Original Paper

Infusion of Valproic Acid Into the Renal Medulla Activates Stem Cell Population and Attenuates Salt-Sensitive Hypertension in Dahl S Rats

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Key Words

CD133 • Inflammation • Interleukin-1 β • Fibroblast growth factor-2 • Sodium excretion

Abstract

Background: Our previous study has detected a stem cell deficiency in the renal medulla in Dahl salt-sensitive (S) rats. This study determined whether infusion of valproic acid (VA), an agent known to stimulate the stem cell function, attenuated salt-sensitive hypertension in Dahl S rats. **Methods:** Uninephrectomized Dahl S rats were infused with vehicle or VA (50mg/kg/d) into the renal medulla and fed with a low (LS) or high salt diet (HS). Stem cell marker and number were analyzed by immunohistochemistry, Real-time RT-PCR and Western blot. Sodium excretion and blood pressure were measured. **Results:** VA significantly increased the mRNA and protein levels of FGF2, a stem cell niche factor, and CD133, a stem cell marker. The number of CD133+ cells was significantly increased in the renal medulla in VA-treated rats. Meanwhile, high salt-induced increases in the mRNA level of proinflammatory factors interleukin-1 β and interleukin-6 were blocked in VA-treated rats. Functionally, sodium excretion in response to the blood pressure increase and acute sodium loading was significantly enhanced, sodium retention attenuated, high salt-induced increase of blood pressure reduced in VA-treated rats. **Conclusion:** Activation of stem cell function by VA inhibits the activation of proinflammatory factors and attenuates salt-sensitive hypertension in Dahl S rats.

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Introduction

It has been demonstrated that many and perhaps all adult organs harbor stem cells [1] including the kidneys [2-4]. Organ-specific stem cells are necessary for organ repair during routine maintenance [5, 6]. Rapid accumulating evidence shows that defects in organ-specific adult stem cells may be a pathogenic mechanism for various diseases [7-10]. This aspect has been under-investigated considering numerous studies on the stem cell-based therapy. Although dysfunctions in progenitor/stem cells have been identified in chronic kidney diseases due to the toxicity of the uremic milieu [11], however, little is known about whether there are stem cell defects as pathogenic mechanisms causing various kidney diseases. In this regard, our recent study has shown that there is a stem cell deficiency with reduced stem cell population in the renal medulla [12] in Dahl salt-sensitive (S) rats, a widely used genetic model of human salt-sensitive hypertension. Given that the renal medullary dysfunction has long been recognized as one of the major mechanisms for the development of hypertension in Dahl S rats [13, 14], the stem cell deficiency in the renal medulla may be associated the renal medullary dysfunction and that correction of this stem cell deficiency may attenuate the hypertension in this rat model. Indeed, transplantation of mesenchymal stem cells into the renal medulla inhibits the high salt-induced inflammation in the renal medulla and improves salt-sensitive hypertension in Dahl S rats [12], suggesting that stem cell defect in the renal medulla may contribute to the pathogenesis of the hypertension in Dahl S rats.

Valproic acid (VA) is an inhibitor of histone deacetylases and shown to regulate several genes that are critical for stem cell functions, such as those in Notch signaling, Wnt signaling and Oct4 [15-18]. VA has been well proven to stimulate the proliferation and differentiation of stem/progenitor cells in many different organs/tissues under a variety of conditions [19-22]. We thus wondered whether VA could stimulate the activation of stem cells and correct the deficiency of stem cells in the renal medulla, thereby improving the salt-sensitive hypertension in Dahl S rats. We infused the VA chronically into the renal medulla and found an improvement in the population of stem cell marker CD133+ cells, which was accompanied with reduced levels of proinflammatory factors and attenuation of hypertension after high salt intake in Dahl S rats.

Materials and Methods

Animals

Experiments used male Dahl S rats (Charles River) and SS-13BN rats (Charles River), weighing 250 to 350 g. Animal procedures were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. SS-13BN rat was used as control because it is considered as one of the best normotensive control strains for Dahl S rat [23]. SS13-BN is a consomic subcolony of Dahl S rat with substitution of chromosome 13 from Brown Norway rat. The differences in genotype between SS-13BN rat and Dahl S rat is 1.95%, which is much smaller than the differences between Dahl S rat and other commonly used "control" strains: Dahl R 30%, Sprague-Dawley 52%, ACI 57%, and BN 77% [23].

