

Exploring oxidative stress and endothelial dysfunction as a mechanism linking bisphenol S exposure to vascular disease in human umbilical vein endothelial cells and a mouse model of postnatal exposure.

Sarah Easson^{a,*}, Radha Singh^{a,b,c,*}, Liam Connors^{a,b}, Taylor Scheidl^{a,b,c}, Larissa Baker^a, Anshul Jadli^{a,b}, Hai-Lei Zhu^a, Jennifer Thompson^{a,b,c}

* Authors contributed equally

^a Department of Physiology and Pharmacology, University of Calgary, 3330 Hospital Dr. NW, Calgary, Alberta, Canada, T2N 1N4

^b Libin Cardiovascular Institute, Cumming School of Medicine, University of Calgary

^c Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary

Corresponding author:

Jennifer Thompson PhD

University of Calgary

Heritage Medical Research Building rm. 78

3330 Hospital Dr. NW

Calgary, Alberta

Canada

T2N 4N1

Email: Jennifer.thompson2@ucalgary.ca

Phone: 403 220-3873

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1 **Abstract**

2 **Background:** Structural analogues used to replace bisphenol A (BPA) since the introduction of
3 new regulatory restrictions are considered emerging environmental toxicants and remain
4 understudied with respect to their biological actions and health effects. Studies reveal a link
5 between BPA exposure and vascular disease in human populations, whereas the vascular effects
6 of BPA substitutes remain largely unknown.

7 **Objectives:** To determine the effect of BPS, a commonly used BPA substitute, on redox balance,
8 nitric oxide (NO) availability and microvascular NO-dependent dilation.

9 **Methods:** In human umbilical vein endothelial cells (HUVEC), production of reactive oxygen
10 species (ROS) and NO after exposure to BPS was measured using fluorescent probes for DCFDA
11 and DAF-FM diacetate, respectively. The contribution of endothelial NO synthase (eNOS)
12 uncoupling to ROS generation was determined by measuring ROS in the presence or absence of
13 an eNOS inhibitor (L-NAME) or eNOS co-factor, BH4, while the contribution of mitochondria-
14 derived ROS was determined by treating cells with mitochondria-specific antioxidants prior to
15 BPS exposure. Bioenergetic profiles were assessed using Seahorse extracellular flux analysis and
16 mitochondria membrane polarization was measured with TMRE and JC-1 assays. In a mouse
17 model of low dose BPS exposure, NO-mediated endothelial function was assessed in pressurized
18 microvessels by inducing endothelium-dependent dilation in the presence or absence of L-NAME.

19 **Results:** BPS exposure (≥ 25 nM) reduced NO and increased ROS production in HUVEC, the
20 latter corrected by treating cells with L-NAME or BH4. BPS exposure led to a loss of mitochondria
21 membrane potential but had no impact on bioenergetic parameters except for a decrease in the
22 spare respiratory capacity. Treatment of HUVEC with mitochondria-specific antioxidants
23 abolished the effect of BPS on NO and ROS. NO-mediated vasodilation was impaired in male
24 mice exposed to BPS.

25 **Discussion:** Exposure to BPS may promote cardiovascular disease by perturbing NO-mediated
26 vascular homeostasis through the induction of oxidative stress.

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31 **Introduction**

32 The Lancet Commission on Pollution and Health estimated that pollution cost 9 million premature
33 deaths per year, 62% due to cardiovascular disease (CVD).^{1,2} One of the most common chemical
34 pollutants in our environment is Bisphenol A (BPA), a plasticizer used in the manufacture of
35 polycarbonate plastics and epoxy resins. In recent years, global production of BPA has reached 5-
36 6 billion pounds annually according to the CDC and is projected to grow over the next five years.
37 Human exposure occurs primarily through direct consumption of BPA monomers leached from
38 food and beverage containers but can also occur through inhalation and skin absorption and
39 indirectly through contamination of soil, water, and air.^{3,4} Following consumption, BPA is
40 absorbed in the gastrointestinal tract, rapidly metabolized in the liver, and excreted in the urine.
41 Despite its short half-life, BPA is detected in over 90% of urine and blood samples as exposure is
42 ubiquitous and continuous.^{5,6} The heavy environmental and health burden of industrial BPA
43 production has made it a target of international efforts to safeguard planetary and public health.

