1 **Cyclic Stretch and Hypertension Increase Retinal Succinate: Potential**
2 **Mechanisms for Exacerbation of Ocular Neovascularization by Mechanical**
3 **Stress**
4
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25

Abstract

Purpose

To investigate succinate metabolism in cells undergoing clinically relevant cyclic stretch and in spontaneously hypertensive rat (SHR) retina.

Methods

ARPE-19 cells were seeded on 6-well BioFlex collagen I-coated, silicone elastomer-bottomed culture plates. Cells were then subjected to pulsatile stretch using a computer-controlled vacuum stretch apparatus. A physiologic stretch frequency of 60 cycles per minute and 5-15% prolongation of the elastomer-bottomed plates were used. Succinate concentration was assessed by enzymatic analysis and high-performance liquid chromatography-mass spectrometry. 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, were also significantly increased compared with controls. BAPTA/AM inhibited this increase. For the in vivo experiments, hypertension increased vitreoretinal succinate and fumarate in SHRs compared with the normotensive WKY controls. When hypertension was reduced using captopril, vitreoretinal succinate returned to baseline levels.

51 **Conclusions**

52 These findings suggest that cyclic stretch and hypertension increased intracellular
53 succinate in cultured retinal pigment epithelial cells and the vitreoretinal succinate of
54 SHRs through a calcium-dependent pathway.
55

56 **Introduction**

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

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

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

76

77 **Methods**

78 ***Reagents***

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

81

82 ***Cell Culture***

83 ARPE-19 cells, a human RPE cell line, were purchased from American Type Culture
84 Collection (ATCC, Manassas, VA). To confirm that the ARPE19 cells were of RPE
85 origin, we first identified the immunohistochemistry using anti-pan cytokeratin (1:100;
86 Sigma; data not shown). Cells were maintained in growth medium that consisted of
87 Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) media with
88 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (all
89 purchased from Gibco, Carlsbad, CA). Cells were cultured in 5% CO_2 at 37°C, with
90 the medium changed every 3 days. Cells were plated at a density of 0.5 to 1.0 × 10^4
91 cells/cm^2 and passaged when confluent (3-6 days). Cells from passages 20-30 were
92 used for the experiments.

93

94 ***Mechanical Stretch***

95 Cells were seeded on 6-well BioFlex collagen I-coated, silicone elastomer-bottomed
96 culture plates (Flexcell Intl. Corp., McKeesport, PA). When the cultures were
97 confluent, the culture medium was replaced with serum free DMEM/F12 for 24
98 hours. Cells were then subjected to uniform radial and circumferential strain in 5%
99 CO_2 at 37°C using a computer-controlled vacuum stretch apparatus (Flexercell
100 Strain Unit; Flexcell Intl. Corp.). Stretch magnitudes are reported as a percent, while
101 the cyclic stretch frequencies are reported as cycles per minute (cpm). A physiologic
102 stretch frequency of 60 cpm and 5-15% prolongation of the elastomer-bottomed
103 plates were used in accordance with a previously described method.[15] For controls,
104 BioFlex collagen I culture plates were prepared in parallel, but not subjected to
105 pulsatile stretch.

106

107 ***Succinate Extraction***

108 The medium was decanted and cells were washed three times with cold phosphate
109 buffered saline and solubilized in 100 µl/well TNE buffer (10 mmol/l Tris-HCl, (pH 7.8)
110 / 1% NP40 / 0.15 mol/l NaCl / 1 mmol/l EDTA / 1.5 µmol/l aprotinin). The suspension
111 was incubated at 4°C for 10 minutes, and then centrifuged at 15,000 rotations per
112 minute (rpm) for 10 minutes. The aqueous phase was transferred to a new tube, and
113 stored at -80°C until needed. Succinate concentration was assessed by enzymatic
114 analysis of succinate and normalized with total protein quantity. Intracellular
115 succinate concentration was determined using a cuvette-based enzymatic assay
116 according to the manufacturer's instructions (Boehringer Mannheim/R-Biopharm AG,
117 Mannheim, Germany).[16] Briefly, the enzymatic reaction measures the conversion of
118 succinate by evaluating succinyl-CoA synthetase, pyruvate kinase, and L-lactate
119 dehydrogenase and the stoichiometric amount of nicotinamide adenine dinucleotide
120 (NADH) oxidized in the reaction. By measuring the absorbance at 340 nm (UV
121 detection), the amount of succinate can be calculated from the amount of NADH
122 oxidized.