Detection of CD133+ cells in the renal medulla

Immunohistochemistry was used to detect these cells in the renal medulla. Percentage of cells with positive staining was calculated using a computer program (Image-Pro Plus) as described previously [24]. In brief, photomicrographs covering medullary area were taken and then the counts of brown-stained positive cells and blue-stained nuclei were used to calculate the % of CD133+ cells: number of brown-stained cells/total number of cells (blue-stained nuclei).

Chronic Infusion of VA into renal medulla

Chronic intra-renal infusion was performed as described previously by us [25] and others [26-28]. In brief, rats were uninephrectomized and catheters were implanted into the renal medulla of remaining left kidneys. The catheters were tunneled to the back of neck and connected to osmotic pumps (ALZET, model

2ML2), which contained either vehicle or VA (50mg/Kg/d) and was implanted subcutaneously. The dose of VA was based on the pump speed/infusion rate of 5 μ l/hr and the dilution at a rate around 30 ml/hr renal medullary plasma flow in a 300g rat, which would generate an intra-renal medullary concentration of VA at 20 μ g/ml, a concentration range showing a significant effect on stem cell proliferation and differentiation [29, 30]. In addition, VA has no toxic effect at doses up to 500mg/kg/d in rats [31]. At the end of experiment, half of the kidney was fixed in 10% neutral buffered formalin and the other half frozen in liquid N₂ and stored in -80°C.

Measurement of pressure natriuresis in response to the elevations of renal perfusion pressure

After 10 day VA infusion, while the rats were maintained on low salt diet, pressure natriuresis was measured in these rats as described previously [32, 33]. In brief, after surgical preparation and equilibration, renal perfusion pressure (RPP) was acutely increased by tying off the celiac and mesenteric arteries. At each RPP level, after a 10-min equilibration period, urine samples were collected during a 20-min clearance period. Urinary sodium excretion were measured and factored per gram kidney weight.

Measurement of urinary sodium excretion in response to acute sodium loading

Additional rats were treated with VA infusion and maintained on low salt diet for 10 days as above. Urinary sodium excretion after acute sodium loading were measured as we described before [32, 33]. In brief, after surgical preparation and equilibration, two 10-min control-period urine samples were collected and then a 5% body weight isotonic saline load was administered intravenously within 30 minutes. Three 10-min samples were collected over 30 minutes and three more 10-min post-control samples were taken. Urinary sodium excretion were measured and factored per gram kidney weight.

Measurement of daily sodium balance

Additional rats were treated the same as above and then housed in metabolic cages. Daily sodium balance was calculated by subtracting sodium excretion from sodium intake. After 1 day of control measurements, rats were fed with 2% NaCl water and daily sodium balance were measured for 3 more days [32, 33].

Monitoring of arterial pressure

Arterial pressure was measured as described before [32, 33] using a telemetry system (Data Science International) for 2 days while the rats remained on a low salt diet, and then 12 more days when rats were on either a low salt diet (0.4%) or a high salt diet (8%). The high salt diet started 2 days after the surgery and the beginning of VA infusion. After the measurement of arterial pressure, the kidneys were saved for immunostaining or protein and RNA isolation.

