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RUNNING HEAD: Flow-mediated dilation and d-sarcoglycan Reduced microvascular flow-mediated dilation in Syrian hamsters lacking d-sarcoglycan is caused by increased oxidative stress

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1 SHORT REPORT

2 RUNNING HEAD: Flow-mediated dilation and δ -sarcoglycan

3 **Reduced microvascular flow-mediated dilation in Syrian**
4 **hamsters lacking δ -sarcoglycan is caused by increased**
5 **oxidative stress**

6

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23

24

25 **ABSTRACT**

26 δ -sarcoglycan mutation reduces mechanotransduction and induces dilated cardiomyopathy with aging.
27 We hypothesized that in young hamsters with δ -sarcoglycan mutation, which do not show
28 cardiomyopathy, flow mechanotransduction might be affected in resistance arteries as the control of local
29 blood flow.

30 Flow-mediated-dilation (FMD) was measured in isolated mesenteric resistance arteries, using 3-months
31 old hamsters carrying a mutation in the δ -sarcoglycan gene (CH-147) and their control littermates.

32 The FMD was significantly reduced in the CHF-147 group. Nevertheless, passive arterial diameter, vascular
33 structure and endothelium-independent dilation to sodium nitroprusside were not modified. Contraction
34 induced by KCl was not modified, whereas contraction due to phenylephrine was increased. The basal NO
35 production and total eNOS expression levels were not altered. Nevertheless, eNOS phosphorylation, FAKs

36 and RhoA expression were reduced in CHF-147. In contrast, p47phox, COX2, iNOS and reactive oxygen
37 species levels were higher in the endothelium of CHF-147 hamsters. Reducing ROS levels using the
38 superoxide dismutase analog Tempol significantly restored the flow-mediated dilation (FMD) levels in
39 CHF-147 hamsters. However, treatment with the COX-2 inhibitor NS-398 showed a non-significant
40 improvement in FMD.

41 **NEW & NOTEWORTHY**

42 This study suggests that the sarcoglycan complex is selectively involved in flow-mediated dilation, thus
43 highlighting its role in endothelial responsiveness to shear stress and amplifying tissue damage in
44 myopathy.

45 **Keywords:** mutation, myopathy, resistance arteries, shear stress

46

47 **INTRODUCTION**

48 The dystrophin-glycoprotein complex (DGC) forms a group of transmembranous and membrane-
49 associated proteins mostly located in the sarcolemma of striated muscle cells, as well as in smooth muscle
50 cells. The complex is composed of dystrophin and several sub-classes of proteins (the dystroglycans [α
51 and β], sarcoglycans [α , β , γ and δ], sarcospan, the syntrophins [$\alpha 1$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$ (+)], and α -
52 dystrobrevin). The complex links the extracellular matrix and cytoskeletal proteins via α -dystroglycan, a
53 laminin-associated protein.

54 Mutations in the sarcoglycan subunit result in muscular dystrophy or sarcoglycanopathy in humans (1, 2).
55 δ -sarcoglycan gene deletion or mutations induce cardiac and skeletal muscle dystrophy characterized by
56 focal necrosis and fibrosis (3). Blood vessels may also be affected by the absence of δ -sarcoglycan (4).
57 Histological analysis of coronary arteries of δ -sarcoglycan -deficient mice shows multiple constrictions
58 along the vascular tree, suggesting the occurrence of vasospasm. In addition, it has been shown that
59 treatment of δ -sarcoglycan deficient mice with a vasodilator restores the smooth appearance of the
60 vascular tree, without focal narrowing of the lumina (5). Although no measurement of blood flow or
61 vascular function was performed in this study, and the vascular defect could possibly be due to smooth
62 muscle and/or endothelial dysfunction.

63 Smooth muscle cells isolated from Syrian hamsters CHF-147, which lack δ -sarcoglycan, show increased
64 calcium permeability, abnormal proliferation, and apoptosis (6). This, and the above observations, led us
65 to hypothesize that vascular tone might be affected in δ -sarcoglycan -deficient animals. We therefore
66 investigated flow-dependent tone, in resistance arteries isolated from the male CHF-147 Syrian hamsters.
67 Resistance arteries play a key role in peripheral vascular resistance and tissue perfusion (7). Equilibrium
68 in vascular tone allows efficient control of local blood flow in peripheral tissues, and provides a
69 background vascular tone, which increases the effectiveness of circulating and locally produced vasoactive
70 agents.

