Cyclic Stretch and Hypertension Increase Retinal Succinate: Potential Mechanisms for Exacerbation of Ocular Neovascularization by Mechanical Stress

Hirofumi Kinoshita,[1] Kiyoshi Suzuma,[1] Toshihide Maki,[2] Yuki Maekawa,[1] Makiko Matsumoto,[1] Mao Kusano,[1] Masafumi Uematsu,[1] and Takashi Kitaoka[1]

[1]Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
[2]Department of Structure Analysis for Chemicals, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Correspondence: Kiyoshi Suzuma, Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan; suzuma@nagasaki-u.ac.jp.

Submitted: December 27, 2013
Accepted: June 1, 2014

Citation: Kinoshita H, Suzuma K, Maki T, et al. Cyclic stretch and hypertension increase retinal succinate: potential mechanisms for exacerbation of ocular neovascularization by mechanical stress. *Invest Ophthalmol Vis Sci*. 2014;55:4320–4326. DOI:10.1167/iovs.13-13839

Purpose. We investigated succinate metabolism in cells undergoing clinically relevant cyclic stretch and in spontaneously hypertensive rat (SHR) retina.

Methods. We seeded ARPE-19 cells on 6-well BioFlex collagen I–coated, silicone elastomer–bottomed culture plates. Cells then were subjected to pulsatile stretch using a computer-controlled vacuum stretch apparatus. A physiologic stretch frequency of 60 cycles per minute and 5% to 15% prolongation of the elastomer-bottomed plates were used. Succinate concentration was assessed by enzymatic analysis and high-performance liquid chromatography-mass spectrometry. The VEGF was measured using enzyme-linked immunosorbent assays. The 12-week-old male SHRs and weight-matched Wistar-Kyoto (WKY) control rats were treated with or without 100 $mg \cdot kg^{-1} \cdot day^{-1}$ captopril for 1 week. The vitreous body and retina of each rat were extracted after 1 week of therapy, and the vitreoretinal succinate concentration was measured.

Results. Cells exposed to cyclic stretch accumulated intracellular succinate in a time- and magnitude-dependent manner, and also accumulated VEGF protein levels. Moreover, BAPTA/AM, an intracellular calcium chelate reagent, significantly inhibited the stretch-induced succinate increase. After cyclic stretch, levels of intracellular fumarate, a citric acid cycle intermediate, also were significantly increased compared to controls. The BAPTA/AM inhibited this increase. For the in vivo experiments, hypertension increased vitreoretinal succinate and fumarate in SHRs compared to the normotensive WKY controls. When hypertension was reduced using captopril, vitreoretinal succinate returned to baseline levels.

Conclusions. These findings suggest that cyclic stretch and hypertension increased intracellular succinate in cultured retinal pigment epithelial cells and the vitreoretinal succinate of SHRs through a calcium-dependent pathway.

Keywords: hypertension, stretch, succinate, retina

Numerous vision-threatening diseases, such as diabetic retinopathy[1–3] and age-related macular degeneration (AMD), are exacerbated by, or associated with, coexistent systemic hypertension. Increased vascular permeability and intraocular neovascularization characterize these conditions, and are complications primarily mediated by VEGF.[4–9]

Citric acid cycle intermediates, such as succinate, accumulate in conditions linked with insufficient oxygen supply.[10,11] Recent studies have reported that succinate accumulates in the hypoxic retina of rodents, and induces VEGF expression and potently mediates vessel growth during normal retinal development and proliferative ischemic retinopathy via its cognate receptor, G protein-coupled receptor-91 (GPR91).[12,13] Moreover, we previously demonstrated that succinate increased in the vitreous fluid of patients with active proliferative diabetic retinopathy (PDR).[14]

To the best of our knowledge, however, the effect of hypertension on succinate metabolism has yet to be determined. Because systemic hypertension increases vascular and tissue stretch, we evaluated succinate levels in RPE cells undergoing clinically relevant cyclic stretch, which mimics systemic hypertension, and in the spontaneously hypertensive rat (SHR) retina.

Methods

Reagents

Reagent GF109203X was purchased from Wako (Osaka, Japan), while LY294002, genistein, PD98059, and BAPTA/AM were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

The ARPE-19 cells, a human RPE cell line, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). To confirm that the ARPE-19 cells were of RPE origin, we first identified the immunohistochemistry using anti-pan cytokeratin (1:100; Sigma-Aldrich; data not shown). Cells were maintained in growth medium that consisted of Dulbecco's

modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F-12) media with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin (all purchased from Gibco, Carlsbad, CA, USA). Cells were cultured in 5% CO_2 at 37°C, with the medium changed every 3 days. Cells were plated at a density of 0.5 to 1.0 × 10^4 cells/cm^2 and passaged when confluent (3–6 days). Cells from passages 20 to 30 were used for the experiments.

Mechanical Stretch

Cells were seeded on 6-well BioFlex collagen I–coated, silicone elastomer–bottomed culture plates (Flexcell Intl. Corp., McKeesport, PA, USA). When the cultures were confluent, the culture medium was replaced with serum-free DMEM/F-12 for 24 hours. Cells then were subjected to uniform radial and circumferential strain in 5% CO_2 at 37°C using a computer-controlled vacuum stretch apparatus (Flexercell Strain Unit; Flexcell Intl. Corp.). Stretch magnitudes are reported as a percent, while the cyclic stretch frequencies are reported as cycles per minute (cpm). A physiologic stretch frequency of 60 cpm and 5% to 15% prolongation of the elastomer-bottomed plates were used in accordance with a previously described method.[15] For controls, BioFlex collagen I culture plates were prepared in parallel, but not subjected to pulsatile stretch.