Molecular biology assays

The mRNA and/or protein levels of FGF2, CD133, interleukin-1 β and IL-6 in renal medullary tissues were detected by Real-time RT-PCR and Western blot analyses as we described previously [34]. In brief, for RT-PCR, total RNA from renal medullary tissues was extracted using TRIzol solution (Life Technologies) and then reverse-transcribed (RT) (cDNA Synthesis Kit, Bio-Rad), the RT products were amplified using a TaqMan Gene Expression Assays kit with predesigned primers and probes (Applied Biosystems), 18S ribosomal RNA was used as an endogenous control, the relative gene expressions were calculated in accordance with the $\Delta\Delta$ Ct method; for Western blot, renal medullary tissues were homogenized in ice-cold HEPES buffer containing (in mmol/L): Na-HEPES, 25; EDTA, 1; and phenylmethylsulfonyl fluoride, 0.1, the homogenate was centrifuged at 6000g for 5 minutes at 4°C and the supernatant was collected, samples (20 μ g protein) were subjected to 10% SDS-PAGE gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes, the membranes were probed with antibodies (1:1,000) against CD133 (Abcam) or FGF2 (Santa Cruz Biotechnology) overnight at 4°C and then horseradish peroxidase-labeled secondary antibodies (1:3,000) for 1 h at room temperature, enhanced chemiluminescence detection solution (ECL, Pierce) was applied onto the membranes and the membranes then exposed to Kodak Omat film, the intensity of the blots was determined using an imaging analysis program (NIH ImageJ). The membranes were stripped and re-probed with β -actin antibody, which was used as internal control.

Statistics

Data are presented as means $\pm$ SE. The significance of differences in mean values within and among multiple groups was evaluated using an ANOVA followed by Tukey's post hoc test. Student's t-test was used to evaluate statistical significance of differences between two groups. Regression slopes for the blood pressure vs. urinary sodium excretion in pressure natriuresis was compared using ANCOVA [35]. $P < 0.05$ was considered statistically significant.

Results

Comparison of the levels of FGF2 and CD133 in the renal medulla between Dahl S and SS-13BN rats in response to high salt intake

Basic fibroblast growth factors (FGF2) is one of the fundamental regulators to support the proliferation and self-renewal of adult stem cells [36-40]. CD133 as a stem cell marker has been widely used to detect stem cells in a variety of organs including the kidneys [4, 41]. The mRNA levels of both FGF2 and CD133 were much lower in Dahl S rats than in SS-13BN rats (Fig. 1). Interestingly, both FGF2 and CD133 gene expressions were increased after high salt intake (2 weeks) in SS-13BN rats. However, there was no difference in FGF2 and CD133 mRNA levels between low and high salt-treated Dahl S rats (Fig. 1). These data indicate that there is a defect in FGF2, a factor that regulates stem cell function [36-40], and that FGF2 deficiency may diminish stem cell response to high salt intake in the renal medulla of Dahl S rats.

Effect of VA on the levels of FGF2 and CD133 in the renal medulla in Dahl S rats

The mRNA and protein levels of both FGF2 and CD133 were significantly higher in animals treated with VA infusion than those treated with vehicle (Fig. 2&3), suggesting

Fig. 1. The mRNA levels of FGF2 and CD133 in the renal medulla from Dahl S and SS-13BN rat by Real-Time RT-PCR analysis. Dahl S (DS) and SS-13BN (BN) rats were treated with a low salt (LS) or high salt (HS) diet for 2 weeks. * $P < 0.05$ vs. other groups (n=6).

Fig. 2. Effect of VA on the mRNA levels of FGF2 and CD133 in the renal medulla from Dahl S by Real-Time RT-PCR analysis. Rats were treated with intra-renal medullary infusion of vehicle or VA and fed with a low salt (LS) or high salt (HS) diet. LS, low salt + vehicle; HS, high salt + vehicle; HS+VA, HS + valproic acid. * $P < 0.05$ vs. other groups (n=6).

Fig. 3. Effect of VA on the protein levels of FGF2 and CD133 in the renal medulla from Dahl S by Western blot analysis. Top: Representative ECL gel documents of Western blot analyses depicting the protein levels of FGF2 and CD133. Bottom: Summarized blot intensities of FGF2 and CD133 (ratio to β-actin). LS, low salt + vehicle; HS, high salt + vehicle; HS+VA, HS + valproic acid. * $P < 0.05$ vs. other groups ($n=6$).

that VA increases the levels of FGF2 and stimulates the activation of stem cells in the renal medulla of Dahl S rats.

Effect of VA on the number of CD133+ cells in the renal medulla in Dahl S rats

Immunostaining showed that the percentage of CD133+ cells was much higher in rats treated with VA infusion than those treated with vehicle (Fig. 4), further suggesting that VA stimulates the activation of stem cells in the renal medulla of Dahl S rats.