44
45 In response to new regulatory restrictions on the use and import of BPA-containing infant products
46 introduced in the late 2000s in Canada, US and Europe, the plastic industry began to substitute
47 BPA with structural analogues including bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF
48 (BPAF). Whether these new synthetic bisphenols are safe alternatives to their predecessor remains
49 unknown because the ‘innocent until proven guilty’ principal of chemical regulation liberates
50 manufacturers from the burden of proving safety before marketing and widespread production.
51 BPA substitutes are considered emerging environmental contaminants and are now detected in
52 over 80% of urine samples.^{7,8} There remains scant data on the biological properties and health
53 effects of BPA analogues, and thus investigation is needed to determine if these new substitutes
54 offer benefits from a public health perspective.

55
56 A large body of evidence demonstrates wide-ranging health effects of BPA, including
57 perturbations in sexual development, reproductive dysfunction, endocrine disorders, and
58 cancer.^{3,9,10} Adverse health effects of BPA and its analogues have largely been attributed to their
59 action as endocrine disrupting chemicals (EDC), synthetic chemicals that interfere with endocrine
60 signaling. Few studies have focused on the cardiovascular effects of BPA or its substitutes;
61 however, data in human populations reveal a potential relationship between BPA exposure and

62 hypertension, coronary artery disease, and atherosclerosis.¹¹⁻¹⁴ Endothelial dysfunction is a key
63 early pathophysiological event in diseases of the heart and blood vessels. In the current study, we
64 determined the impact of BPS on vascular redox balance, nitric oxide (NO) availability and NO-
65 mediated endothelial function.

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68 **Methods**

69 **Cell Culture**

70 Ea.hy926 human umbilical vein endothelial cells (HUVEC, ATCC) were cultured in DMEM
71 containing 10% fetal bovine serum (FBS), 1% HT Supplement and 1% penicillin streptomycin, at
72 37°C and 5% CO₂ and passaged 5-10 times. Once 75% confluency was reached, HUVEC were
73 starved in 2% FBS 24 hrs prior to being exposed to various concentrations (2.5 nM to 25 µM) of
74 BPS (Sigma) dissolved in DMSO.

75

76 **Animal model**

77 Male and female C57BL6 mice (Jackson Laboratories) were housed in cages absent of plastic
78 enrichment, fed a phytoestrogen-low diet (Teklab 2020), and maintained on a 12-hr light-dark
79 cycle. At the time of weaning, littermates were randomly assigned to BPS (250 nM) or vehicle
80 (DMSO), administered through the drinking water in glass bottles (Lab Products LLC). The
81 concentration of 250 nM in the drinking water reflects a human equivalent estimated daily intake
82 (EDI) of approximately 8 nmol BPS/kg body weight/day. This dose falls below the current
83 tolerable daily intake (TDI) of 18 nmol BPA/kg body weight/day and 219 nmol BPA/kg body
84 weight/day set by the European Food Safety Authority (EFSA) and the US. Food and Drug
85 Administration (FDA), respectively.¹⁵ Currently, there are no TDI guidelines set for BPA
86 analogues such as BPS. At 12 weeks of age, glucose tolerance was assessed in fasted mice by
87 taking serial measurements of blood glucose from the tail with a glucometer after IP injection of
88 glucose (2g/kg body weight). Mice were induced with 5% isoflurane with oxygen (1L/min) before
89 euthanizing by decapitation. Serum was collected from trunk blood and the mesentery collected
90 for dissection of microvessels. All animal procedures were approved by the University of Calgary
91 Animal Care Committee (protocol #: AC19-0006) and conducted in accordance with the Canadian
92 Council on Animal Care Ethics and the ARRIVE guidelines.