71

72

73 MATERIALS AND METHODS

74 *Animals*

75 This study was performed on mesenteric resistance arteries (MRAs), from 63 young (three month-old)
76 male δ -sarcoglycan deficient Syrian hamsters (CHF-147, Institut de Myologie, Paris, France) and 63 young
77 (3 month-old) male wild-type Syrian hamsters (RjHan:Aura, Elevage Janvier, France). CHF-147 Syrian
78 hamsters are characterized by a mutation in the δ -sarcoglycan gene that results in the absence of the
79 protein (8, 9). For the FMD study, mice were fed by gavage with SOD-mimetic tempol (4-hydroxy-2,2,6,6-
80 tetramethyl piperidinoxyl) at a dose of 20 mg kg⁻¹ per day for 8 days.

81 The clinical feature of the δ -sarcoglycan is a “ Duchenne like “ myopathy (CPK rate very high and EMG confirmed
82 myopathic syndrome). Thus, for this first study, we only used male CHF-147 hamsters because the Duchenne
83 Muscular Dystrophy (DMD) gene responsible for the disease is located on the X chromosome

84

85 The animals were sacrificed by CO₂ inhalation, and the MRA were gently dissected in physiological solution
86 (PSS) composed of NaCl 130 mM, KCL 78.8 mM, MgSO₄ (7H₂O) 1.2 mM, NaHCO₃ 14.9 mM, HEPES 5 mM,
87 kH₂PO₄ 1.2 mM, D-glucose 11 mM D-glucose) at 4°C with a physiological pH of 7.4 (\pm 0.2).

88 **Ethics Statement:**

89 The procedure followed in the care and euthanasia of the study animals was in accordance with the
90 European Community Standards on the Care and Use of Laboratory Animals (Ministère de l’Agriculture,
91 France, authorization No. 6422) and with the principles of laboratory animal care (NIH publication no. 85–
92 23, revised 1985). The protocol was approved by the ethical committee (Protocol CEEA PdL #2008.10).

93

94 *Pressure myography - Flow-dependent tone in mesenteric arteries*

95 Segments of MRAs were cannulated at both ends and mounted in a video-monitored perfusion system,
96 as previously described (10). Briefly, cannulated arterial segments were bathed in a 5 ml organ bath
97 containing the PSS solution with a pH of 7.4, a pO₂ maintained at 160 mmHg, and a pCO₂ maintained at
98 37 mmHg (10). The arterial diameter was recorded continuously using the video monitoring system (Living
99 System Instrumentation Inc., Burlington, VT). Intraluminal pressure and flow rate could be changed
100 independently. Arteries were submitted to a pressure of 75 mmHg and further contracted with
101 phenylephrine (PE, 50% of initial diameter) to maintain a stable and reproducible tone in the different
102 groups. Intraluminal flow was then increased step by step (3 μ l/min to 100 μ l/min) and the diameter
103 measured. At the end of each experiment, arteries were perfused and superfused with a Ca²⁺-free PSS
104 containing ethylenebis-(oxyethylenenitrolo) tetra-acetic acid (EGTA, 2 mmol/L), sodium nitroprusside (10
105 μ mol/L) and papaverin (10 μ mol/L). Pressure (ranging from 10 to 150 mmHg) was then increased step by
106 step to determine the passive diameter of the vessel, i.e., in the absence of smooth muscle tone. Pressure
107 and diameter measurements were collected using a Biopac data acquisition system (Biopac MP 100, La
108 Jolla, CA, USA) and analyzed (Acqknowledge® software, Biopac). FMD was calculated as percentage of
109 passive diameter (10). NS-398 was used at 10⁻⁵ M in MRA flow-mediated dilation. Before each experiment,
110 the contractility of the muscle was tested using PE (10 μ mol/L), and the integrity of the endothelium was
111 assessed by testing the relaxing effect of acetylcholine (ACh, 1 μ mol/L) (10).

112 ***Wire myography - Pharmacological properties of resistance arteries***

113 Segments of MRAs (2 mm long) were dissected in PSS and mounted on a wire-myograph (DMT, Aarhus,
114 DK) as previously described (10). Briefly, 2 tungsten wires (25 µm diameter) were inserted into the artery
115 lumina; one wire was fixed to a force transducer, the other to a micrometer. Arteries were bathed in a
116 PSS as described above and maintained at 37°C, pH 7.4, pO₂ 160 mm Hg, pCO₂ 37 mm Hg. Wall tension
117 was applied as previously described (11). Vessels were then allowed to stabilize for one hour. Artery
118 viability was tested using a potassium-rich solution (80mol/L, 80K PSS). The endothelium was considered
119 functional when an 80% ACh-induced relaxation (10⁻⁶M) was obtained after PE-induced precontraction
120 (50% of maximal contraction with KCL 80 mM). A cumulative concentration-response curve (CRC) to PE
121 (10⁻⁹-10⁻⁴ M) was constructed. Finally, a CRC to sodium nitroprusside (SNP, 10⁻⁹-10⁻⁵M) after PE-induced
122 precontraction (10⁻⁶ M) was constructed.

