They were then stained with 0.1% picrosirius red for 1 h and washed twice with 0.5% acetic acid. Images were obtained with a computerized digital camera (SPOT, Diagnostic Instruments), and collagen was examined under a microscope (Nikon E600) using normal light and analyzed with SigmaScan Pro (Jandel Scientific).

Western blot for detection of galectin-3 in the LV and kidney. In all three groups, galectin-3 protein expression in myocardial tissue was detected by Western blot. A total of 60 μg protein from the LV extract

Fig. 1. FACS analysis to compare expression of F4/80 by macrophages. A: surface expression of F4/80 by macrophages obtained by treating bone marrow stem cells (BMSC) with macrophage growth medium (MGM). B: isotype control for A. C: surface F4/80 expression in cells treated with MGM + N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP; 10 nM). D: isotype control for C. FL1-H, fluorescence intensity on the FL1, (FITC) channel; M₁ marker used to define positive events.
was subjected to 12% SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane. The primary antibody was a mouse monoclonal antibody against full-length recombinant human galectin-3 (1 μg/ml, BioReagents). The membrane was incubated with the primary antibody at 4°C overnight, followed by anti-mouse IgG, HRP-linked antibody at room temperature for 1 h. ECL Western kit (GE Healthcare) was used to visualize the bands. Bands were quantified with a bioscanner, and the results were normalized to actin and expressed as fold increase compared with sham.

Statistical analyses

Data are means ± SE. A paired t-test followed by Hochberg’s adjustment was used to judge statistical significance between treatment groups, with $P < 0.05$ considered as significant.

RESULTS

Effect of Ac-SDKP on Differentiation of BMSC to Macrophages

BMSC isolated from mouse bones were treated with Ac-SDKP. Cells treated with MGM developed podocytic processes and strongly expressed the mature macrophage-specific marker F4/80 after 7 days (Fig. 1 A). Adding 10-nM Ac-SDKP to the medium every 24 h for 7 days significantly reduced surface expression of F4/80 (Fig. 1 C; Fig. 2), while maintaining the number of viable cells, suggesting that Ac-SDKP inhibits differentiation of BMSC to mature macrophages (geometric mean of F4/80 positivity: vehicle, 14.09 ± 1.06 MFI; Ac-SDKP, 10.63 ± 0.35 MFI; $P < 0.05$).

Effect of Ac-SDKP on Macrophage Migration

Exogenous recombinant galectin-3 (3 μM) increased macrophage migration (Fig. 3 A). Ac-SDKP at increasing concentrations caused significant inhibition of galectin-3-induced macrophage migration, except the 100-nM dose, at which the effects of Ac-SDKP were lessened (Fig. 3 A). Similarly, compared with vehicle-treated cells, mouse recombinant M-CSF at a concentration of 100 ng/ml showed an increase in macrophage migration, which was significantly reduced by Ac-SDKP (Fig. 3 B).

Effect of Ac-SDKP on TNF-α Release by Activated Macrophages

To test for changes in TNF-α release by LPS-activated macrophages in culture, we treated macrophages with bacterial LPS for 48 h and measured TNF-α in the supernatant by quantitative ELISA. As shown in Fig. 4, LPS increased TNF-α release (TNF-α: vehicle, 721.8 ± 66.1 pg/ml; LPS, 1,853.9 ± 132.2 pg/ml; $P = 0.001$), and 10 nM Ac-SDKP significantly decreased LPS-induced TNF-α release (1,289.8 ± 34.6 pg/ml; $P = 0.006$).
Effect of Ac-SDKP on Blood Pressure, Cardiac Hypertrophy, and Infiltration of Activated Macrophages Into the Myocardium of ANG II Hypertensive Mice

We treated mice with vehicle, ANG II, or ANG II/ Ac-SDKP. As expected, ANG II significantly increased systolic blood pressure, heart rate, and LV weight, which were not altered by Ac-SDKP (Table 1). We also found that in mice treated with ANG II/H Ac-SDKP the collagen deposition in the LV was significantly decreased compared with mice given ANG II/vehicle (Fig. 5). Subsequently, we measured Mac-2 (galectin-3) protein levels in the myocardium of ANG II-treated hypertensive mice and controls by Western blotting. Galectin-3 expression was increased in the myocardium of ANG II-treated mice. Ac-SDKP infusion significantly decreased ANG II-induced galectin-3 production in the myocardium (Fig. 6). Decreased galectin-3 expression in the myocardium after Ac-SDKP treatment suggested decreased infiltration of activated macrophages.

DISCUSSION

We have evidence of the anti-inflammatory and antifibrotic properties of Ac-SDKP when administered chronically in a rat model of hypertension; however, the mechanism(s) of the anti-inflammatory effect of Ac-SDKP has not yet been established. In the present study, we found that in vitro Ac-SDKP inhibits 1) differentiation of BMSC to macrophages (F4/80 expression), 2) macrophage migration, and 3) macrophage activation (TNF-α). We also found that in mice with ANG II-induced hypertension Ac-SDKP inhibits galectin-3 (Mac-2) expression and cardiac collagen deposition without affecting blood pressure or cardiac hypertrophy.

Inflammation, scarring, and target organ damage are common consequences of hypertension. Typically, an inflamma-

| Table 1. Blood pressure, heart rate, left ventricular hypertrophy, and plasma Ac-SDKP in rats infused with vehicle, ANG II, or ANG II + Ac-SDKP |
|-----------------|-----------------|------------------|
| SBP, mmHg       | 110±6           | 156±6*           |
| HR, beats/min   | 574±7           | 583±23           |
| LVW/BW          | 31.2±1          | 41.1±2.1*        |
| Plasma Ac-SDKP, nM | 1.67±0.21      | 1.88±0.11        |

Values are means ± SE. Ac-SDKP, N-acetyl-Ser-Asp-Lys-Pro; SBP, systolic blood pressure; HR, heart rate, LVW/BW, ratio of left ventricular weight to body weight. *P ≤ 0.005 vs. control; †P = 0.014 vs. control.