123

124 ***Succinate Quantitation Using High-Performance Liquid Chromatography-Mass***
125 ***Spectrometry (HPLC/MS)***

126 Intracellular succinate levels were also quantified using a previously described
127 selective ion monitoring mode of HPLC/MS, with slight modifications made in order
128 to additionally confirm the intracellular succinate concentration using enzymatic
129 analysis. To ensure we achieved optimal performance during the quantification, we
130 performed ion exclusion column chromatography using 0.1% formic acid as the

131 eluent and negative mode detection with electrospray ionization mass spectrometry.

132

133 ***Quantitative VEGF***

134 VEGF protein levels were measured using enzyme-linked immunosorbent assays
135 (ELISA; R&D Systems, Minneapolis, MN, USA).

136

137 ***In Vivo Studies***

138 All animal experiments were conducted in accordance with the ARVO Statement for
139 the Use of Animals in Ophthalmic and Vision Research, and the rules and
140 regulations to animal experiments at Nagasaki University (approval number;
141 1010250881). The 12-week-old male SHRs and weight-matched Wistar-Kyoto
142 (WKY) control rats were obtained from KBT Oriental Co., Ltd. (Tosu, Japan) and
143 allowed to become accustomed to their new surroundings for 1 week. Systolic blood
144 pressure was measured in each animal using a tail cuff sensor and monitoring
145 system (MK-2000; Muromachi Kikai, Tokyo, Japan). Animals were then treated with
146 or without 100 $mg \cdot kg^{-1} \cdot day^{-1}$ captopril for 1 week. The drugs were administered in
147 the animals' drinking water. Blood pressure measurements were repeated after 1
148 week of therapy. Before extraction of the vitreous body and retina of each rat, the
149 animals were deeply anesthetized with intraperitoneal pentobarbital (100 mg/kg) and
150 then killed by a pentobarbital overdose. Whole enucleated eyes were cut in half
151 equatorially behind the ora serrate and lenses, with the capsules then carefully
152 removed. The vitreoretinal complexes were mechanically teased apart with micro
153 forceps, were separated from the choroid-sclera carefully. Extracted vitreoretinal
154 complexes of the right and left eyes were solubilized in 750 μl TNE buffer and
155 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 ± standard deviation (S.D.). 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

180 hours. As seen in Fig. 1B, significant increases were observed for the average
181 intracellular succinate after a 10% (25.25 ± 5.72) and 15% cyclic stretch (24.49 ±
182 5.16) (P < 0.05). We also investigated intracellular succinate after cyclic stretch by
183 using HPLC/MS to confirm the amount of succinate. As seen in Fig. 1C, the
184 HPLC/MS results were similar to those found for the enzymatic analysis, with
185 significant increases noted in the intracellular succinate after a cyclic stretch for 2
186 hours (3.65 ± 1.49-fold compared to control, P < 0.05). These results suggest that
187 cyclic stretch increased the intracellular succinate in a time- and magnitude-
188 dependent manner. Moreover, to confirm the correlation between succinate and
189 VEGF, VEGF protein levels from the same samples were also investigated. As seen
190 in Fig. 1D, significant increases were observed for the average VEGF protein levels
191 after a cyclic stretch for 2 hours (430.67 ± 46.57 pg/ml) and 3 hours (418.17 ±
192 87.65) (control; 187.33 ± 34.54, P < 0.01).
193
194 ***Mechanistic Evaluation of Stretch-Induced Succinate Increase***
195 To determine the mechanism by which stretch increased intracellular succinate,
196 inhibitors of classical/novel protein kinase C (PKC) isoforms (GF109203X, 5 μmol/l),
197 phosphatidylinositol (PI) 3-kinase (LY294002, 50 μmol/l), tyrosine phosphorylation
198 (genistein, 100 μmol/l), mitogen-activated protein kinase/extracellular signal-
199 regulated kinase kinase (MEK) 1 (PD98059, 20 μmol/l) and intracellular calcium
200 (BAPTA/AM, 10 μmol/l) were evaluated (Fig. 2A). In all experiments, 2 hours of
201 10%/60 cpm cyclic stretch induced intracellular succinate metabolism. Furthermore,
202 the cyclic stretch significantly increased the intracellular succinate (31.89 ± 12.87, P
203 < 0.01) compared to controls. However, inhibitors of MEK1 that used PD98059 had