123 ***Western blot analysis***

124 Western blot analysis was performed on MRAs as previously described (12). Briefly, pooled MRAs (3 per
125 animal) were homogenized using a lysis buffer (1% sodium dodecyl sulfate [SDS], 10 mmol/L Tris-HCl [pH
126 7.4], 1 mmol/L sodium orthovanadate, 2.5 mg/L leupeptin and 5 mg/L aprotinin). Extracts were incubated
127 at 4°C for 30 minutes and then centrifuged (14 000 rpm, 15 minutes, 14°C). The protein concentration
128 was determined using the Micro BCA Protein Assay Kit (Pierce). After denaturation at 95°C for 5 minutes,
129 equal amounts of protein (25 µg) were loaded onto a 9% polyacrylamide gel and transferred to
130 nitrocellulose membranes for 90 minutes (100 V, 4°C). Membranes were blocked with 5% bovine albumin
131 (BSA) in Tris-buffered saline Tween 20 (TBST) (20 mmol/L Tris [pH 8.0], 150 mmol/L NaCl, and 0.1% Tween-
132 20) for 1 hour; they were then incubated with the primary antibody in 5% BSA in TBST overnight at 4°C.
133 After extensive washing in T-TBS at room temperature, membranes were then incubated with secondary
134 antibody for 90 minutes at room temperature. After 3 washes with TBST, immunocomplexes were
135 detected by chemiluminescent reaction (ECL-kit; Amersham Pharmacia Biotech) using a computer-based
136 imaging system (Fuji LAS 3000 plus; Fuji Medical System). Quantification was performed by densitometric
137 analysis and normalized by β-actin signal.

138 Antibodies were used as follows: eNOS 1/1000 (9572 Cell signaling); phospho-eNOS Ser1177 1/1000 (9571
139 Cell signaling); Akt 1/1000 (9272 Cell signaling); phospho-Akt Ser473 1/1000 (4060 Cell signaling); PI3Kp85
140 1/500 (4292 Cell signaling); P47 1/500 (PA1-9073 Thermofisher); focal adhesion kinase (FAK) 1/500
141 (ab40794 Abcam); β-actine 1/1000 (A5316 Sigma-Aldrich); anti-rabbit 1/4000 (NA934 Amersham
142 Pharmacia Biotech, Orsay, France) or anti-mouse 1/4000 horseradish peroxidase antibody (NA931
143 Amersham Pharmacia Biotech, Orsay, France).

144 ***Histomorphometric analysis***

145 Segments of MRAs were fixed in 4% paraformaldehyde at 75 mmHg and were mounted in embedding
146 medium (Tissu-Tek, Miles, Inc), frozen in isopentane pre-cooled in liquid nitrogen, and stored at -80°C.
147 Transverse sections (7 µm thick) of MRA were stained with orcein solution. Media thickness, and internal
148 and external medial circumferences were measured. The media-to-lumen ratio was calculated as
149 previously described (10).

150

151

152 ***Immunofluorescence analysis***

153 Segments of 7 μm thick cross sections of MRAs were mounted in embedding medium (Miles, Inc), frozen
154 in isopentane pre-cooled in liquid nitrogen, and stored at -80°C . Sections were incubated with
155 cyclooxygenase (COX)-2 or iNOS (1/200 each, overnight, 4°C), rinsed for five minutes three times, and
156 then incubated with the secondary antibody (Alexa 488 nm, 1/200) for 90 minutes. Fluorescent staining
157 was visualized using confocal microscopy (Solamere Technology, Salt Lake City, UT, USA). Image analysis
158 was performed using Histolab (Microvision, France). Briefly, pixel quantification was performed after
159 separating the endothelial layer.