Effect of VA on the mRNA levels of interleukin (IL)-1β and IL-6 in the renal medulla in Dahl S rats

The mRNA levels of IL-1β and IL-6 were significantly increased after high salt challenge in vehicle-treated rats, which was blocked in VA-treated rats (Fig. 5), suggesting that VA-induced activation of stem cell function inhibits the proinflammatory response to high salt challenge in the renal medulla in Dahl S rats.

Effects of VA on sodium excretion in response to renal perfusion pressure in Dahl S rats

The urinary sodium excretion (U·Na) were increased in response to the elevation of blood pressure. However, these pressure natriuretic responses were remarkably improved in VA-treated rats compared with the controls (Fig. 6A).

Fig. 4. Effect of VA on the number of CD133+ cells in the renal medulla by immunohistochemistry. Top: Representative photomicrographs showing immunostaining of CD133+ cells. Bottom: Percentage of CD133-positive stained cells. * $P < 0.05$ vs. other groups ($n=6$).

Fig. 5. Effect of VA on the mRNA levels of IL-1β and IL-6 in the renal medulla from Dahl S by Real-time analysis. LS, low salt + vehicle; HS, high salt + vehicle; HS+VA, HS + valproic acid. * $P < 0.05$ vs. other groups ($n=6$).

Fig. 6. Effects of VA on sodium excretion (U·Na) in response to the elevation of blood pressure (A) and acute sodium loading (B) as well as the sodium balance after high salt intake (C & D). * $P < 0.05$ vs. control ($n = 5$). A, comparison of regression slopes by ANCOVA; B, C and D, comparison of mean values by ANOVA.

Fig. 7. Effects of VA on the mean arterial pressure (MAP) in Dahl S rats. LS, low salt + vehicle; HS, high salt + vehicle; HS+VA, HS + valproic acid. * $P < 0.05$ vs. other groups.

Effects of VA on urinary sodium excretion after acute sodium loading in Dahl S rats

Acute sodium loading increased U·Na. These increases in U·Na were significantly enhanced in VA-treated rats compared with controls (Fig. 6B).

Effects of VA on salt balance after chronic sodium loading in Dahl S rats

High salt intake produced a positive daily and cumulative salt balance. The daily positive salt balances were progressively increased in the first two days and decreased on the third day of high salt intake. The positive salt balances were significantly attenuated in VA-treated rats compared with controls (Fig. 6C&D).

Effect of VA on high salt induced increase in blood pressure in Dahl S rats

High salt intake remarkably increased mean arterial pressure (MAP) in vehicle-treated rats. However this high salt-induced increase in MAP was significantly reduced in VA-treated rats (Fig. 7), demonstrating that infusion of VA into renal medulla attenuated salt sensitive hypertension in Dahl S rats. The changes of systolic and diastolic pressure showed the exactly same pattern as MAP and that there was no change or difference in heart rate among different groups (data not shown).

Discussion

The present study demonstrated that high salt intake increased the levels of FGF2 and CD133 in the renal medulla in control rats but not in Dahl S rats. Infusion of VA significantly increased the levels of FGF2 and CD133 as well as the number of CD133⁺ cells in the renal medulla in Dahl S rats. Infusion of VA also suppressed high salt-induced increase in the

mRNA levels of IL-1 β and IL-6 in the renal medulla, promoted the sodium excretion, reduced sodium retention and attenuated high salt-induced increase in blood pressure in Dahl S rats.

We have recently demonstrated that there is a defect of stem cells in the renal medulla in Dahl S rats [12]. The present study additionally found that there were activations in the stem cell regulator FGF2 and stem cell marker CD133 in the renal medulla after high salt intake in normotensive rats and that this high salt-induced activation of stem cell-associated factors was absent in Dahl S rats. These results further suggest that the normal activation of stem cell function in response to high salt intake is also impaired, which is associated with the impaired response of stem cell regulator; in Dahl S rats. VA, a known agent to stimulate stem cell function, recovered the impaired response of FGF2 and CD133 to the high salt intake. As we showed before that the transplantation of MSC to correct the stem cell defect in the renal medulla reduced sodium retention and attenuated salt sensitive hypertension in Dahl S rats [12], we wondered if using VA to activate the stem cell function in the renal medulla would also be able to attenuate hypertension in Dahl S rats. Significant promotion of sodium excretion, reduction of sodium retention and attenuation of salt-sensitive hypertension in VA-treated Dahl S rats indicates that VA achieves a similar effect as stem cell transplantation to lowering blood pressure in this hypertensive model. Stem cell therapy has been used in the treatment of different forms of diseases and emerged as a new exciting therapeutic option for a variety of pathological conditions [42]. The stimulator effects of VA on the levels of FGF2 and CD133 as well as the number of CD133+ cells suggest that an agent that activates stem cell function can be used as an alternative approach to correct the stem cell defect in the management of different pathological conditions, such as renal medullary dysfunction and salt-sensitive hypertension.