93

94 **Detection of ROS**

95 HUVEC cultured in 96-well black plates were incubated in a 25 μ M solution of 2',7'-
96 dichlorodihydrofluorescein diacetate (DCFDA, Sigma, D6883) at 37°C for 30 min, and
97 subsequently exposed to various concentrations of BPS or vehicle. The ROS generator, menadione
98 (Sigma) was used as a positive control. After 30 min of exposure, ROS production was quantified
99 by measuring the relative fluorescent intensity (RFI) of DCFDA (485/535 nm) on a SpectraMax
100 M2 plate reader (Molecular Devices) and subsequently normalized to protein concentration.
101 Oxidative stress was assessed in HUVEC exposed to BPS for 24 or 48 hrs by staining with 5 μ M
102 CellROX Deep Red™ (Invitrogen, C10422) for 30 min at 37°C. CellROX (640/665 nm) staining
103 was quantified by plate reader or flow cytometry using an Attune® Acoustic Focusing Cytometer
104 (Thermo Fisher Scientific) after cells were dissociated from the culture dish using 5 mM EDTA in
105 Hanks balanced salt solution (HBSS), centrifuged at 500g and resuspended in HBSS. Images of
106 HUVEC stained with CellROX and the Hoechst 33342 nuclear stain were captured on a Nikon Ti
107 Eclipse Widefield Microscope. Mitochondria-derived ROS were identified with MitoSOX Red™
108 (Invitrogen, M36008) after 30 min of BPS exposure and quantified by fluorescence spectroscopy
109 (510/580 nm) and flow cytometry. Three biological replicates were used for each assay.

110

111 **Production of NO and eNOS uncoupling**

112 Production of NO was measured in HUVEC exposed to BPS for 30 or 90 min by loading with a
113 DAF-FM Diacetate probe (Invitrogen, D23844) for 45 min at 37°C and quantifying on a
114 SpectraMax M2 plate reader or flow cytometer. The endothelial NO synthase (eNOS) inhibitor,
115 L-N^G-Nitro arginine (L-NAME, Sigma), was used as a positive control. To assess the contribution
116 of uncoupled eNOS to excess ROS production, cells were pre-incubated in L-NAME or
117 tetrahydrobiopterin (BH4, Sigma), a cofactor that stabilizes the eNOS dimer, prior to measurement
118 of acute ROS production by the DCFDA fluorescent probe. The contribution of mitochondria-
119 derived ROS to NO availability and vascular oxidative stress was determined by incubating cells
120 with mitochondria-targeted antioxidants, MitoTempol (500 nM, Cayman Chemical) or SS31 (100
121 nM), for 1 hr prior to exposing cells to BPS. Three biological replicates were used for each assay.

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123

124 **Antioxidant activity**

125 Superoxide dismutase (SOD) activity was assessed via a commercially available kit (Cayman
126 Chemical). In brief, after 48 hrs exposure of HUVEC to 2.5 uM BPS, cells were detached,
127 centrifuged and the pellet was homogenized in cold HEPES digestion buffer (1 mM EGTA, 210
128 mM mannitol, 70mM sucrose). The digested samples were centrifuged, and the supernatant stored
129 at -80°C until assaying. Samples and standards were plated in triplicate. A radical detector and
130 xanthine oxidase were added according to the manufacturer's instructions. Following 30 min
131 incubation at room temperature, absorbance was quantified using on a plate reader at 450nm and
132 normalized to protein concentration.

133

134 **Gene expression analysis**

135 After 48 hrs exposure to BPS or vehicle, total RNA was isolated from HUVEC using a RNeasy
136 Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of RNA was
137 quantified with an N50 Nanophotometer (Implen Inc.) and RNA integrity evaluated with a
138 TapeStation RNA assay (Agilent). Following DNase treatment, cDNA was generated using a
139 High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). cDNA was mixed with
140 Powerup SYBR green master mix (Applied Biosystems) and primer pairs were run in triplicates
141 on a QuantStudio 5 Real-time PCR instrument (Applied Biosystems). Primers were designed using
142 the NCBI/Primer Blast tool (Table 1) and tested for specificity using melting curve analysis. Target
143 mRNA expression was normalized to ribosomal protein lateral stalk subunit (RPLP0) and fold
144 change calculated using the $2^{-\Delta\Delta C_t}$ method. Expression data were further verified using beta-actin
145 as a second internal control for normalization.