160 ***Reactive oxygen species (ROS) detection***

161 Superoxide anion detection was performed on 7 μm thick artery cross sections incubated with
162 dihydroethidine (DHE) as previously described (13, 14). Positive staining was visualized using confocal
163 microscopy and QED-image software (Solamere Technology, Salt Lake City, UT). Image analysis was
164 performed using Histolab (Microvision, France). Pixel quantification was carried out as previously
165 described (14). A positive control was performed using sections of MRA treated with lipopolysaccharide
166 in vitro in the PSS at 37°C for 2 hours. A negative control was obtained by omitting DHE or by adding
167 tempol to the sections 15 min before DHE.

168 ***Statistical analysis***

169 Results are expressed as mean \pm SEM. Significance of the differences between groups was determined by
170 analysis of variance (2-way ANOVA for consecutive measurements, when appropriate) or Mann Whitney
171 test for two groups analysis. P values less than 0.05 were considered as significant.

172 **RESULTS**

173 ***Flow-mediated dilation is decreased in mesenteric arteries from CHF-147 hamsters***

174 When pressure was maintained at 75 mmHg and after 50% of basal diameter contraction with PE,
175 increasing flow step-by-step resulted in dilation in control arteries (Figure 1). In CHF-147 hamsters, flow
176 (shear stress)-induced dilation was significantly attenuated in mesenteric resistance arteries (Figure 1).

177 ***The δ -sarcoglycan deficiency does not impact vascular structure and reactivity of mesenteric arteries***

178 No significant change was found in passive arterial diameter (Figure 2A), lumen area, media area internal
179 diameter or media to lumen ratio area in mesenteric resistance arteries, reflecting no vascular remodeling
180 (Figure 2B). Moreover, no significant change was observed between the two groups in endothelium-
181 dependent dilation (Figure 2C), endothelium-independent dilation (Figure 2D and E), concentration-
182 dependent inhibition of NO synthesis by L-NAME (Figure 2E) as well as KCL-induced contraction (Figure
183 2F). However, phenylephrine induced a significant increase in MRAs contraction in CHF-147 hamster
184 (Figure 2G).

185 ***The δ -sarcoglycan deficiency induce a decrease in NO and FAK pathway***

186 To understand the mechanism that modifies vascular reactivity, the proteins expression of the eNOS
187 pathway was studied in MRAs (Figure 3). Globally, the shear stress pathway indicators were not modified
188 in the δ -sarcoglycan deficient MRAs. However, the phosphorylation of eNOS Ser1177 was significantly

189 decreased (Figure 3G and I). In addition, δ -sarcoglycan deficient MRAs were characterized by significant
190 decrease in the FAK and RhoA expression (Figure 3B and 3J, respectively).

191 ***The decrease in FMD due to δ -sarcoglycan deficiency is caused by increased oxidative stress, iNOS and***
192 ***COX-2***

193 The strong attenuation of mechanotransduction properties of the MRAs led us to study related oxidative
194 stress and inflammatory factors. We tested the possibility that the diminished FMD could be caused by
195 the production of reactive oxygen species (ROS) or cyclooxygenase (COX). In the whole tissue, MRA
196 presented a significant increase in p47phox expression (Figure 4A). In addition, when treated by DHE for
197 ROS observation, MRAs isolated from the δ -sarcoglycan deficient hamsters showed a greater signal in the
198 endothelium compared to control (Figure 4B). Endothelial cells also presented a significant increase in
199 COX-2 and iNOS expression (Figure 4C and D, respectively). Furthermore, the SOD mimetic Tempol (Figure
200 4E) significantly improved FMD in CHF-147 hamsters, while the COX-2 inhibitor NS398 (Figure 4F) showed
201 a non-significant improvement.

202

203 **DISCUSSION**

204 This study demonstrated selective alteration of flow (shear stress)-dependent mechanotransduction in
205 the mesenteric resistance arteries of young CHF-147 hamsters without cardiomyopathy (δ -sarcoglycan-
206 deficient hamster) due to increased oxidative stress.