Succinate Extraction

The medium was decanted and cells were washed three times with cold PBS and solubilized in 100 µL/well TNE buffer (10 mmol/L Tris-HCl [pH 7.8], 1% NP40, 0.15 mol/L NaCl, 1 mmol/L EDTA, and 1.5 µmol/L aprotinin). The suspension was incubated at 4°C for 10 minutes, and then centrifuged at 15,000*g* for 10 minutes. The aqueous phase was transferred to a new tube, and stored at –80°C until needed. Succinate concentration was assessed by enzymatic analysis of succinate and normalized with total protein quantity. Intracellular succinate concentration was determined using a cuvette-based enzymatic assay according to the manufacturer's instructions (Boehringer Mannheim/R-Biopharm AG, Mannheim, Germany).[16] Briefly, the enzymatic reaction measures the conversion of succinate by evaluating succinyl-CoA synthetase, pyruvate kinase, and L-lactate dehydrogenase, and the stoichiometric amount of nicotinamide adenine dinucleotide (NADH) oxidized in the reaction. By measuring the absorbance at 340 nm (UV detection), the amount of succinate can be calculated from the amount of NADH oxidized.

Succinate Quantitation Using HPLC-Mass Spectrometry (HPLC/MS)

Intracellular succinate levels also were quantified using a previously described selective ion monitoring mode of HPLC/MS, with slight modifications made to additionally confirm the intracellular succinate concentration using enzymatic analysis. To ensure we achieved optimal performance during the quantification, we performed ion exclusion column chromatography using 0.1% formic acid as the eluent and negative mode detection with electrospray ionization mass spectrometry.

Quantitative VEGF

The VEGF protein levels were measured using ELISA (R&D Systems, Minneapolis, MN, USA).

In Vivo Studies

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the rules and regulations to animal experiments at Nagasaki University (approval number, 1010250881). The 12-week-old male SHRs and weight-matched Wistar-Kyoto (WKY) control rats were obtained from KBT Oriental Co., Ltd. (Tosu, Japan) and allowed to become accustomed to their new surroundings for 1 week. Systolic blood pressure was measured in each animal using a tail cuff sensor and monitoring system (MK-2000; Muromachi Kikai, Tokyo, Japan). Animals then were treated with or without 100 $mg \cdot kg^{-1} \cdot day^{-1}$ captopril for 1 week. The drugs were administered in the animals' drinking water. Blood pressure measurements were repeated after 1 week of therapy. Before extraction of the vitreous body and retina of each rat, the animals were deeply anesthetized with intraperitoneal pentobarbital (100 mg/kg) and then killed by a pentobarbital overdose. Whole enucleated eyes were cut in half equatorially behind the ora serrata and lenses, with the capsules then removed carefully. The vitreoretinal complexes were mechanically teased apart with micro forceps, and separated from the choroid-sclera carefully. Extracted vitreoretinal complexes of the right and left eyes were solubilized in 750 µL TNE buffer and thoroughly ground up using a pestle. After homogenization, samples were stored at –80°C until needed.

Statistical Analysis

All experiments were repeated at least three times unless otherwise indicated. Results are expressed as mean ± SD. Statistical analysis used either a Dunnett test or Tukey test to compare the quantitative data populations with normal distributions and equal variance. A P value of <0.05 was considered statistically significant.

Results

Cyclic Stretch Increased Intracellular Succinate

To determine whether cardiac-profile cyclic stretch at 60 cpm was sufficient for increasing the intracellular succinate in ARPE-19 cells, intracellular succinate after cyclic stretch was investigated by enzymatic analysis. In the first step, we determined that the maximum magnitude of the cyclic stretch was 15%. The average amount of succinate for the control (which corresponds to the unstretched cells) was 18.20 ± 8.20 mg (succinate)/g (total protein). The average amounts of succinate for the 1-, 2-, 3-, 6-, 9-, and 24-hour stretches were 24.87 ± 10.35, 38.00 ± 7.03 ($P = 0.047$), 35.84 ± 11.64 ($P = 0.023$), 38.19 ± 14.12 ($P = 0.027$), 39.32 ± 13.07 ($P = 0.019$), and 38.35 ± 12.12 ($P = 0.009$), respectively (Fig. 1A). A significant increase was observed for the average amount of succinate after a cyclic stretch of 2 or more hours. Based on these results, we subsequently investigated intracellular succinate after further changes in the magnitude of the cyclic stretch for 2 hours. Confluent cultures of ARPE-19 cells were subjected to 5%, 10%, and 15% cyclic stretch for 2 hours. As seen in Figure 1B, significant increases were observed for the average intracellular succinate after a 10% (25.25 ± 5.72) and 15% (24.49 ± 5.16) cyclic stretch ($P < 0.05$). We also investigated intracellular succinate after cyclic stretch by using HPLC/MS to confirm the amount of succinate. As seen in Figure 1C, the HPLC/MS results were similar to those found for the enzymatic analysis, with significant increases noted in the intracellular succinate after a cyclic stretch for 2 hours (3.65 ± 1.49-fold compared to control, $P < 0.05$). These results