146

147 **Western blot**

148 Whole cell lysates were homogenized in RIPA buffer (Invitrogen) and protein quantified with a
149 BCA protein assay (Thermo Scientific). Protein (40 µg) was loaded into pre-casted NuPAGE 4-
150 12% Bis-Tris gels (Invitrogen) and separated by electrophoresis. After transferring separated
151 protein onto PVDF membranes (GE Healthcare Life Sciences), membranes were blocked in 5%
152 (w/v) non-fat dry milk (Biorad) diluted in TBST for 1 hour. Membranes were probed overnight in
153 primary antibodies [phospho-eNOS (Cell Signaling, rabbit 9571); eNOS (Cell Signaling, rabbit
154 32027); phospho-Akt (Cell Signaling, rabbit 4060); Akt (Cell Signaling, rabbit 4691); GAPDH

155 (Cell Signaling, rabbit 2118) that were pre-incubated in the blocking buffer for 1 hour. Following
156 washing of the membrane and incubation in secondary anti-rabbit antibody, immunoreactive
157 proteins were imaged on an iBright 1500 Imaging System (Molecular Probes) using enhanced
158 chemiluminescence (ECL).

159

160 **Mitochondria membrane potential**

161 HUVEC were exposed to BPS for 48 hrs and assessed for mitochondria function. Mitochondria
162 membrane polarization was measured by loading cells with 2 μ M JC-1 (5, 5', 6, 6'-tetrachloro-1,
163 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide, Invitrogen, 15003) at 37°C for 15 min. After
164 washing, fluorescence was measured at 514/529 (green, monomer) and 514/590 (red, aggregate)
165 using the SpectraMax M2 plate reader. The mitochondria membrane potential was also assessed
166 in cells stained with 50 nM tetramethyl rhodamine ethyl ester (TMRE, ThermoFisher, T669) and
167 subsequently collected in HBSS containing 5 mM EDTA, washed and resuspended in media. The
168 TMRE fluorescent signal was quantified on a plate reader and flow cytometer. The mitochondria
169 uncoupler, FCCP, was used as a positive control. Three biological replicates were used for each
170 assay.

171

172 **Mitochondria bioenergetics**

173 Extracellular flux analysis was performed with HUVEC exposed to 2.5 μ M BPS or vehicle using
174 a Seahorse XFp Analyzer (Agilent Technologies). Cells were plated at 5000 cells/well on Seahorse
175 XFp miniplates. After 48 hrs of exposure, cells were switched to Seahorse XF assay media (non-
176 buffered DMEM containing 2 mM L-glutamine, 1mM sodium pyruvate and 10 mM glucose) and
177 incubated for 45 min. The oxygen consumption rate (OCR), extracellular acidification rate
178 (ECAR) and other bioenergetic parameters were measured using the Agilent Seahorse XFp Mito
179 stress test kit (Agilent, 103010-100). Baseline and stressed OCR and ECAR were determined prior
180 and post-injection of stressor compounds, oligomycin (1.5 μ M), FCCP (0.5 μ M) and
181 rotenone/antimycin A (0.5 μ M), respectively. The data were normalized to protein content from
182 individual wells. Data were analyzed using Wave software version 2.6 (Agilent Technologies).

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186 **Isolation of microvessels and arterial pressure myography**

