Angiotensin-converting enzyme inhibition improves defective angiogenesis in the ischemic limb of spontaneously hypertensive rats

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Abstract

Objectives: Natural angiogenesis has been shown to be impaired in spontaneously hypertensive rats (SHR). The purpose of this study was to determine whether pathological angiogenesis in the setting of tissue ischemia is also impaired in SHR, and to what extent it is modified by angiotensin-converting enzyme (ACE) inhibition. Methods: Ischemia was induced in the hindlimb of SHR by excision of the femoral artery, after which the animals were randomly assigned to receive low-dose perindopril (sub-antihypertensive, 0.2 mg/kg/day), high-dose perindopril (antihypertensive, 2.0 mg/kg/day), or vehicle for 3 weeks. Wistar–Kyoto rats (WKY) with femoral artery excision served as a control group. Results: Tissue ACE activity in SHR was significantly increased compared to WKY (49.4±6.2 vs. 34.0±14.2 IU/mg, P<0.01). Administration of perindopril significantly reduced ACE activity in SHR (low dose: 12.4±2.3; high dose: 11.0±2.1 IU/mg, P<0.005). Angiogenesis of the ischemic limb muscles was significantly impaired at 4 weeks in SHR versus WKY as indicated by the lower capillary density in the former (364.5±43.0 vs. 463.8±63.0/mm², P<0.05) as well as the reduced hindlimb perfusion assessed by laser Doppler imaging (0.86±0.08 vs. 1.03±0.09, P<0.05). Administration of perindopril significantly augmented both the capillary density (low dose: 494.3±69.8; high dose: 543.9±76.9/mm², P<0.005) and the limb perfusion (low dose: 1.06±0.15; high dose: 1.05±0.12, P<0.05) of the ischemic limb in SHR. Conclusions: These findings indicate that pathological angiogenesis in the setting of tissue ischemia is impaired in SHR compared with WKY, and that this impairment can be reversed by ACE inhibition. The angiogenic properties of an ACE inhibitor may benefit patients with essential hypertension presenting with lower limb vascular insufficiency.

Keywords: ACE inhibitors; Angiogenesis; Capillaries; Collateral circulation; Hypertension; Ischemia

1. Introduction

The ability of organisms to spontaneously develop collateral vessels represents an important response to vascular occlusive disease that determines the severity of residual tissue ischemia. Recent investigations have indicated that certain cardiovascular risk factors, including hyperlipidemia [1] and diabetes [2], adversely affect collateral development in animal models of limb ischemia. Hypertension is another major risk factor for cardiovascular diseases. Previous studies have suggested that natural angiogenesis is impaired in human and animal models of hypertension [3,4]. However, whether pathological angiogenesis in the setting of tissue ischemia is similarly affected in individuals prone to develop primary hypertension has not yet been studied.

Angiotensin-converting enzyme (ACE) inhibitors have been shown to augment natural angiogenesis in normotensive [5] and hypertensive [6] animals. ACE inhibitors have also been shown to enhance collateral vessel development in the ischemic limb of normotensive animals [7]. Taken together, these findings suggest that ACE inhibitors may
have a favorable impact upon collateral vessel development in hypertensive individuals.

Accordingly, in the current study, we investigated whether angiogenesis is impaired in the ischemic limb of spontaneously hypertensive rats (SHR), and to what extent it is modified by ACE inhibition. The outcome of this in vivo study, performed in a rat model of limb ischemia, indicates that pathological angiogenesis in the setting of tissue ischemia is impaired in hypertensive individuals, and that this impairment can be reversed by ACE inhibition. The angiogenic properties of an ACE inhibitor may have clinical utility in the treatment of lower limb vascular insufficiency associated with essential hypertension.

2. Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.1. Ischemic hindlimb model

Pathological angiogenesis in the setting of tissue ischemia was studied using a rat model of hindlimb ischemia described previously [8–10]. Briefly, 4-week-old male SHR (SHR/Izm, Funabashi Farm, Chiba, Japan) and Wistar–Kyoto rats (WKY/Izm, Funabashi Farm) [11] were anesthetized with sodium pentobarbital (40 mg/kg i.p.; Nembutal, Abbott Laboratories, North Chicago, IL), following which the left femoral artery was surgically excised to induce limb ischemia. The animals were then divided into four groups: WKY treated with vehicle (n=8); SHR treated with vehicle (SHR-vehicle, n=8); SHR treated with low-dose perindopril (0.2 mg/kg/day, SHR-low, n=8); SHR treated with high-dose perindopril (2.0 mg/kg/day, SHR-high, n=8). The doses of perindopril used here were determined based on previous animal studies [12,13]. Perindopril dissolved in water was administered orally once a day for 3 weeks. Animals were weighed daily to adjust the administered dose.

Blood pressure was measured once a week using a tail-cuff probe connected to a pressure monitor (BP-98A, Softron, Tokyo, Japan). At 4 weeks (1 week after discontinuation of perindopril), following blood pressure measurement, the animals were anesthetized to determine hindlimb blood flow (see below). An interval of 1 week between the time of perindopril discontinuation and blood flow measurement was incorporated to obviate a direct vasodilator effect of perindopril.

2.2. Hindlimb blood flow

At 4 weeks (1 week after the discontinuation of perindopril), hindlimb blood flow was measured using a laser Doppler perfusion imager (LDPI, Lisca Inc., Linköping, Sweden) [1,2]. The laser beam sequentially scans the tissue surface to a depth of several hundred microns. During the scanning procedure, the moving blood cells shift the frequency of incident light based on the Doppler principle. A photodiode collected the back-scattered light, and variations in the original light intensity are transformed into voltage variations (range: 0 to 10 V). Hindlimb blood flow is expressed as the ratio of perfusion in the ischemic versus non-ischemic limb.

2.3. Capillary density

Capillary density, as an anatomic index of angiogenesis, was examined by measuring the number of capillaries in light microscopic sections taken from both the non-ischemic and the ischemic limbs as previously described [7,14–16]. Briefly, at 4 weeks, animals were sacrificed after blood flow measurement by LDPI. Tissue samples were obtained as transverse sections from the rectus femoris and adductor muscles. These two muscles were chosen because (1) they are the major muscles of the thigh, and (2) they were originally perfused by the excised femoral artery. Harvested muscles were embedded in O.C.T. compound (Miles, Elkhart, IN), and snap-frozen in liquid nitrogen. Multiple frozen sections (5 μm in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion. Tissue sections were stained for alkaline phosphatase using an indoxyl-tetrazolium method to detect capillary endothelial cells [17], and counterstained with eosin. A total of 20 different fields from two muscles (10 fields each) was randomly selected by a single observer blinded to the treatment regimen. Care was taken so that the selected fields were distributed equally over the whole sample area. Capillaries were counted under a 10× objective to determine the capillary density (mean number of capillaries per mm²).

2.4. Tissue ACE activity

An additional four groups of rats (n=8 for each) underwent femoral artery excision, and were treated for 1 week with perindopril, following which the animals were sacrificed. Since one muscle was often not large enough for the measurement, rectus femoris and adductor muscles were harvested, and homogenized together in saline. After centrifugation, the supernatant was used for the measurement of ACE activity, based on a colorimetric method with p-hydroxybenzoyl-glycyl-l-histidyl-l-leucine as a substrate (Fujirebio Inc., Tokyo, Japan) [18]. ACE activity is expressed as international units per mg of tissue (IU/mg).