Fig. 5. Effect of Ac-SDKP on ANG II-induced interstitial collagen deposition in the mice heart. A: representative images of the picrosirios-stained sections of the heart. B: quantification of the interstitial collagen contents in the picrosirios-stained sections. ANG II significantly increased interstitial collagen volume fraction in mice heart, which was inhibited by Ac-SDKP; n = 5–7.
part the inhibitory effect of Ac-SDKP on macrophage infiltration seen in the heart, kidneys, and arteries of hypertensive animals (25–27, 32). Although the exact mechanism of chemokine-directed cell migration is still under investigation, local activation of cellular tyrosine kinase has been implicated (38). We recently demonstrated that Ac-SDKP binds specifically to binding sites located in the membranes of cardiac fibroblasts, enabling us to further postulate that Ac-SDKP may exert its anti-inflammatory effects via a receptor possibly expressed in the macrophages that have not yet been identified (43).

Inflammation in cardiovascular diseases is associated with activation of and infiltration by macrophages, which express and secrete proinflammatory cytokines and chemokines (21, 37). The term “activated macrophages” denotes those macrophages that have acquired a heightened ability to perform specific immune functions. Typically, activated macrophages show higher levels of major histocompatibility complex class II, CD11b (Mac-1), and galectin-3 (Mac-2) gene expression. Previously, we observed macrophage infiltration and excess of galectin-3 expression in the heart of a transgenic rat model with excessive ANG II-induced hypertension [TGR (mREN2); ref. 37]. Chronic infusion of galectin-3 into the pericardial space caused cardiac fibrosis and LV dysfunction (37). We also reported that inflammation and fibrosis were decreased in Ac-SDKP-treated rats with 2-kidney-1 clip hypertension (35). These studies, along with our current data generated in an ANG II infusion model of hypertension, have advanced our previous findings, since we now know that the anti-inflammatory and antifibrotic effects of Ac-SDKP can be attributed at least in part to its inhibitory effects of macrophage activation and galectin-3 expression. The current study also supports our previous findings that Ac-SDKP does not have any effects on blood pressure or cardiac hypertrophy. The antifibrotic effects of this novel tetrapeptide can be attributed in part to its anti-inflammatory properties.

It is known that Ac-SDKP acts as a physiological regulator of hematopoiesis and inhibits S-phase entry of murine and human hematopoietic stem cells (23). Recently, we have shown that decreased basal levels of Ac-SDKP in rats treated with the POP inhibitor are associated with increased cardiac and renal perivascular fibrosis and renal glomerulosclerosis. This suggests that Ac-SDKP plays an important physiological role at basal concentrations, preventing organ collagen accumulation (7).

Several in vitro inhibitory effects of Ac-SDKP have been described, including inhibition of cardiac and renal fibroblast proliferation, collagen synthesis, and mesangial growth (13–15, 30, 34, 34, 40). Thus the anti-inflammatory and antifibrotic effects of Ac-SDKP may be complex, since Ac-SDKP influences a broad range of physiological activities both in vivo and in vitro by its inhibitory effects on inflammation, cell proliferation, and collagen synthesis, in addition to its neo-angiogenesis effects (9, 25, 28, 32–35, 41, 42). We have also shown that part of the anti-inflammatory effect of ACEi is mediated by Ac-SDKP (26, 29).

Study Limitations

BSCMC differentiation and macrophage migration studies were performed in vitro. It is well understood that pharmacology of cells in vitro does not completely mimic the complex

![Fig. 6. Measurement of left ventricular (LV) galectin-3 levels in mice treated with vehicle (n = 5), ANG II (n = 7), or ANG II + Ac-SDKP (n = 7) for 8 wk. A: representative galectin-3 immunoblot on the protein extracted from mice heart treated with vehicle, ANG II, or ANG II + Ac-SDKP. Actin immunoblot was performed on the same sample concurrently to normalize the galectin-3 levels. B: quantification of galectin-3 levels in the immunoblots. ANG II treatment increased LV galectin-3 expression levels, which was significantly reduced by Ac-SDKP; n = 5–7.](image-url)
in vivo cell environment in a whole animal. On the other hand, the studies in vitro have allowed us to determine that Ac-SDKP has a direct effect on stem cell maturation into macrophages, macrophage migration, and cytokine release. The present data support in part the studies in vivo; however, in the intact animals additional factors, such as inhibition of expression of cell adhesion molecules, fibroblast proliferation, and collagen synthesis, could also have a significant effect on the antifibrotic effects of Ac-SDKP.

In conclusions, Inflammation and fibrosis in the heart, kidney, and arteries are among the known consequences of long-standing hypertension. Excess inflammatory cell infiltration and increased circulating proinflammatory cytokine levels are consistently reported in hypertensive end-organ damage. This study shows that Ac-SDKP can exert its anti-inflammatory effects at multiple stages of macrophage morphogenesis, including BMSC differentiation, migration, activation, and cytokine secretion. Since it is well documented that ACEi increases plasma and tissue Ac-SDKP, it can be concluded that the multiple effects of Ac-SDKP demonstrated in this study may point to a new direction in our understanding the mechanism by which ACEi counteracts the adverse outcomes of long-standing hypertension.

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