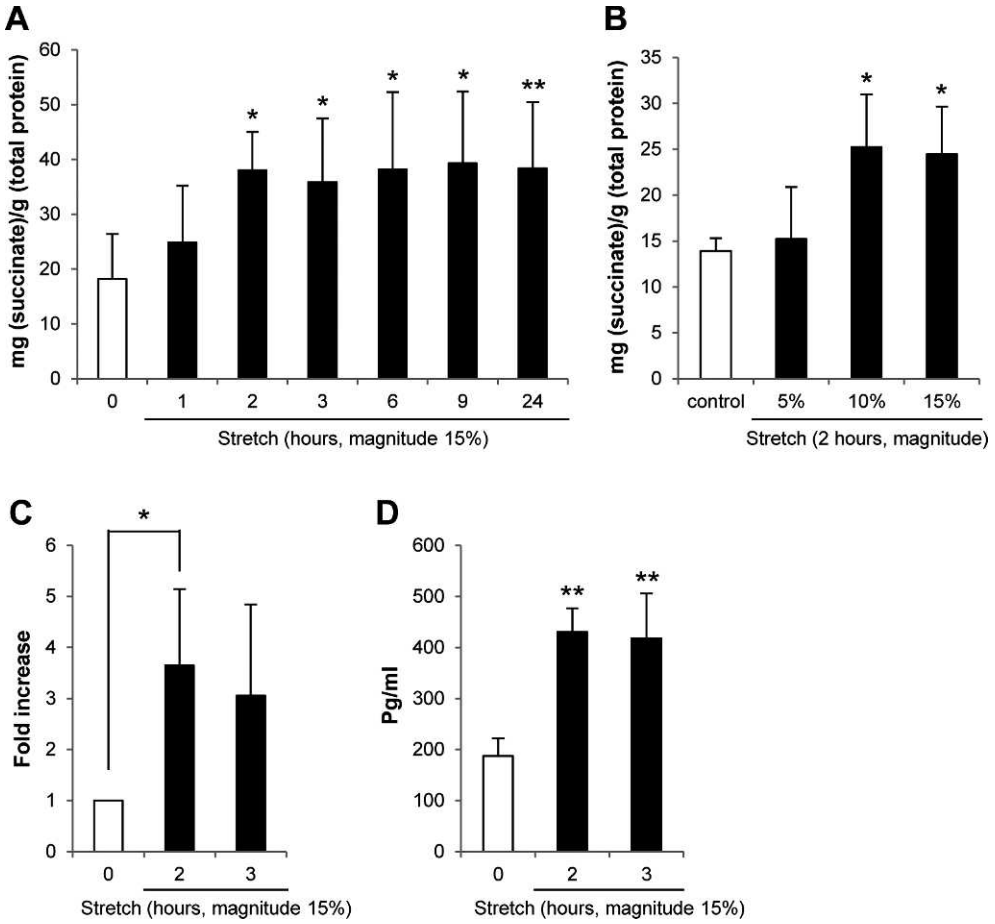

FIGURE 1. Cyclic stretch increases intracellular succinate in a stretch time- and magnitude-dependent manner. (**A**) Intracellular succinate after cyclic stretch was investigated by enzymatic analysis. The *vertical axis* corresponds to the corrected amount of succinate when using the total protein, while the *horizontal axis* corresponds to the stretch time. Average amount of succinate after 15%/60 cpm cyclic stretch significantly increased after 2 or more hours compared to control (*$P < 0.05$, **$P < 0.01$, Dunnett test). (**B**) Confluent cultures of ARPE-19 cells were subjected to 5%, 10%, and 15% cyclic stretch for 2 hours. Significant increases were observed for the average intracellular succinate after 10% and 15% cyclic stretch (*$P < 0.05$, Dunnett test). (**C**) Intracellular succinate after cyclic stretch also was investigated by HPLC/MS to confirm the amount of succinate. Results of HPLC/MS were similar to the enzymatic analysis, with the results showing a significant increase in the intracellular succinate after 2 hours of cyclic stretch (*$P < 0.05$, Dunnett test). (**D**) To confirm the relationship between succinate and VEGF, ELISA was used to investigate the VEGF protein levels after cyclic stretch. Significant increases were observed for the average VEGF in the cell lysate after cyclic stretch for 2 and 3 hours (**$P < 0.01$, Dunnett test). *$P < 0.05$, **$P < 0.01$.

suggested that cyclic stretch increased the intracellular succinate in a time- and magnitude-dependent manner. Moreover, to confirm the correlation between succinate and VEGF, VEGF protein levels from the same samples also were investigated. As seen in Figure 1D, significant increases were observed for the average VEGF protein levels after a cyclic stretch for 2 (430.67 ± 46.57 pg/mL) and 3 (418.17 ± 87.65) hours (control, 187.33 ± 34.54; $P < 0.01$).