207 Resistance arteries, subjected to mechanical forces, develop a highly autoregulated tone to locally adapt
208 the blood flow supply in order to meet organ needs (15). Flow-mediated dilation plays a key role in the
209 control of resistance artery tone, and consequently, in the control of local blood flow (16). A change in
210 the balance between vasodilator and vasoconstrictor influences may alter the blood flow supply and
211 induce tissue damage, such as that observed in δ -sarcoglycan-deficient hamsters (5). In this latter study,
212 histological analysis suggested the presence of coronary vasospasm, which was suppressed by vasodilator
213 treatment. This study provides a potential explanation for these observations. The vasoconstrictor tone
214 in MRAs was modified with an increased alpha-adrenergic tone. Flow-mediated dilation was severely
215 reduced without a change in the dilatory capacity of smooth muscle cells (sodium nitroprusside-induced
216 dilation) or in the basal production of NO. Decreased FMD is the hallmark of endothelial dysfunction, and
217 an early decrease in FMD has been described in most cardiovascular diseases (such as coronary disease
218 (17) and hypertension (18)), metabolic syndrome (19) and diabetes (20). Thus, our findings provide a
219 rational explanation for the vascular disorder accompanying cardiomyopathy in old animals in δ -
220 sarcoglycan-deficient hamsters. In addition, the early decline in FMD occurring before any alteration in
221 cardiac function, play certainly a role in the development of cardiac disorders.

222 We previously showed that dystrophin plays a role in FMD. Indeed, in mdx mice lacking functional
223 dystrophin, FMD is strongly and selectively attenuated (21) and treatment with gentamicin (which allows
224 recovery of the protein) restores FMD to a normal level (22). In addition, vascular remodeling due to a
225 chronic increase in blood flow is attenuated in mdx mice (10). The results of the present study strengthen
226 the assumption that the mechanotransduction of shear stress by endothelial cells involves the
227 sarcoglycan-dystrophin complex. Nevertheless, the link between this complex and eNOS activation
228 remains unclear. In fact, the PI3 kinase-Akt pathway selectively activates endothelial cell NO production

229 in response to shear stress (23, 24). Nevertheless, in the present study, this pathway was not affected by
230 the absence of δ -sarcoglycan. The main differences between the studies are the type of cells used
231 (cultured cells in the referenced studies versus whole blood vessels with quiescent cells in our present
232 study) and the type of response observed with a direct measurement of dilation in our present study.
233 Although eNOS expression was not lower in δ -sarcoglycan-deficient hamsters, its phosphorylation level
234 was decreased. Arteries were rapidly collected from the hamsters after sacrifice and quickly frozen before
235 analysis. Thus, our finding that eNOS phosphorylation was low in CHF-147 hamsters is in agreement with
236 our observation that FMD was reduced. Flow (shear stress) is the main endogenous regulator of eNOS
237 expression. We also found that oxidative stress, COX-2 and iNOS expression were increased in MRAs from
238 δ -sarcoglycan-deficient hamsters. This is in agreement with previous studies that have shown the
239 involvement of ROS in the pathogenesis of muscular dystrophy (25). iNOS is associated with increased
240 catabolic activity and co-expressed with pro-inflammatory cytokines and adhesion molecules during
241 muscle injury and wasting (26). Unlike other forms of NOS, this isoform is not regulated by the calcium
242 ion, but is primarily induced by ROS and inflammatory cytokines via the activation of NF κ B and the
243 MAPKinase pathway in skeletal muscle (27). In the endothelium and due to the decrease in FMD, high
244 levels of NO are required to compensate for this decrease, which can lead to the formation of
245 peroxynitrite, a highly reactive species that contributes to oxidative damage. Under these conditions of
246 increased oxidative stress, it has been described that the generation of ROS and the formation of
247 peroxynitrite further activate COX-2, explaining this increase in hamsters deficient for δ -sarcoglycan (28,
248 29).-In addition, we demonstrated an increase of p47phox in CHF-147 endothelial cells. P47phox is a
249 subunit of the NADPH oxidase (NADPHox) complex, involved in the production of H₂O₂. But the
250 mechanism by which δ -sarcoglycan mutation increases p47phox level is not clear. We can hypothesize
251 that the δ -sarcoglycan mutation may disrupt cellular signaling pathways, leading to increased
252 transcription of the gene encoding p47phox (transcriptional regulation) (30). Then, mutation may affect
253 the stability of p47phox mRNA or its translation rate (post-transcriptional regulation). Finally, the δ -
254 sarcoglycan mutation may alter protein degradation mechanisms, leading to accumulation of p47phox
255 (post-translational regulation). Another hypothesis is that δ -sarcoglycan mutations may increase oxidative
256 stress (31) and inflammation in endothelial cells. This could trigger a compensatory response, increasing
257 the expression of components of the NADPHox system, including p47phox. Finally, δ -sarcoglycan
258 mutations affect the stability of the plasma membrane (32).

259 This could indirectly influence the regulation of p47phox, which is involved in the function of NADPHox at
260 the membrane (disruption of membrane stability). Further research would be needed to elucidate the
261 precise mechanism.