187 Fourth order mesenteric arteries were carefully excised from the mesentery under a dissecting
188 microscope and cleared of perivascular fat in ice-cold Krebs solution (120 mM NaCl, 25mM
189 NaHCO₃, 4.8 mM KCl, 11 mM glucose, 0.27 mM EDTA, 1.2 mM NaH₂PO₄, 1.2 mM of MgSO₄,
190 and 2.5 mM CaCl₂; pH 7.4). The vessels were mounted and secured with sutures onto glass
191 cannulas diametrically opposed in a pressure myograph chamber (Living Systems), which was
192 placed on the stage of an inverted microscope (Nikon) equipped with live video recording. The
193 pressure myograph chamber was aerated with 5% CO₂/95% O₂ air gas mixture and cannulated
194 vessels maintained at 37°C at a constant intraluminal pressure of 60 mmHg under no flow
195 conditions with a servo-controlled pressure device (Living Systems). Vessel diameter was
196 recorded using IonOptix software. After equilibration for 30 min, arterial viability was assessed
197 by stimulation with a dose of saturated KCL solution. Contractile responses were assessed by
198 adding cumulative concentrations (10⁻¹⁰ M to 10⁻⁴ M) of the α -adrenergic agonist, phenylephrine
199 (Phe). After pre-contraction with 10⁻⁶ M Phe, NO-dependent dilation was determined by
200 generating concentration response curves to methacholine (Mch, 10⁻¹¹ M to 10⁻⁵ M). To determine
201 the contribution of NO to Mch-induced dilation, a subset of vessels was incubated with L-NAME
202 for 30 min prior to generating Mch concentration-response curves. Endothelium-independent
203 dilation was assessed by measuring dilation in response to 10⁻⁴ M sodium nitroprusside in pre-
204 contracted vessels. After concentration response curves were generated, the Krebs solution was
205 replaced with Ca²⁺-free buffer and equilibrated for 45 min before recording passive diameter at 60
206 mmHg. Contractile and dilatory responses were normalized to passive diameter and expressed as
207 a percentage of maximum KCL-induced contraction or pre-contraction, respectively.

208

209 **Statistical analyses**

210 Statistical analyses were carried out using GraphPad Prism version 9.3.1. Two-tailed student's t-
211 test or One-way ANOVA with Dunnett's or Tukey's multiple comparison test were used to
212 evaluate differences between groups for *in vitro* experiments. Two-way ANOVA was performed
213 to evaluate differences in concentration response curves. For calculation of the EC₅₀,
214 concentrations were converted to log form, data were normalized and the EC₅₀ was compared by
215 a nonlinear regression using the least squares method. Differences were considered statistically
216 significant if $p < 0.05$ and data are presented as mean \pm SEM.

217 **Results**

218 **ROS production in HUVEC exposed to BPS**

219 Production of ROS was increased in HUVEC acutely exposed to 25 nM to 25 μ M BPS for 30 min
220 (Figure 1A). After 24 and 48 hrs of BPS exposure, an increase in CellROX staining was observed
221 at BPS concentrations as low as 2.5 nM (Figure 1B-E). HUVEC exposed to BPS exhibited
222 decreased mRNA expression of key genes involved in antioxidant defences against oxidative stress
223 (Figure 1F), including superoxide dismutase 3 (SOD3), heme oxygenase (HO-1), glutathione
224 peroxidase 1 (GPX1), and NADPH quinone 1 dehydrogenase (NQO1). The activity of SOD was
225 reduced in HUVEC exposed to 2.5 μ M for 48 hrs (Figure 1G).

226

227 **NO availability and the contribution of eNOS uncoupling in BPS-exposed HUVEC**

228 When the production of the free radical, superoxide (O_2^-) exceeds antioxidant capacity, O_2^- reacts
229 with NO to reduce its bioavailability. NO production in HUVEC was reduced when exposed to
230 BPS for 30 min (Figure 2A) or 90 min (Figure 2B). The phosphorylation of eNOS at serine 1177
231 was reduced in HUVEC exposed to 2.5 μ M BPS for 48 hrs, while BPS had no impact on
232 phosphorylation of Akt at serine 473 or total eNOS expression (Figure C & D). Under conditions
233 of oxidative stress, eNOS becomes uncoupled from L-arginine oxidation and produces O_2^- rather
234 than NO, contributing to a vicious cycle of excess ROS production and NO depletion. In HUVEC
235 exposed to BPS for 48 hrs, inhibition of eNOS with L-NAME abolished the observed differences
236 in DCFDA staining between BPS and vehicle (Figure 2E & G). BH4 is a critical cofactor that
237 stabilizes the active eNOS dimer and its oxidation by ROS contributes to eNOS uncoupling.
238 Supplementation with BH4 decreased DCFDA staining in BPS-exposed HUVEC but had no
239 impact on control cells (Figure 2F & G), providing further evidence for eNOS uncoupling. Thus,
240 heightened ROS production and uncoupled eNOS in BPS-exposed endothelial cells lead to
241 reduced bioavailability of NO.