Mechanistic Evaluation of Stretch-Induced Succinate Increase

To determine the mechanism by which stretch increased intracellular succinate, inhibitors of classical/novel protein kinase C (PKC) isoforms (GF109203X, 5 μmol/L), phosphatidylinositol (PI) 3-kinase (LY294002, 50 μmol/L), tyrosine phosphorylation (genistein, 100 μmol/L), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) 1 (PD98059, 20 μmol/L), and intracellular calcium (BAPTA/AM, 10 μmol/L) were evaluated (Fig. 2A). In all experiments, 2 hours of 10%/60 cpm cyclic stretch induced intracellular succinate metabolism. Furthermore, the cyclic stretch significantly increased the intracellular succinate (31.89 ± 12.87, $P < 0.01$) compared to controls. However, inhibitors of MEK1 that used PD98059 had little effect on the stretch-induced succinate metabolism. Similarly, there were no alterations of the intracellular succinate metabolism after inhibition of the PKC classical/novel isoforms using GF109203X, PI 3-kinase using LY294002, or tyrosine phosphorylation using genistein. In contrast, use of BAPTA/AM to inhibit the intracellular calcium resulted in marked inhibition of the stretch-induced intracellular succinate (17.24 ± 6.24, $P < 0.01$). Subsequently, we then evaluated the activity of the citric acid cycle during the intracellular succinate increase by using HPLC/MS to measure fumarate, which is the metabolite of succinate in the citric acid cycle. Results indicated that levels of intracellular fumarate were similar to those for the intracellular succinate during the cyclic stretch. As shown in Figure 2B, we observed a significant increase in the average amount of fumarate (3.39 ± 2.57-fold, $P < 0.05$) after 2 hours of 10%/60 cpm cyclic stretch compared to control, and a marked inhibition of stretch-induced intracellular fumarate (1.29 ± 1.07-fold, $P < 0.05$) after BAPTA/AM inhibition of the intracellular calcium. These results suggested that intracellular

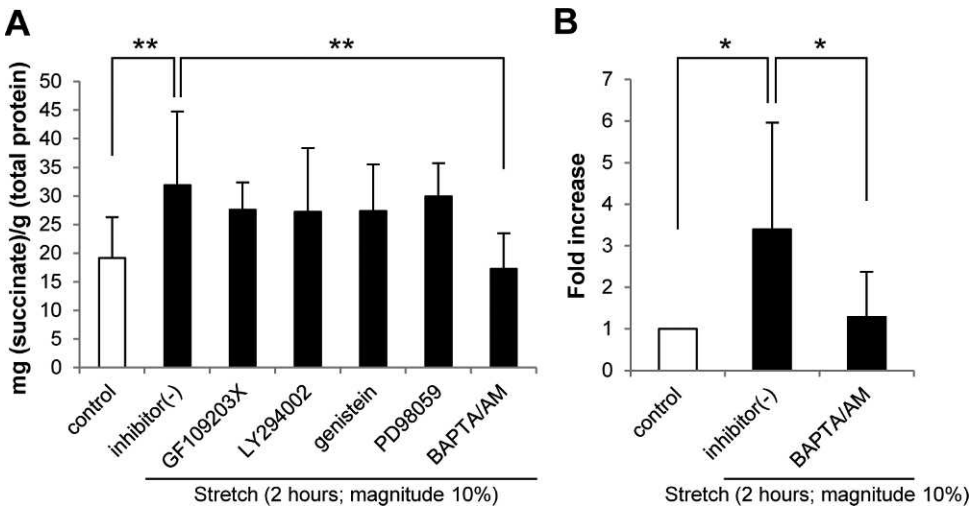

FIGURE 2. Effect of various inhibitors on stretch-induced succinate and fumarate metabolism. Confluent cultures of ARPE-19 cells were exposed to 10% cyclic stretch at 60 cpm for 2 hours in the presence of the PKC classical/novel isoform inhibitor GF109203X (5 μmol/L), PI 3-kinase inhibitor LY294002 (50 μmol/L), tyrosine kinase inhibitor genistein (100 μmol/L), MEK1 inhibitor PD98059 (20 μmol/L), and an intracellular calcium chelate reagent BAPTA/AM (10 μmol/L). (**A**) Stretch-induced succinate was examined in confluent cultures of ARPE-19 cells after treatment with various pharmacologic inhibitors. A significant increase was observed for the 2-hour stretch treatment compared to control (**$P < 0.01$, Tukey test). After treatment with BAPTA/AM, a decrease was observed compared to the 2-hour stretch results (**$P < 0.01$, Tukey test). (**B**) To evaluate how active the citric acid cycle was during the intracellular succinate increase, fumarate, which is the subsequent succinate metabolite in the citric acid cycle, was measured by HPLC/MS. After cyclic stretch, the intracellular fumarate was similar to the succinate. Average amount of fumarate after 10% cyclic stretch for 2 hours was significantly increased compared to control (*$P < 0.05$, Tukey test), while it was significantly decreased after treatment with BAPTA/AM (*$P < 0.05$, Tukey test). *$P < 0.05$, **$P < 0.01$.

succinate metabolism involves a calcium-dependent pathway, with fumarate exhibiting a parallel reaction with succinate in the citric acid cycle.