204 little effect on the stretch-induced succinate metabolism. Similarly, there were no
205 alterations of the intracellular succinate metabolism after inhibition of the PKC
206 classical/novel isoforms using GF109203X, PI 3-kinase using LY294002, or tyrosine
207 phosphorylation using genistein. In contrast, use of BAPTA/AM to inhibit the
208 intracellular calcium resulted in marked inhibition of the stretch-induced intracellular
209 succinate (17.24 ± 6.24, $P < 0.01$). Subsequently, we then evaluated the activity of
210 the citric acid cycle during the intracellular succinate increase by using HPLC/MS to
211 measure fumarate, which is the metabolite of succinate in the citric acid cycle.
212 Results indicated that levels of intracellular fumarate were similar to those for the
213 intracellular succinate during the cyclic stretch. As shown in Fig. 2B, we observed
214 both a significant increase in the average amount of fumarate (3.39 ± 2.57-fold, $P <$
215 0.05) after 2 hours of 10%/60 cpm cyclic stretch compared to control, and a marked
216 inhibition of stretch-induced intracellular fumarate (1.29 ± 1.07-fold, $P < 0.05$) after
217 BAPTA/AM inhibition of the intracellular calcium. These results suggest that
218 intracellular succinate metabolism involves a calcium-dependent pathway, with
219 fumarate exhibiting a parallel reaction with succinate in the citric acid cycle.
220
221 ***Vitreoretinal Succinate and Fumarate in WKY SHR Rats***
222 To determine if hypertension induced an increase in the vitreoretinal succinate, 12-
223 week-old SHRs (derived from WKY rats) and weight-matched WKY control animals
224 were treated orally for 1 week with or without the angiotensin converting enzyme
225 inhibitor, captopril. SHRs had elevated baseline systolic blood pressures ($P < 0.001$)
226 compared to the WKY controls (Table). Systolic blood pressure was reduced in
227 response to the captopril therapy as compared to the untreated SHRs ($P < 0.05$). As
228 seen in Fig. 3A, increased vitreoretinal succinate was observed in the SHRs (16.53 ±

229 3.33, $P < 0.05$) compared with the normotensive WKY control animals (12.71 ±
230 3.29). After using captopril to reduce the hypertension in the SHRs, vitreoretinal
231 succinate (12.44 ± 1.67) decreased to the same levels seen in the normotensive
232 WKY controls. Similarly, increased vitreoretinal fumarate was also observed in the
233 SHRs (5.95 ± 6.00-fold, $P < 0.05$) compared with normotensive WKY control animals
234 (Fig. 3B). Overall, these results suggest that systemic hypertension induces an
235 increase of the vitreoretinal succinate and fumarate, while blood pressure control
236 reduces the vitreoretinal succinate and fumarate.
237
238 **Discussion**
239 The present study demonstrated that cyclic stretch, which mimics systemic
240 hypertension, induced the production of succinate by the RPE cells in vitro. In
241 addition, systemic hypertension induced increases of vitreoretinal succinate.
242 Mechanical stress has recently been shown to be an important regulator of
243 gene expression, protein synthesis, growth, and differentiation of many cell
244 types.[17,18] Although VEGF is a potent angiogenic mitogen that is secreted by tumor
245 cells and by cells exposed to hypoxia, mechanical stretch has been shown to induce
246 VEGF expression in rat ventricular myocardium,[19] rat cardiac myocytes,[20] human
247 mesangial cells[21] and rat RPE cells.[22] Moreover, recent reports that succinate can
248 induce cellular signaling events through GPR91 has raised the possibility that its
249 physiological properties are beyond its traditional role as a citric acid cycle
250 metabolite.[12,13] Therefore, in order to confirm the hypothesis that intracellular
251 succinate may be increased by mechanical stretch under hypertensive conditions,
252 we investigated intracellular succinate in the RPE cells after cyclic stretch. Our
253 findings showed that the cyclic stretch mimicked the cardiac cycle in terms of

254 frequency, magnitude, and stress contour, thereby resulting in an accumulation of
255 intracellular succinate and VEGF. After 2 or more hours of cyclic stretch or exposure
256 to 10% and 15% cyclic stretch, significant increases in the average amount of
257 succinate were observed.