262 In skeletal muscles, ROS contribute to membrane protein nitrosylation and muscle cell inflammation. ROS
263 and COX-2 are also associated with alterations in the endothelium and smooth muscle-dependent tone in
264 the vascular wall. Although ROS levels were higher in CHF-147 hamsters than in control animals, the main
265 difference between hamster types was found in FMD. Decreasing ROS levels with SOD-mimetic tempol
266 significantly improved FMD in CHF-147 hamsters without affecting the wild-type. However, specific
267 inhibition of COX-2 by NS-398 did not significantly improve FMD in CHF-147 hamsters although ROS and
268 COX-2 derivatives showed an increase in CHF-147 hamsters and as previously shown in the context of
269 arterial dysfunction (33–36), demonstrating their involvement in reducing FMD.

270 In conclusion, the present study demonstrated that specific vascular disorders affect
271 mechanotransduction in CHF-147 hamster MRAs, with a reduction in flow-mediated dilation. The
272 involvement of ROS and inflammation in resistance arteries endothelium may be of interest for the
273 treatment of vascular disorders and ischemic events in patients with muscular dystrophy or
274 sarcoglycanopathy.

275 **GLOSSARY**

276 Ach : Acetylcholine
277 COX : Cyclooxygenase
278 eNOS : Endothelial nitric oxide synthase
279 FMD : Flow-mediated dilation
280 iNOS : Inducible nitric oxide synthase
281 MRA : Mesenteric resistance arteries
282 PE : Phenylephrine
283 ROS : Reactive oxygen species
284 SNP : Sodium nitroprusside
285

286 **DATA AVAILABILITY**

287 The data, methods used in the analysis, and materials used to conduct the study are available from the
288 corresponding author upon reasonable request. Requests to access the datasets should be directed to
289 laurent.loufrani@inserm.fr.

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314 The funders had no role in study design, data collection and analysis, decision to publish, or preparation
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316 **DISCLAIMERS**

317 None

318 **AUTHOR CONTRIBUTIONS**

319 Alexis Richard: prepared figures, drafted manuscript, approved final version of manuscript.
320 Arnaud Bocquet: performed experiments, analyzed data, prepared figures, approved final version of
321 manuscript.
322 Eric Belin de Chantemèle: performed experiments, analyzed data, prepared figures, approved final version
323 of manuscript.
324 Kevin Retailleau: performed experiments, analyzed data, prepared figures, approved final version of
325 manuscript.
326 Bertrand Toutain: performed experiments, analyzed data, approved final version of manuscript.
327 Héloïse Mongue-Din: performed experiments, analyzed data, approved final version of manuscript.
328 Anne-Laure Guihot: performed experiments, analyzed data, approved final version of manuscript.
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331 Daniel Henrion: edited and revised manuscript, approved final version of manuscript.
332 Yves Fromes: edited and revised manuscript, approved final version of manuscript.
333 Laurent Loufrani: Conceived and designed research, interpreted results of experiments, prepared figures,
334 drafted manuscript, edited and revised manuscript, approved final version of manuscript.

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450 **FIGURE LEGENDS**

451 **Figure 1. Flow-induced dilation is attenuated in mesenteric arteries from CHF-147 hamsters.** Changes in
452 diameter in response to flow changes when pressure was maintained at 75 mmHg and after 50% of basal
453 diameter contraction with PE, in mesenteric resistance arteries from control and CHF-147 hamsters (N=11
454 WT, N=8 CHF-147). Values are mean \pm SEM. 2-way RM-ANOVA was used.

455 **Figure 2. Mesenteric arteries from CH-147 hamsters did not show any changes in their vascular structure**
456 **and reactivity.** (A) Pressure-diameter (passive arterial diameter) relationship determined in mesenteric
457 resistance arteries (N=11 WT, N=8 CHF-147); (B) Media cross sectional area in mesenteric resistance
458 arteries, which were measured under a pressure of 75 mmHg. Arteries were isolated from control and

459 CHF-147 hamsters (N=9 WT, N=4 CHF-147). (C) Endothelium dependent and (D) independent dilation after
460 phenylephrine precontraction (10^{-6} M) (N=9 WT, N=4 CHF-147); (E) Concentration-dependent inhibition
461 of NO synthesis by L-NAME after phenylephrine precontraction (10^{-6} M) (N=9 WT, N=4 CHF-147); (F) KCL
462 (N=9 WT, N=4 CHF-147) and (G) phenylephrine induced contraction (10^{-9} - 10^{-4} M) (N=9 WT, N=4 CHF-147).
463 Values are mean \pm SEM. 2-way RM-ANOVA was used for curves, and Mann Whitney test for histograms.