Vitreoretinal Succinate and Fumarate in WKY SHR Rats

To determine if hypertension induced an increase in the vitreoretinal succinate, 12-week-old SHRs (derived from WKY rats) and weight-matched WKY control animals were treated orally for 1 week with or without the angiotensin-converting enzyme inhibitor, captopril. The SHRs had elevated baseline systolic blood pressures ($P < 0.001$) compared to the WKY controls (see Table). Systolic blood pressure was reduced in response to the captopril therapy as compared to the untreated SHRs ($P < 0.05$). As seen in Figure 3A, increased vitreoretinal succinate was observed in the SHRs (16.53 ± 3.33, $P < 0.05$) compared to the normotensive WKY control animals (12.71 ± 3.29). After using captopril to reduce the hypertension in the SHRs, vitreoretinal succinate (12.44 ± 1.67) decreased to the same levels seen in the normotensive WKY controls. Similarly, increased vitreoretinal fumarate also

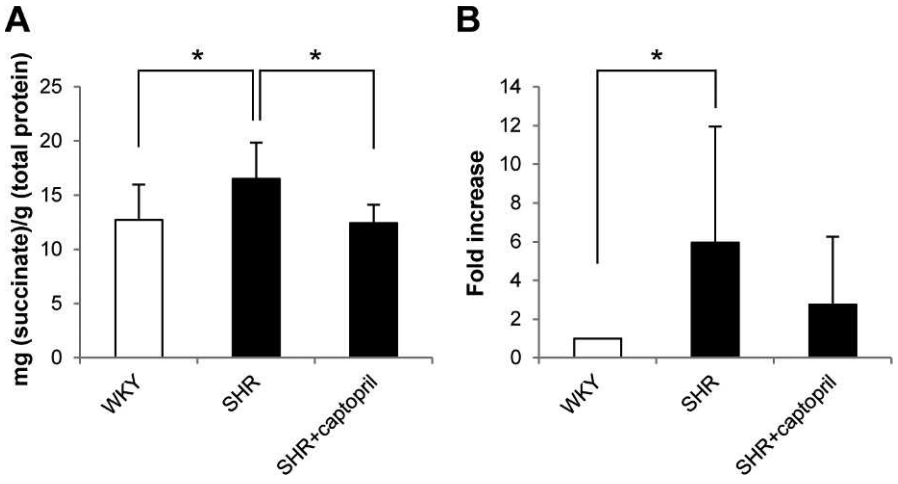

FIGURE 3. Vitreoretinal succinate and fumarate levels in the Wistar-Kyoto (WKY) rat and spontaneously hypertensive rat (SHR). After 12-week-old SHRs (derived from WKY rats) and weight-matched WKY control animals were treated orally for 1 week with or without the ACE inhibitor, captopril, vitreoretinal succinate (**A**), and fumarate (**B**) were measured. As compared to the normotensive WKY control animals, vitreoretinal succinate was increased in the SHRs (*$P < 0.05$, Tukey test). After using captopril to reduce the hypertension in the SHRs, vitreoretinal succinate decreased to the same levels observed in the normotensive WKY controls (*$P < 0.05$, Tukey test). Similar results were observed for fumarate, with increased vitreoretinal fumarate levels seen in the SHRs compared to the normotensive WKY control animals (*$P < 0.05$, Tukey test). *$P < 0.05$, **$P < 0.01$.

Table. Systolic Blood Pressure for Each Experimental Group

mm Hg	WKY Rats	SHRs	SHRs + Captopril
Before	132.81 ± 9.08	187.75 ± 13.78	193.50 ± 11.89
After	125.27 ± 10.60	204.69 ± 24.05	165.43 ± 35.45

Data are means ± SD.

was observed in the SHRs (5.95 ± 6.00-fold, $P < 0.05$) compared to normotensive WKY control animals (Fig. 3B). Overall, these results suggested that systemic hypertension induces an increase of the vitreoretinal succinate and fumarate, while blood pressure control reduces the vitreoretinal succinate and fumarate.

Discussion

The present study demonstrated that cyclic stretch, which mimics systemic hypertension, induced the production of succinate by the RPE cells in vitro. In addition, systemic hypertension induced increases of vitreoretinal succinate.

Mechanical stress recently has been shown to be an important regulator of gene expression, protein synthesis, growth, and differentiation of many cell types.[17,18] Although VEGF is a potent angiogenic mitogen that is secreted by tumor cells and by cells exposed to hypoxia, mechanical stretch has been shown to induce VEGF expression in rat ventricular myocardium,[19] rat cardiac myocytes,[20] human mesangial cells,[21] and rat RPE cells.[22] Moreover, recent reports that succinate can induce cellular signaling events through GPR91 has raised the possibility that its physiological properties are beyond its traditional role as a citric acid cycle metabolite.[12,13] Therefore, to confirm the hypothesis that intracellular succinate may be increased by mechanical stretch under hypertensive conditions, we investigated intracellular succinate in the RPE cells after cyclic stretch. Our findings showed that the cyclic stretch mimicked the cardiac cycle in terms of frequency, magnitude, and stress contour, thereby resulting in an accumulation of intracellular succinate and VEGF. After 2 or more hours of cyclic stretch or exposure to 10% and 15% cyclic stretch, significant increases in the average amount of succinate were observed.

Previously, Folbergrova et al.[10] and Hoyer et al.[11] reported that during conditions linked with insufficient oxygen supply to the rat cerebral cortex, succinate accumulated as an end product of anaerobic glucose catabolism. In addition, succinate accumulation also has been reported to occur extracellularly in the peripheral tissues during specific pathophysiologic states where the energy and oxygen supply/demand are unbalanced.[23] However, to the best of our knowledge, the effect of stretch on succinate metabolism has not been evaluated previously. In the current study, we demonstrated for the first time that mechanical stretch also could induce a succinate increase in the RPE cells.