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

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

276 The mechanism by which cellular stretch is detected and translated into
277 intracellular signaling has yet to be completely understood. Stretch rapidly activates
278 a plethora of second messenger pathways including tyrosine kinases, $p21^{ras}$,

279 extracellular signal-regulated kinase (ERK), S6 kinase, PKC, phospholipases C
280 (PLC) and D, and the P450 pathway.[25,26] Mechanical stretch can also regulate
281 protein synthesis and the activity of numerous factors including NO,[27] endothelin-1,[28]
282 platelet-derived growth factor,[29] fibroblast growth factor,[30,31] and angiotensin II.[32]
283 Although ERK has been reported to be important for VEGF expression,[33-38] another
284 previous report suggested that stretch-induced VEGF expression is mediated by PI
285 3-kinase and PKC-ζ in a manner that is independent of ERK1/2, Akt, or Ras.[39] In
286 order to determine the mechanism by which stretch increased the intracellular
287 succinate, the present study evaluated inhibitors of the classical/novel PKC isoforms
288 (GF109203X), PI 3-kinase (LY294002), tyrosine phosphorylation (genistein), MEK1
289 (PD98059) and calcium chelator (BAPTA/AM). Inhibition of intracellular calcium
290 using BAPTA/AM resulted in marked inhibition of the stretch-induced intracellular
291 succinate metabolism. However, other types of inhibition did not alter the intracellular
292 succinate metabolism. Thus, these results suggest that calcium is required for any
293 signals involved in the intracellular succinate metabolism.

294 Calcium increases in the inner ear hair cells[40] and endothelial cells,[41] and
295 during stretch-induced injury in astroglia,[42] neurons[43] and Müller cells,[44] have been
296 shown to indicate the mechanosensitivity of these different cell types. However, the
297 specific mechanism responsible for these calcium increases has yet to be
298 investigated in detail. Current reports suggest that ATP receptors[45] and
299 mechanosensitive channels[46] play a part in the kinetics of the calcium transients.
300 Calcium regulates mitochondrial function, movement, and viability. Like the
301 endoplasmic reticulum, mitochondria can also store calcium and thus, there is
302 stimulation of the calcium-sensitive dehydrogenases of the citric acid cycle,[47] as the
303 increased mitochondrial calcium boosts ATP production. Consequently, this activity

304 can potentially induce an increase of the succinate metabolism. Conversely, since
305 BAPTA/AM induces a decrease of the intracellular calcium, this may inactivate the
306 citric acid cycle and lead to a decrease in the succinate metabolism.

307 To determine whether hypertension induced an increase in intracellular
308 succinate in vivo, we investigated vitreoretinal succinate in SHRs using previously
309 described methods.[15] Moreover, we used captopril rather than a calcium channel
310 blocker in order to make it possible to investigate the effect of normalizing
311 hypertension itself. Our results showed that vitreoretinal succinate was increased in
312 the SHRs compared with the normotensive WKY control animals. When blood
313 pressure was controlled in the SHRs through the use of captopril, there was a
314 reduction in the vitreoretinal succinate to levels similar to those found in the
315 normotensive WKY controls. These results suggested that not only hypertension-
316 induced cyclic stretch in vitro but also systemic hypertension in vivo induced
317 increased succinate metabolism. Moreover, short periods of blood pressure control
318 can also reduce vitreoretinal succinate. Similarly, a previous report showed that
319 hypertension increased VEGF expression while captopril reduced VEGF expression
320 to control levels.[15] In addition, not only has succinate been reported to induce VEGF
321 expression,[13] it has also been suggested that a positive feedback mechanism exists
322 between succinate and VEGF.[14] As hypertension induces succinate as well as
323 VEGF, the interaction of these molecules may exacerbate diabetic retinopathy, AMD,
324 and hypertensive retinopathy itself. Furthermore, fumarate increased both
325 hypertension-induced cyclic stretch in vitro and systemic hypertension in vivo, which
326 suggests that the signal regulation is not related to the inhibition of succinate
327 dehydrogenase in the citric acid cycle.