2.5. Statistics

Results are expressed as mean±standard deviation.
Statistical significance was evaluated by ANOVA followed by Scheffe’s procedure using the StatView application (version 5.0 for Windows, SAS Institute Inc., Cary, NC). A value of \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Tissue ACE activity

ACE activity in the SHR-vehicle group was significantly higher than in the WKY group (49.4±6.2 vs. 34.0±14.2 IU/mg, \( P < 0.01 \)). Administration of perindopril significantly inhibited ACE activity in the SHR-low (12.4±2.3 IU/mg) and SHR-high (11.0±2.1 IU/mg) groups, which were lower than in SHR-vehicle and WKY groups (\( P < 0.005 \) for both). No statistically significant differences in ACE activity were found between SHR-low and SHR-high groups (Fig. 1A).

3.2. Systolic blood pressure

At baseline, the systolic blood pressure was significantly higher in SHR groups (SHR-vehicle: 119.3±8.0 mmHg, SHR-low: 118.9±5.4 mmHg, SHR-high: 118.8±13.3 mmHg) than in the WKY group (100.7±6.3 mmHg, \( P < 0.005 \) for all). During treatment, the systolic blood pressure in the SHR-vehicle group gradually increased (1-week: 128.9±5.2 mmHg, 2-week: 134.3±7.3 mmHg, 3-week: 152.5±12.7 mmHg), which was significantly higher than in the WKY group (1-week: 107.8±8.1 mmHg, 2-week: 108.4±5.8 mmHg, 3-week: 110.4±6.8 mmHg, \( P < 0.005 \) at each time point). Administration of high-dose perindopril lowered blood pressure significantly in the SHR-high group (1-week: 105.7±6.1 mmHg, 2-week: 113.8±5.3 mmHg, 3-week: 109.5±15.5 mmHg, \( P < 0.005 \) vs. SHR-vehicle at each time point). In contrast, low-dose perindopril did not affect systolic blood pressure in the SHR-low group (1-week: 128.5±5.5 mmHg, 2-week: 133.8±8.1 mmHg, 3-week: 150.6±6.3 mmHg), the level of which was similar to that in the SHR-vehicle group, and significantly higher than those in the SHR-high and WKY groups (\( P < 0.005 \) for both, Fig. 1B).

At 4 weeks (1 week after discontinuation of perindopril), the systolic blood pressure of the SHR-vehicle (158.3±9.4 mmHg) and SHR-low groups (157.8±8.8 mmHg) continued to be higher than that of the WKY group (111.1±4.5 mmHg, \( P < 0.005 \) for both). No signifi-

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Fig. 1. Effects of perindopril on (A) tissue angiotensin-converting enzyme (ACE) activity (*\( P < 0.01 \) vs. WKY; †\( P < 0.005 \) vs. SHR-low, SHR-high), (B) systolic blood pressure (*\( P < 0.005 \) vs. WKY, SHR-high; †\( P < 0.005 \) vs. WKY), (C) limb blood flow (*\( P < 0.05 \) vs. SHR-vehicle), and (D) capillary density (*\( P < 0.05 \), †\( P < 0.005 \) vs. SHR-vehicle). WKY, Wistar–Kyoto rats; SHR-vehicle, spontaneously hypertensive rats (SHR) treated with vehicle; SHR-low, SHR treated with low-dose perindopril (0.2 mg/kg/day); SHR-high, SHR treated with high-dose perindopril (2.0 mg/kg/day).
cant differences were observed in blood pressure between the SHR-vehicle and SHR-low groups. The systolic blood pressure of the SHR-high group increased to 139.6 ± 8.9 mmHg at 4 weeks, which was significantly higher than that of the WKY group (P < 0.005), but still lower than those of the SHR-vehicle and SHR-low groups (P < 0.005 for both) (Fig. 1B).

3.3. Hindlimb blood flow

Limb perfusion was assessed by LDPI at 4 weeks. The blood flow ratio in the SHR-vehicle group (0.86 ± 0.08) was significantly lower than that in the WKY group (1.03 ± 0.09, P < 0.05). Treatment with perindopril significantly increased limb blood flow in both the SHR-low (1.06 ± 0.15) and SHR-high groups (1.05 ± 0.12) compared with the SHR-vehicle group (P < 0.05 for both; Figs. 1C and 2A–D). No significant difference in flow was observed between SHR-low and SHR-high groups.