Sapieha et al.[13] used immunohistochemistry to demonstrate that GPR91 was strongly expressed and predominantly localized in the cell bodies of the ganglion cell layer and, to a lesser extent, in the cells of the inner nuclear layer and outer retina. Gnana-Prakasam et al.[24] further reported finding expression of GPR91 mRNA in the RPE as well as in the neural retina. The results of their GPR91 expression analysis showed that there were positive signals throughout the retina, including the RPE cell layer. Consistent with these previous data, our current findings also suggested that cyclic stretch-induced accumulation of succinate in the RPE cells may have a role in retinal and choroidal neovascularization.

The mechanism by which cellular stretch is detected and translated into intracellular signaling has yet to be understood completely. Stretch rapidly activates a plethora of second messenger pathways, including tyrosine kinases, $p21^{ras}$, extracellular signal-regulated kinase (ERK), S6 kinase, PKC, phospholipases C (PLC) and D, and the P450 pathway.[25,26] Mechanical stretch also can regulate protein synthesis and the activity of numerous factors, including nitric oxide (NO),[27] endothelin-1,[28] platelet-derived growth factor,[29] fibroblast growth factor,[30,31] and angiotensin II.[32] Although ERK has been reported to be important for VEGF expression,[33–38] another previous report suggested that stretch-induced VEGF expression is mediated by PI 3-kinase and PKC-ζ in a manner that is independent of ERK1/2, Akt, or Ras.[39] To determine the mechanism by which stretch increased the intracellular succinate, the present study evaluated inhibitors of the classical/novel PKC isoforms (GF109203X), PI 3-kinase (LY294002), tyrosine phosphorylation (genistein), MEK1 (PD98059) and calcium chelator (BAPTA/AM). Inhibition of intracellular calcium using BAPTA/AM resulted in marked inhibition of the stretch-induced intracellular succinate metabolism. However, other types of inhibition did not alter the intracellular succinate metabolism. Thus, these results suggested that calcium is required for any signals involved in the intracellular succinate metabolism.

Calcium increases in the inner ear hair cells[40] and endothelial cells,[41] and during stretch-induced injury in astroglia,[42] neurons,[43] and Müller cells,[44] have been shown to indicate the mechanosensitivity of these different cell types. However, the specific mechanism responsible for these calcium increases has yet to be investigated in detail. Current reports suggest that ATP receptors[45] and mechanosensitive channels[46] have a part in the kinetics of the calcium transients. Calcium regulates mitochondrial function, movement, and viability. Like the endoplasmic reticulum, mitochondria also can store calcium and, thus, there is stimulation of the calcium-sensitive dehydrogenases of the citric acid cycle,[47] as the increased mitochondrial calcium boosts ATP production. Consequently, this activity potentially can induce an increase of the succinate metabolism. Conversely, since BAPTA/AM induces a decrease of the intracellular calcium, this may inactivate the citric acid cycle and lead to a decrease in the succinate metabolism.

To determine whether hypertension induced an increase in intracellular succinate in vivo, we investigated vitreoretinal succinate in SHRs using previously described methods.[15] Moreover, we used captopril rather than a calcium channel blocker to make it possible to investigate the effect of normalizing hypertension itself. Our results showed that vitreoretinal succinate was increased in the SHRs compared to the normotensive WKY control animals. When blood pressure was controlled in the SHRs through the use of captopril, there was a reduction in the vitreoretinal succinate to levels similar to those found in the normotensive WKY controls. These results suggested that not only hypertension-induced cyclic stretch in vitro, but also systemic hypertension in vivo induced increased succinate metabolism. Moreover, short periods of blood pressure control also can reduce vitreoretinal succinate. Similarly, a previous report showed that hypertension increased VEGF expression while captopril reduced VEGF expression to control levels.[15] In addition, not only has succinate been reported to induce VEGF expression,[13] it also has been suggested that a positive feedback mechanism exists between succinate and VEGF.[14] As hypertension induces succinate as well as VEGF, the interaction of these molecules may exacerbate diabetic retinopathy, AMD, and hypertensive retinopathy itself. Furthermore, fumarate increased hypertension-induced cyclic stretch in vitro and systemic hypertension

in vivo, which suggests that the signal regulation is not related to the inhibition of succinate dehydrogenase in the citric acid cycle.

Severe systemic hypertension can induce not only vascular and tissue stretch, but also can lead to an insufficient oxygen supply due to an irreversible change of the vessels that results from angiospasm and occlusion. Severe hypertension can induce stretch and ischemia via VEGF and/or succinate and, thus, lead to exacerbation of retinal vascular diseases.

Investigations in our present study used ARPE-19, which is a human RPE cell line. However, it is possible that the cell characteristics for this cell line may not be capable of exhibiting original RPE characteristics or mature RPE characteristics when using the current experimental setup. Therefore, these types of investigations may achieve better results if rat primary culture cells, human primary culture cells, or differentiated cells are used. In addition, it may be important to use a different coated dish, such as collagen IV or laminin-coated culture plates to ensure conditions are as close as possible to the in vivo environment. Moreover, to definitively clarify the stretch-induced succinate function in the retina, further studies that examine vessel components, such as endothelial cells or pericytes, or cells in the neural retina, such as astrocytes and Müller cells, will need to be undertaken. Detailed investigations of vitreoretinal succinate in patients with systemic hypertension also will need to be examined in future studies.