328 Severe systemic hypertension can induce not only vascular and tissue

329 stretch, but can also lead to an insufficient oxygen supply due to an irreversible
330 change of the vessels that results from angiospasm and occlusion. Severe
331 hypertension can induce both stretch and ischemia via VEGF and/or succinate and
332 thus, lead to exacerbation of retinal vascular diseases.

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

346 Our data suggest that a novel molecular mechanism might account for the
347 exacerbation of retinal vascular diseases by concomitant hypertension. Furthermore,
348 these findings may also partially explain the principal clinical manifestations of
349 hypertensive retinopathy itself. Our results additionally suggest the possibility that a
350 similar process may be involved in hypertension’s effect on nonocular conditions. At
351 the current time, anti-VEGF therapies are the standard treatment for ocular
352 neovascular diseases such as AMD, PDR and other retinal vascular diseases. Our
353 data imply that succinate therapies as well as anti-VEGF therapies may prove

354 therapeutically effective for hypertensive retinopathy and may ameliorate the
355 deleterious effects of coexistent hypertension on numerous succinate-associated
356 disorders.
357

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360

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483 Table

484 Systolic blood pressure for each experimental group

	WKY rats	SHRs	SHRs + Captopril
Before (mmHg)	132.81 ± 9.08	187.75 ± 13.78	193.50 ± 11.89
After (mmHg)	125.27 ± 10.60	204.69 ± 24.05	165.43 ± 35.45

485 Data are means ± S.D.

486 WKY rats; Wistar Kyoto rats, SHRs; spontaneously hypertensive rats.

487 Legends

488 Figure 1.

489 Cyclic stretch increases intracellular succinate in a stretch time- and magnitude-
490 dependent manner. (A) Intracellular succinate after cyclic stretch was investigated by
491 enzymatic analysis. The vertical axis corresponds to the corrected amount of
492 succinate when using the total protein, while the horizontal axis corresponds to the
493 stretch time. Average amount of succinate after 15%/60 cpm cyclic stretch
494 significantly increased after 2 or more hours compared to control (*$P < 0.05$, ** $P <$
495 0.01 Dunnett test). (B) Confluent cultures of ARPE-19 cells were subjected to 5, 10,
496 and 15% cyclic stretch for 2 hours. Significant increases were observed for the
497 average intracellular succinate after 10% and 15% cyclic stretch (*$P < 0.05$ Dunnett
498 test). (C) Intracellular succinate after cyclic stretch was also investigated by
499 HPLC/MS to confirm the amount of succinate. Results of HPLC/MS were similar to
500 the enzymatic analysis, with the results showing a significant increase in the
501 intracellular succinate after 2 hours of cyclic stretch (*$P < 0.05$ Dunnett test). (D) To
502 confirm the relationship between succinate and VEGF, ELISA was used to
503 investigate the VEGF protein levels after cyclic stretch. Significant increases were
504 observed for the average VEGF in the cell lysate after cyclic stretch for 2 and 3
505 hours (**$P < 0.01$ Dunnett test). Asterisk indicates *; $P < 0.05$, **; $P < 0.01$.