3.4. Capillary density

Histologic analysis of ischemic limb muscles demonstrated a decrease in the capillary density in the SHR-vehicle group (364.5 ± 43.0/ mm²) versus the WKY group (463.8 ± 63.0/ mm², P < 0.05). Administration of perindopril increased the capillary density in both the SHR-low (494.3 ± 69.8/ mm²) and SHR-high groups (543.9 ± 76.9/ mm²), which were significantly greater than in the SHR-vehicle group (P < 0.005 for both). No statistically significant difference was observed in capillary density between SHR-low and SHR-high groups (Figs. 1D and 2E–H). Analysis of capillary density was also performed in muscles of the non-ischemic limb. Capillary density in the SHR-vehicle group (378.7 ± 47.4/ mm²) was significantly lower than that in the WKY group (467.3 ± 35.1/ mm², P < 0.005). Treatment with high-dose perindopril significantly augmented the capillary density in the SHR-high group (514.7 ± 45.5/ mm², P < 0.001 vs. SHR-vehicle), while low-dose perindopril failed to do so in the SHR-low group (438.2 ± 81.8/ mm², P = 0.08 vs. SHR-vehicle).

4. Discussion

The present study is, to our knowledge, the first to document that pathological angiogenesis in the setting of limb ischemia is impaired in hypertensive animals and that such impaired angiogenesis in hypertensive animals can be improved by ACE inhibition even at doses too low to antagonize the development of hypertension.

4.1. Impaired angiogenesis in the ischemic limb of SHR

Although hypertension is a major risk factor for cardiovascular diseases, the extent to which it may modulate angiogenesis in the setting of tissue ischemia has not been studied. We used a model of hindlimb ischemia in SHR, which develops a form of hypertension with clinical features similar to those of human essential hypertension, and documented impaired angiogenesis in the ischemic limb of SHR compared with WKY. There are several possible mechanisms which could be responsible for impaired angiogenesis in SHR. Firstly, angiogenic potential is impaired in SHR compared with WKY [4,19]. The decrease in the number of arterioles and capillaries has been shown to be the most consistent change in the microcirculation in human and animal models of hypertension, so-called rarefaction [4].

As observed in the non-ischemic limb of SHR in the current study, rarefaction occurs even in the very early stages of hypertension in SHR [20] and in normotensive young adults with a genetic predisposition to hypertension [3]. These findings suggest that rarefaction in the non-ischemic limb as well as the decreased angiogenic response in the ischemic limb observed in the current study may be the result of inherently decreased angiogenic potential in individuals prone to develop primary hypertension, rather than an adaptive remodeling of the vascular network due to altered mechanical stresses in hypertension. Secondly, angiogenesis is a complex process that involves activation, migration, and proliferation of endothelial cells. Previous observations that endothelial function is impaired in SHR [21,22] as well as in patients with essential hypertension [23] imply that the defective endothelial function may contribute to impaired angiogenesis in SHR. Lastly, expression of certain angiogenic factors are decreased in SHR versus WKY. Using a chick embryo chorio-allantoic membrane, le Noble et al. demonstrated that angiogenic capacity of serum derived from SHR was less than that from WKY [19] Nakano et al. also demonstrated that the expression of hepatocyte growth factor (HGF) was decreased in nonischemic limb muscles of SHR compared to WKY [24]. It is possible that such decreased expression of angiogenic factors may have contributed to impaired angiogenesis in SHR observed in the current study.

4.2. Effects of perindopril on angiogenesis in the ischemic limb of SHR

The selection of the two oral doses of perindopril used in the present study was based on perindopril’s effect on systemic blood pressure. Specifically, we used 0.2 mg/kg/day, which did not alter blood pressure, and 2.0 mg/kg/day, which decreased systolic pressure by 40 mmHg. Treatment with either dose of perindopril significantly augmented angiogenesis of the ischemic limb in SHR. The fact that low-dose perindopril did not alter the blood pressure suggests that the beneficial effect of ACE inhibition upon angiogenesis in the ischemic limb is independent of changes in blood pressure.