Our data suggested that a novel molecular mechanism might account for the exacerbation of retinal vascular diseases by concomitant hypertension. Furthermore, these findings also may partially explain the principal clinical manifestations of hypertensive retinopathy itself. Our results additionally suggested the possibility that a similar process may be involved in hypertension's effect on nonocular conditions. At the current time, anti-VEGF therapies are the standard treatment for ocular neovascular diseases, such as AMD, PDR, and other retinal vascular diseases. Our data implied that succinate therapies as well as anti-VEGF therapies may prove therapeutically effective for hypertensive retinopathy and may ameliorate the deleterious effects of coexistent hypertension on numerous succinate-associated disorders.

Acknowledgments

The authors thank Yumiko Tsunenari for her technical assistance.

Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture (21592234, 21592235).

Disclosure: **H. Kinoshita**, None; **K. Suzuma**, None; **T. Maki**, None; **Y. Maekawa**, None; **M. Matsumoto**, None; **M. Kusano**, None; **M. Uematsu**, None; **T. Kitaoka**, None

References

1. Klein R, Klein BE, Moss SE, Cruickshanks KJ. The Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVII. The 14-year incidence and progression of diabetic retinopathy and associated risk factors in type 1 diabetes. *Ophthalmology*. 1998;105:1801–1815.
2. Wan Nazaimoon WM, Letchuman R, Noraini N, et al. Systolic hypertension and duration of diabetes mellitus are important determinants of retinopathy and microalbuminuria in young diabetics. *Diabetes Res Clin Pract*. 1999;46:213–221.
3. Agardh CD, Agardh E, Torffvit O. The association between retinopathy, nephropathy, cardiovascular disease and long-term metabolic control in type 1 diabetes mellitus: a 5 year follow-up study of 442 adult patients in routine care. *Diabetes Res Clin Pract*. 1997;35:113–121.
4. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*. 1994; 331:1480–1487.
5. Frank RN. Growth factors in age-related macular degeneration: pathogenic and therapeutic implications. *Ophthalmic Res*. 1997;29:341–353.
6. Amin RH, Frank RN, Kennedy A, Eliott D, Puklin JE, Abrams GW. Vascular endothelial growth factor is present in glial cells of the retina and optic nerve of human subjects with nonproliferative diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 1997;38:36–47.
7. Ishibashi T, Hata Y, Yoshikawa H, Nakagawa K, Sueishi K, Inomata H. Expression of vascular endothelial growth factor in experimental choroidal neovascularization. *Graefes Arch Clin Exp Ophthalmol*. 1997;235:159–167.
8. Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. *Invest Ophthalmol Vis Sci*. 1996;37:1929–1934.
9. Pe'er J, Folberg R, Itin A, Gnessin H, Hemo I, Keshet E. Vascular endothelial growth factor upregulation in human central retinal vein occlusion. *Ophthalmology*. 1998;105:412–416.
10. Folbergrová J, Ljunggren B, Norberg K, Siesjö BK. Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex. *Brain Res*. 1974;80:265–279.
11. Hoyer S, Krier C. Ischemia and aging brain. Studies on glucose and energy metabolism in rat cerebral cortex. *Neurobiol Aging*. 1986;7:23–29.
12. He W, Miao FJ, Lin DC, et al. Citric acid cycle intermediates as ligands for orphan g-protein-coupled receptors. *Nature*. 2004; 429:188–193.
13. Sapieha P, Sirinyan M, Hamel D, et al. The succinate receptor GPR91 in neurons has a major role in retinal angiogenesis. *Nat Med*. 2008;14:1067–1076.
14. Matsumoto M, Suzuma K, Maki T, et al. Succinate increases in the vitreous fluid of patients with active proliferative diabetic retinopathy. *Am J Ophthalmol*. 2012;153:896–902.
15. Suzuma I, Hata Y, Clermont A, et al. Cyclic stretch and hypertension induce retinal expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2: potential mechanisms for exacerbation of diabetic retinopathy by hypertension. *Diabetes*. 2001;50:444–454.
16. Toma I, Kang JJ, Sipos A, et al. Succinate receptor GPR91 provides a direct link between high glucose levels and renin release in murine and rabbit kidney. *J Clin Invest*. 2008;118: 2526–2534.
17. Owens GK. Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am J Physiol*. 1989; 257:H1755–H1765.
18. Riser BL, Ladson-Wofford S, Sharba A, et al. TGF-beta receptor expression and binding in rat mesangial cells: modulation by glucose and cyclic mechanical strain. *Kidney Int*. 1999;56: 428–439.
19. Li J, Hampton T, Morgan JP, Simons M. Stretch-induced VEGF expression in the heart. *J Clin Invest*. 1997;100:18–24.
20. Seko Y, Seko Y, Takahashi N, Shibuya M, Yazaki Y. Pulsatile stretch stimulates vascular endothelial growth factor (VEGF) secretion by cultured rat cardiac myocytes. *Biochem Biophys Res Commun*. 1999;254:462–465.
21. Gruden G, Thomas S, Burt D, et al. Mechanical stretch induces vascular permeability factor in human mesangial cells: mechanisms of signal transduction. *Proc Natl Acad Sci U S A*. 1997;94:12112–12116.
22. Seko Y, Seko Y, Fujikura H, Pang J, Tokoro T, Shimokawa H. Induction of vascular endothelial growth factor after applica-

tion of mechanical stress to retinal pigment epithelium of the rat in vitro. *Invest Ophthalmol Vis Sci*. 1999;40:3287–3291.