242

243 **The contribution of mitochondria function to NO availability in BPS-exposed HUVEC**

244 Dysfunctional mitochondria are a major source of excess ROS and contribute to the oxidative
245 stress that drives endothelial dysfunction. A loss of membrane potential in BPS-exposed HUVEC
246 was apparent by lower formation of J-aggregates when JC-1 enters the mitochondria, relative to
247 cationic JC-1 monomers, (Figure 3A & B). Likewise, lower accumulation of the cationic dye,

248 TMRE (Figure C-E) indicates mitochondria membrane depolarization. Mitochondria-derived ROS
249 were increased in HUVEC exposed to 25 nM to 25 μ M of BPS (Figure 3F-H). Figure 4 shows
250 bioenergetic parameters measured by extracellular flux analysis in HUVEC exposed to BPS for
251 48 hrs. There were no differences in basal respiration or maximal respiration, but spare respiratory
252 capacity was reduced in BPS-exposed HUVEC (Figure 4E), suggesting a compromised ability of
253 mitochondria to respond to stress. Treating cells with mitochondria-targeted antioxidants prior to
254 BPS exposure rescued NO production and prevented oxidative stress (Figure 5), suggesting that
255 mitochondria-derived ROS contribute importantly to perturbed redox balance and NO signaling in
256 BPS-exposed endothelial cells.

257

258 **Endothelium-dependent dilation in intact microvessels isolated from BPS-exposed mice**

259 To determine if NO-dependent vasodilation is impaired in a mouse model of BPS exposure, male
260 and female C57BL6 mice were exposed to the human equivalent of 8 nM BPS/kg body weight/day
261 from 3 to 12 weeks of age. Endothelium-dependent dilation was assessed *ex vivo* in 4th order
262 mesenteric arteries by measuring diameter after adding cumulative doses of Mch after pre-
263 contraction with Phe, while contractile responses were subsequently evaluated by adding
264 cumulative doses of Phe (Supplementary Figure 1). BPS exposure had no impact on arterial
265 contractile or dilatory responses in females (Figure 6A & B). Similar to females, there were no
266 differences in contractile responses to the α 1-adrenergic agonist, Phe, between control and BPS-
267 exposed males (Figure 6C). However, microvessels isolated from BPS-exposed males exhibited
268 reduced endothelium-dependent vasodilatory responses to the muscarinic agonist, Mch (Figure 6D
269 & E). Endothelium-independent vasodilatory responses to the smooth muscle relaxant, SNP, were
270 similar between groups. Pre-incubation with L-NAME blunted Mch responses in both vehicle and
271 BPS-exposed males (Figure 6D & E). The NO-sensitive component of endothelium-dependent
272 dilation was 63% in control males vs. 27% in BPS-exposed males, while the non-NO component
273 of vasodilation was 37% in control and 73% in exposed males (Figure 6F). The EC₅₀ values of
274 Mch and Phe concentration response curves, as well as passive diameter and maximum responses
275 to KCL are shown in Table 2. The passive diameter of mesenteric arteries measured in Ca²⁺-free
276 conditions was decreased in BPS-exposed female mice, but not in BPS-exposed male mice. No
277 differences in glucose tolerance were observed in either male or female mice exposed to. BPS
278 (Supplementary Figure 2).