The evaluation of limb perfusion by LDPI was per-
Fig. 2. Laser Doppler imaging and alkaline phosphatase staining. Bright colors (red, yellow, green) in the left ischemic limb (arrows) indicated the perfusion comparable to the right non-ischemic limb in (A) WKY, (C) SHR-low, and (D) SHR-high groups. In contrast, blue color in the left ischemic limb (arrow) indicated decreased perfusion in (B) SHR-vehicle group. Histologic analysis documented decreased number of capillaries in the ischemic limb of (F) SHR-vehicle group compared with (E) WKY, (G) SHR-low, and (H) SHR-high groups. Counterstained with eosin. Bar=100 μm. WKY, Wistar–Kyoto rats; SHR-vehicle, spontaneously hypertensive rats (SHR) treated with vehicle; SHR-low, SHR treated with low-dose perindopril (0.2 mg/kg/day); SHR-high, SHR treated with high-dose perindopril (2.0 mg/kg/day).
formed 1 week after the discontinuation of perindopril. An interval of 1 week between the time of discontinuation and blood flow measurement was incorporated to obviate a direct as well as an indirect vasodilator effect of perindopril, which includes, for example, inhibition of endothelin-1, a potent vasoconstrictor that may be modified by ACE inhibition. Therefore, increased limb perfusion observed in the perindopril-treated animals was not simply the result of a transient, pharmacologically-mediated improvement in endothelium-dependent blood flow. Rather, increased perfusion may reflect persistent modification of the hind-limb vasculature. In fact, necropsy examination documented a significant increase in vascularity at the capillary level in the perindopril-treated animals, consistent with the classical definition of angiogenesis espoused by Klagsbrun and Folkman [25].

The mechanisms responsible for augmented angiogenesis in response to ACE inhibition remain to be elucidated. Fabre et al. hypothesized that ACE inhibition might accelerate angiogenesis indirectly by increasing nitric oxide production, a critical regulator of angiogenesis [7]. ACE inhibition blocks degradation of bradykinin, a potent activator of the l-arginine-NO pathway, which has been shown to promote growth of endothelial cells from post-capillary venules via B1 receptors [26]. The observation that increased capillary length density after ACE inhibition is abolished by chronic bradykinin B2-receptor blockade constitutes inferential evidence that bradykinin may mediate angiogenesis in response to ACE inhibition. Zimmermann et al. reported that expression of vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen shown to promote collateral formation in the ischemic limb of rats [9,10], was increased in cardiac tissue following ramipril treatment [27]. It has also been shown that angiotensin II is a strong inhibitor of HGF adeno-VEGF. Am J Pathol 1999;154:355±363.

4.3. Effects of perindopril on angiogenesis in the non-ischemic limb of SHR

The current study confirmed previous observations that ACE inhibition augments angiogenesis in the non-ischemic tissue of SHR [6]. It must be noted, however, that a higher dose of perindopril was required to augment angiogenesis when compared to the contralateral ischemic limb. Whether such differences in the dose of perindopril may be a distinct consequence of pathological angiogenesis of the ischemic limb versus natural angiogenesis of the non-ischemic limb remains to be determined.

4.4. Study limitations

In this study, we evaluated the angiogenic potential of perindopril using a young SHR at the early stage of hypertension. It must be acknowledged that such angiogenic impact of perindopril cannot be extrapolated to other ACE-inhibitors [7,28], to older animals [29] at different stages of hypertension, and to other diseases such as proliferative retinopathy [30].

5. Conclusions

The current study indicates that angiogenesis of the ischemic limb is impaired in SHR and that this impairment can be reversed by ACE inhibition. The angiogenic potential of ACE inhibitors may benefit patients with essential hypertension presenting with lower limb vascular insufficiency.

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