23. Hebert SC. Physiology: orphan detectors of metabolism. *Nature*. 2004;429:143–145.
24. Gnana-Prakasam JP, Ananth S, Prasad PD, et al. Expression and iron-dependent regulation of succinate receptor GPR91 in retinal pigment epithelium. *Invest Ophthalmol Sci*. 2011;52: 3751–3758.
25. Ishida T, Takahashi M, Corson MA, Berk BC. Fluid shear stress-mediated signal transduction: how do endothelial cells transduce mechanical force into biological responses? *Ann N Y Acad Sci*. 1997;811:12–23, discussion 23–24.
26. Sadoshima J, Izumo S. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J*. 1993;12:1681–1692.
27. Ziegler T, Silacci P, Harrison VJ, Hayoz D. Nitric oxide synthase expression in endothelial cells exposed to mechanical forces. *Hypertension*. 1998;32:351–355.
28. Yamazaki T, Komuro I, Kudoh S, et al. Endothelin-1 is involved in mechanical stress-induced cardiomyocyte hypertrophy. *J Biol Chem*. 1996;271:3221–3228.
29. Hu Y, Böck G, Wick G, Xu Q. Activation of PDGF receptor alpha in vascular smooth muscle cells by mechanical stress. *FASEB J*. 1998;12:1135–1142.
30. Park JM, Borer JG, Freeman MR, Peters CA. Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells. *Am J Physiol*. 1998;275:C1247–C1254.
31. Marrero MB, Schieffer B, Paxton WG, et al. Direct stimulation of JAK/STAT pathway by the angiotensin II AT1 receptor. *Nature*. 1995;375:247–250.
32. Tamura K, Umemura S, Nyui N, et al. Activation of angiotensinogen gene in cardiac myocytes by angiotensin II and mechanical stretch. *Am J Physiol*. 1998;275:R1–R9.
33. Jung YD, Nakano K, Liu W, Gallick GE, Ellis LM. Extracellular signal-regulated kinase activation is required for up-regulation of vascular endothelial growth factor by serum starvation in human colon carcinoma cells. *Cancer Res*. 1999;59:4804–4807.
34. Okajima E, Thorgeirsson UP. Different regulation of vascular endothelial growth factor expression by the ERK and p38 kinase pathways in v-ras, v-raf, and v-myc transformed cells. *Biochem Biophys Res Commun*. 2000;270:108–111.
35. Shih SC, Mullen A, Abrams K, Mukhopadhyay D, Claffey KP. Role of protein kinase C isoforms in phorbol ester-induced vascular endothelial growth factor expression in human glioblastoma cells. *J Biol Chem*. 1999;274:15407–15414.
36. Pal S, Datta K, Khosravi-Far R, Mukhopadhyay D. Role of protein kinase Czeta in Ras-mediated transcriptional activation of vascular permeability factor/vascular endothelial growth factor expression. *J Bio Chem*. 2001;276:2395–2403.
37. Pedram A, Razandi M, Hu RM, Levin ER. Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion. *J Bio Chem*. 1997;272:17097–17103.
38. Pal S, Claffey KP, Dvorak HF, Mukhopadhyay D. The von Hippel-Lindau gene product inhibits vascular permeability factor/vascular endothelial growth factor expression in renal cell carcinoma by blocking protein kinase C pathways. *J Bio Chem*. 1997;272:27509–27512.
39. Suzuma I, Suzuma K, Ueki K, et al. Stretch-induced retinal vascular endothelial growth factor expression is mediated by phosphatidylinositol 3-kinase and protein kinase C (PKC)-zeta but not by stretch-induced ERK1/2, Akt, Ras, or classical/novel PKC pathways. *J Biol Chem*. 2002;277:1047–1057.
40. Vollrath MA, Kwan KY, Corey DP. The micromachinery of mechanotransduction in hair cells. *Ann Rev Neurosci*. 2007; 30:339–365.
41. Nauli SM, Kawanabe Y, Kaminski JJ, Pearce WJ, Ingber DE, Zhou J. Endothelial cilia are fluid shear sensors that regulate calcium signaling and nitric oxide production through polycystin-1. *Circulation*. 2008;117:1161–1171.
42. Rzigalinski BA, Weber JT, Willoughby KA, Ellis EF. Intracellular free calcium dynamics in stretch-injured astrocytes. *J Neurochem*. 1998;70:2377–2385.
43. Ahmed SM, Weber JT, Liang S, et al. NMDA receptor activation contributes to a portion of the decreased mitochondrial membrane potential and elevated intracellular free calcium in strain-injured neurons. *J Neurotrauma*. 2002;19:1619–1629.
44. Lindqvist N, Liu Q, Zajadacz J, Franze K, Reichenbach A. Retinal glial (Müller) cells: sensing and responding to tissue stretch. *Invest Ophthalmol Vis Sci*. 2010;51:1683–1690.
45. Weick M, Wiedemann P, Reichenbach A, Bringmann A. Resensitization of P2Y receptors by growth factor-mediated activation of the phosphatidylinositol-3 kinase in retinal glial cells. *Invest Ophthalmol Vis Sci*. 2005;46:1525–1532.
46. Ingber DE. Cellular mechanotransduction: putting all the pieces together again. *FASEB*. 2006;20:811–827.
47. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev*. 1990;70:391–425.

Investigative Ophthalmology & Visual Science