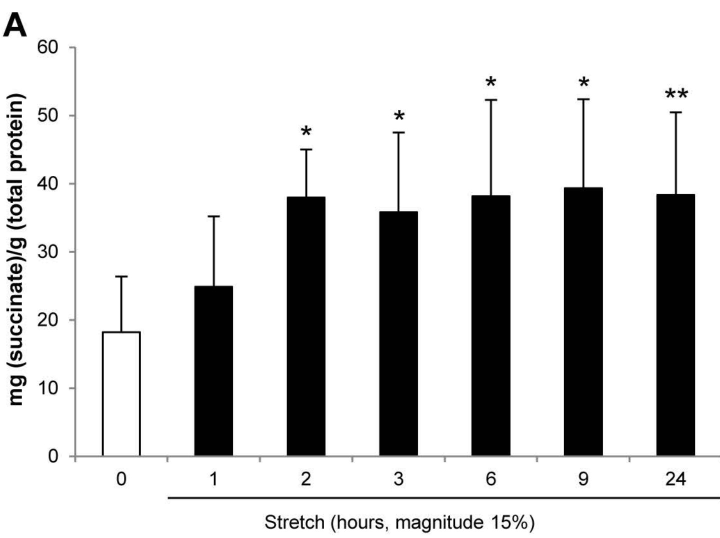
506

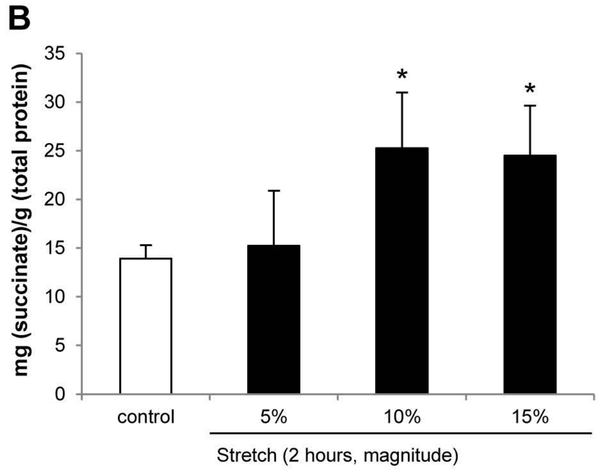
507 Figure 2.

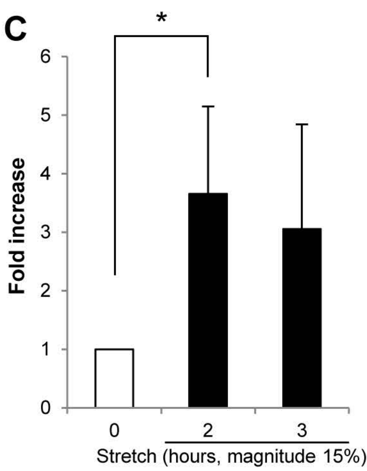
508 Effect of various inhibitors on stretch-induced succinate and fumarate metabolism.
509 Confluent cultures of ARPE-19 cells were exposed to 10% cyclic stretch at 60 cpm
510 for 2 hours in the presence of the PKC classical/novel isoform inhibitor GF109203X
511 (5 µmol/l), PI 3-kinase inhibitor LY294002 (50 µmol/l), tyrosine kinase inhibitor

512 genistein (100 μmol/l), MEK1 inhibitor PD98059 (20 μmol/l) and an intracellular
513 calcium chelate reagent BAPTA/AM (10 μmol/l). (A) Stretch-induced succinate was
514 examined in confluent cultures of ARPE-19 cells after treatment with various
515 pharmacological inhibitors. A significant increase was observed for the 2-hour stretch
516 treatment compared to control (** $P < 0.01$ Tukey test). After treatment with
517 BAPTA/AM, a decrease was observed compared to the 2-hour stretch results (** $P <$
518 0.01 Tukey test). (B) To evaluate how active the citric acid cycle was during the
519 intracellular succinate increase, fumarate, which is the subsequent succinate
520 metabolite in the citric acid cycle, was measured by HPLC/MS. After cyclic stretch,
521 the intracellular fumarate was similar to the succinate. Average amount of fumarate
522 after 10% cyclic stretch for 2 hours was significantly increased compared to control (*
523 $P < 0.05$ Tukey test), while it was significantly decreased after treatment with
524 BAPTA/AM (* $P < 0.05$ Tukey test). Asterisk indicates *; $P < 0.05$, **; $P < 0.01$.
525
526 Figure 3.
527 Vitreoretinal succinate and fumarate levels in the Wistar-Kyoto (WKY) rat and
528 spontaneously hypertensive rat (SHR). After 12-week-old SHRs (derived from WKY
529 rats) and weight-matched WKY control animals were treated orally for 1 week with or
530 without the ACE inhibitor, captopril, vitreoretinal succinate (A) and fumarate (B) were
531 measured. As compared to the normotensive WKY control animals, vitreoretinal
532 succinate was increased in the SHRs (* $P < 0.05$ Tukey test). After using captopril to
533 reduce the hypertension in the SHRs, vitreoretinal succinate decreased to the same
534 levels observed in the normotensive WKY controls (* $P < 0.05$ Tukey test). Similar
535 results were observed for fumarate, with increased vitreoretinal fumarate levels seen
536 in the SHRs compared with the normotensive WKY control animals (* $P < 0.05$ Tukey

537 test). Asterisk indicates *; P < 0.05, **; P < 0.01.