It has been recognized that the beneficial effects of stem cell therapy are predominantly mediated by indirect paracrine mechanisms [43, 44]. In particular, it has been well documented that adult stem cells possess immunomodulatory and anti-inflammatory functions [44, 45]. Renal inflammation plays a pivotal role in salt-sensitive hypertension including that in Dahl S rats [46, 47]. We further evaluated whether VA-induced activation of stem cell function was associated with inhibition of the pro-inflammatory response to high salt challenge in the renal medulla. IL-1 β is closely associated with renal inflammation [48]. IL-6 has also been shown to participate in the salt-sensitive hypertension and renal injury in Dahl S rats [49]. Our results showed that high salt intake significantly increased the mRNA levels of pro-inflammatory factors IL-1 β and IL-6 and that VA blocked high salt-induced increases in pro-inflammatory factors in the renal medulla in Dahl S rats. These data suggest that VA improves stem cell function and corrects the impaired anti-inflammatory mechanisms associated with stem cell deficiency in the renal medulla, thereby attenuating hypertension after high salt challenge in Dahl S rats. These results are consistent with previous findings by us and by others showing that injection/transplantation of stem cells blocks the increase of pro-inflammatory factors in the renal medulla and attenuates hypertension in different models [12, 50].

It should be pointed out that there are limitations in the current study. First, although the present study was focused on the anti-inflammatory action of stem cells, stem cells possess other functions that may also contribute to the anti-hypertensive action. For example, in addition to the immunomodulation, stem cells have been shown to protect the kidneys by actions on cell proliferation/anti-apoptosis, angiogenesis, scavenger effect of oxidative stress, release of microvesicles/exosomes containing cytokines and mRNA/microRNA [51, 52]. Another limitation is the possible involvement of actions by VA that may be independent of stem cell-stimulating effect. For example, VA can inhibit histone deacetylases (HDAC), whereas HDAC is involved in epigenetic modulation [53, 54] and shown to mediate angiotensin II-induced cardiac hypertrophy [55]. VA also inhibits many other enzymes, such as epoxide hydrolase, glycogen synthase kinase-3 β and P450 [56, 57], and inhibits sodium channel as well [58]. All these effects of VA further complicate the possible interpretation of the blood pressure-lowering effects observed in the present study. Further, there may be potential influence of uninephrectomy on the stem cell activation. The

uninephrectomy is a routine strategy to study the role of kidney in long-term regulation of blood pressure, especially when intra-renal manipulation is needed. However, it would lead to kidney hypertrophy and could prime the stem cells for activation that might not occur if two kidneys were present. Nevertheless, the present study showed the activation of stem cells and the attenuation of inflammation in the kidney, which are known anti-hypertensive mechanisms and probably contribute to the blood pressure-lowering effect of VA.

In summary, the present study revealed a deficient response of stem cells to a high salt diet in the renal medulla in Dahl S rats and that chronic infusion of VA, an agent known to stimulate stem cell, increased the levels of stem cell-associated factors and the number of stem cells in the renal medulla and attenuated the salt-sensitive hypertension, which was associated with inhibition of high salt-induced increase of pro-inflammatory factors in the renal medulla. It is concluded that approaches to stimulate the activation of stem cell recovers the stem cell deficiency in the renal medulla and may serve as a new therapeutic strategy for salt-sensitive hypertension.

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Disclosure Statement

The authors declare no conflict of interest.

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