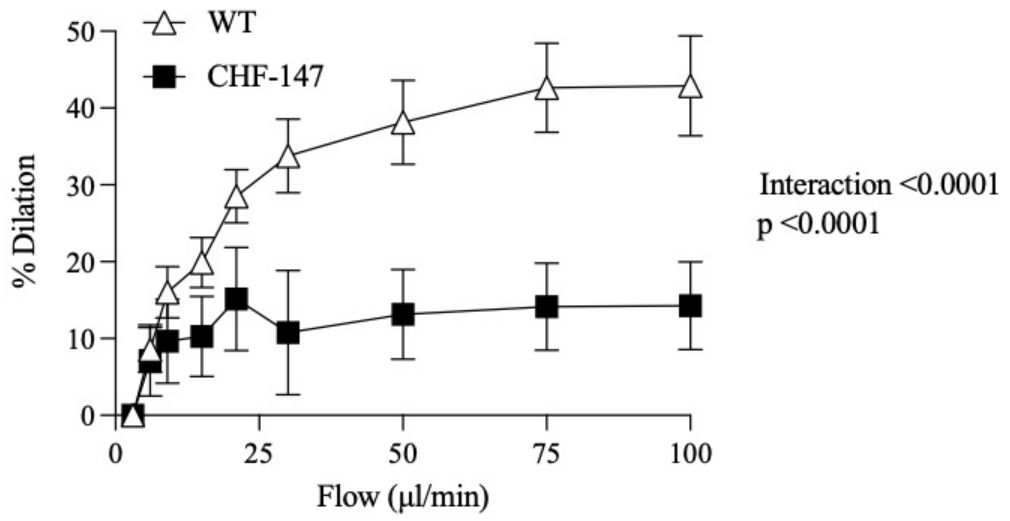
464 **Figure 3. NO and FAK pathway are decreased in mesenteric arteries from CH-147 hamsters. (A)**
465 Immunoblots of focal adhesion kinase (FAK), PI3K, AKT, eNOS, RhoA, and the phosphorylated form (p) of
466 eNOS and Akt in mesenteric resistance arteries from control and CHF-147 hamsters. (B) FAK (N=5 WT, N=3
467 CHF-147), (C) PI3K (N=3 WT, N=3 CHF-147), (D) p-AKT (N=4 WT, N=3 CHF-147), (E) AKT (N=7 WT, N=6 CHF-
468 147), (F) p-AKT/AKT ratio (N=7 WT, N=3 CHF-147), (G) p-eNOS (N=6 WT, N=3 CHF-147), (H) eNOS (N=6 WT,
469 N=3 CHF-147), (I) p-eNOS/eNOS ratio (N=6 WT, N=3 CHF-147), (J) RhoA (N=4 WT, N=4 CHF-147) relative
470 expression to β -actin (same loading control as presented in Figure 4). Values are mean \pm SEM. Mann
471 Whitney test was used.

472 **Figure 4. Increased endothelial superoxide production associated with COX-2 and iNOS expression**
473 **induce an alteration in flow-mediated dilation in mesenteric arteries from CHF-147 hamsters. (A)**
474 p47phox protein quantification in whole mesenteric artery (N=5 WT, N=4 CHF-147), relative expression
475 to β -actin (same loading control as presented in Figure 3). (B) Reactive oxygen species (ROS) visualized
476 using DHE staining (N=4 WT, N=4 CHF-147), (C) COX-2 (N=4 WT, N=4 CHF-147) and (D) iNOS
477 immunofluorescence staining in endothelium of mesenteric arteries from control and CHF-147 hamsters
478 (N=5 WT, N=5 CHF-147). (E) Flow-induced dilation in mesenteric arteries in hamsters treated chronically
479 with tempol or water (N=5 WT, N=5 CHF-147, N=5 WT+Tempol, N=5 CHF-147+Tempol). (F) Flow-induced
480 dilation in isolated mesenteric arteries treated with NS-398 (COX-2 inhibitor) (N=3 WT, N=4 CHF-147, N=4
481 WT+NS-398, N=4 CHF-147+NS-398). Values are mean \pm SEM. Mann Whitney test was used for A-D panels,
482 2-way RM-ANOVA was used for E and F panels.