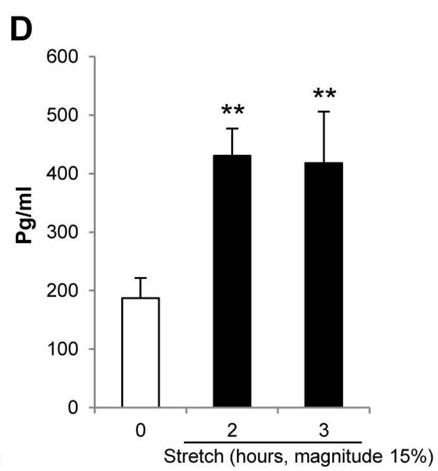
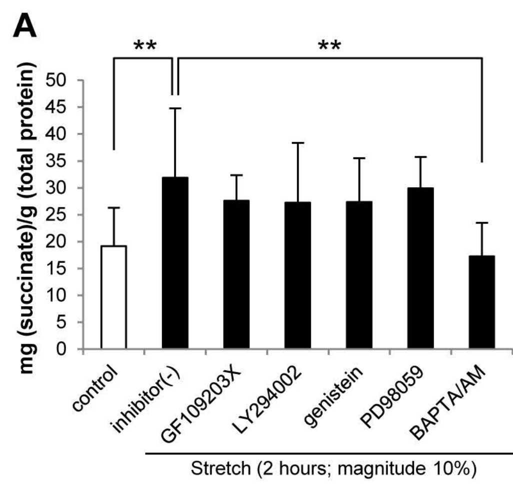


Figure 1



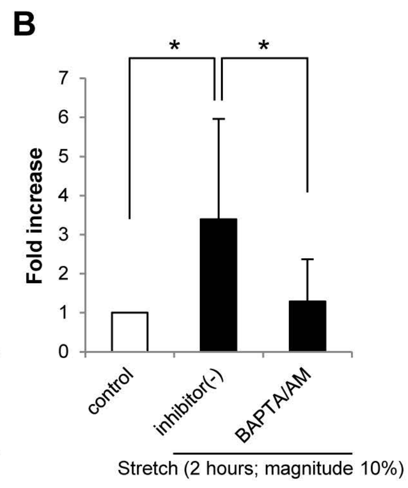
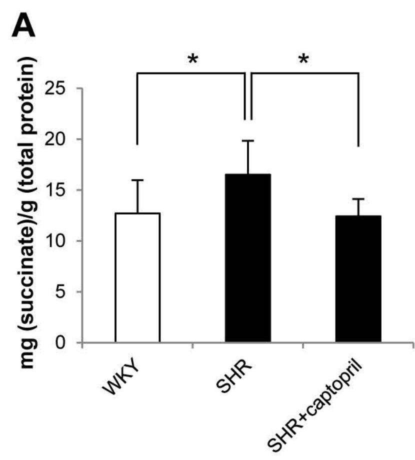


Figure 2



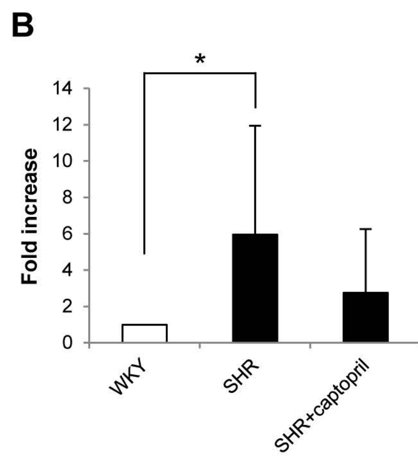


Figure 3