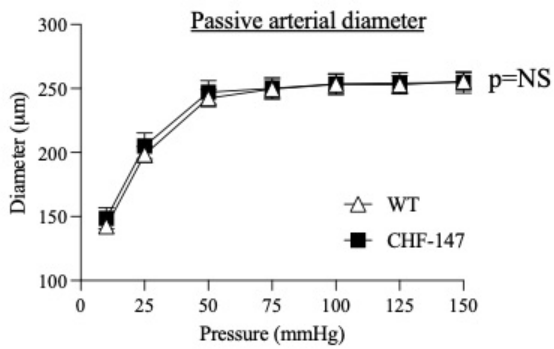
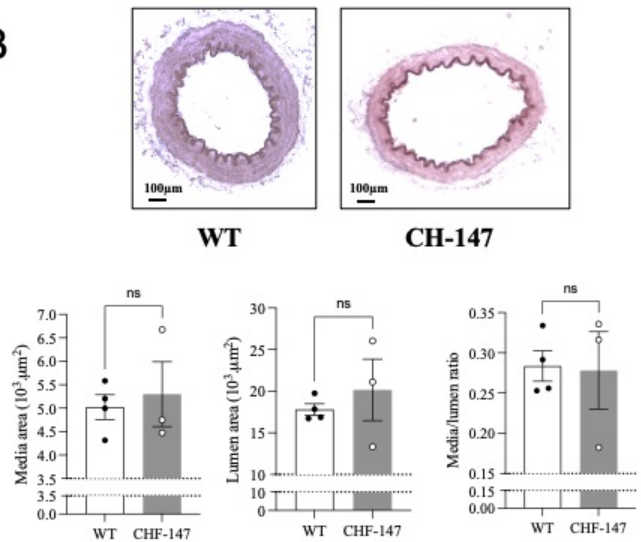
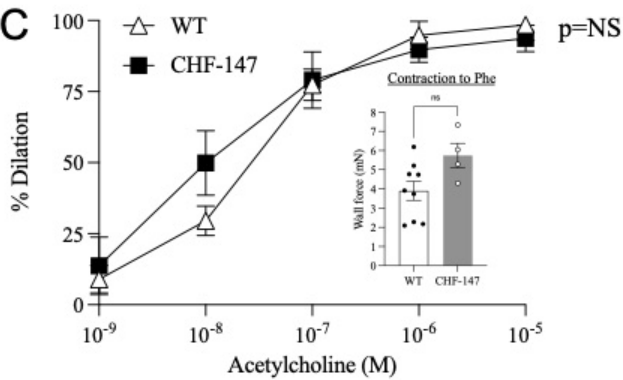
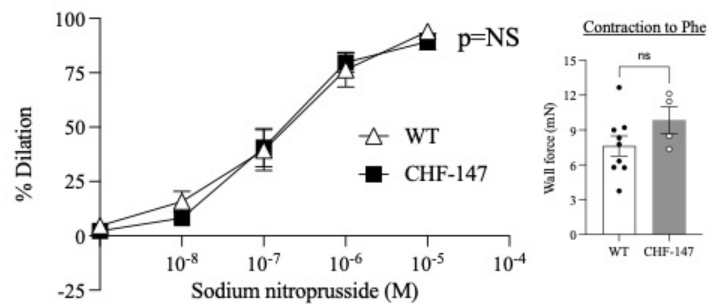
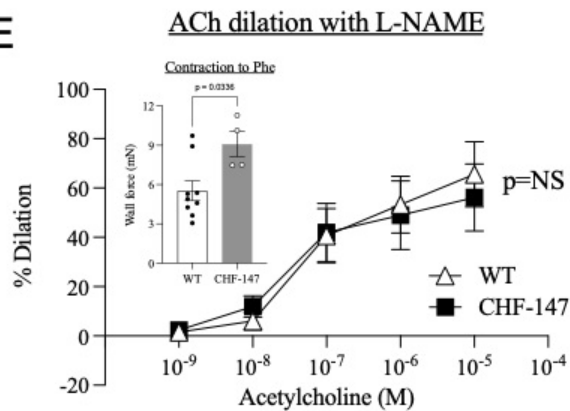
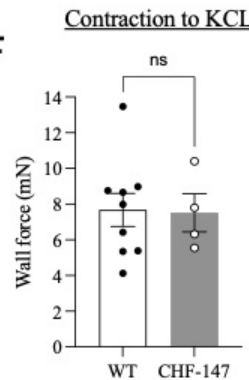
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Flow-mediated dilation



Orcein staining

A**B****C****D****E****F****G**