279 Discussion

280 Structural analogues of BPA are increasingly used by manufacturers and marketed as safer
281 substitutes, yet their biological actions and health effects remain largely unknown. Findings from
282 the current study show that exposure to BPS, a common substitute for BPA, leads to sex-dependent
283 impairments in microvascular function and reveal reduced NO availability due to oxidative stress
284 as a probable underlying mechanism. Endothelium-dependent vasodilatory responses were
285 impaired in intact microvessels isolated from male mice exposed to BPS, while BPS exposure had
286 no impact on microvascular endothelial function in female mice. Pre-incubating vessels with the
287 eNOS inhibitor, L-NAME, revealed a shift to higher contributions of NO-independent signaling
288 in BPS-exposed males, suggesting a compensatory recruitment of other vasodilators such as
289 prostaglandin. Production of NO by the vascular endothelium is not only critical in maintaining
290 vascular tone and blood pressure, but plays a key role in promoting vascular homeostasis as NO
291 exhibits anti-inflammatory, anti-thrombotic and anti-atherogenic properties.^{16,17} Blunted NO
292 production is a hallmark of endothelial dysfunction, a common pathophysiological mechanism in
293 the development of hypertension, atherosclerosis and other diseases of the heart and vasculature.¹⁷
294 Epidemiological studies using data from the National Health and Nutrition Examination Survey
295 (NHANES) show that urinary BPA levels predict the risk for hypertension, coronary artery disease
296 and other CVD.^{13,18} Far fewer studies have focused on the new structural analogues of BPA.
297 Recently, cross-sectional studies examining populations in China and the USA reported positive
298 associations between BPA and BPS exposure with hypertension or CVD, but no effect of BPF,
299 another common substitute for BPA.^{19,20} Our study reveals endothelial dysfunction as a key
300 mechanism linking BPS to the development of CVD. To our knowledge, no other study has
301 examined the effect of *in vivo* BPS exposure on endothelial function in intact vessels; however, a
302 study by Saura *et al.* showed that exposure of mice to 400 nM BPA through the drinking water
303 increased systolic and diastolic blood pressure and impaired endothelium-dependent relaxation in
304 carotid arteries.²¹ The sex of mice used in the above-mentioned study is not noted; therefore, it is
305 unknown if sex-dependent vascular effects are also observed with BPA exposure. The current
306 study shows that males are particularly susceptible to BPS-induced endothelial dysfunction,
307 highlighting the importance of considering sex differences in the health effects of EDC.

308

309 The dose of BPS used for *in vivo* exposure to test the impact on endothelial function in intact
310 microvessels was 250 nM in the drinking water, which is approximately a human equivalent of 1.9
311 $\mu\text{g}/\text{kg}$ body weight/day or 8 nM/kg body weight/day. Although there are no TDI established for
312 BPA analogues, the TDI for BPA currently set by the EFSA is 4 $\mu\text{g}/\text{kg}$ body weight/day or 18
313 nM/kg body weight per day.²² Recently published results from the Canadian Total Diet Study
314 (TDS) reveal that estimated ingestion of BPS from contaminated foodstuffs, a major source of
315 human exposure, ranged between 5.74 to 56.9 ng/kg body weight/day in the provinces of China,
316 and exceeded that of BPA intake.²³ In a cohort of pregnant women recruited for the APrON study
317 in Alberta, Canada, daily 24 hr intake of BPS reached up to 14 nM/kg body weight, approaching
318 the TDI of BPA.²⁴ It is well established that bisphenol exposure is higher in young children;
319 ingestion rates have been reported to be ~ 4-fold higher in toddlers compared to adults.²⁵ Therefore,
320 the dose used in the present study is below the TDI and is environmentally relevant with respect
321 to human exposure. Although several human and animal studies have demonstrated a relationship
322 between bisphenol exposure and insulin resistance,¹⁰ the low dose of BPS used in the present study
323 compromised microvascular endothelial function without influencing glucose homeostasis. These
324 findings highlight that the endothelium of the microvasculature may be particularly susceptible to
325 bisphenol exposure

326
327 Using an *in vitro* HUVEC model, we show that exposure to BPS as low as 2.5 nM induces
328 increased production of ROS. These findings are consistent with other studies showing that excess
329 ROS production in multiple cell types arises from exposure to BPA or its analogues, suggesting
330 that oxidative stress may be an important cellular mechanism that links bisphenol exposure to
331 various diseases and disorders. Vascular oxidative stress decreases the availability of NO and is a
332 common cause of microvascular endothelial dysfunction.^{17,26} When the production of O_2^- in the
333 vasculature exceeds antioxidant defenses, O_2^- reacts with NO, reducing its bioavailability and
334 leading to the production of peroxynitrite (ONNO^-), a highly reactive molecule that oxidizes
335 macromolecules, contributing to cellular dysfunction. Oxidative degradation of BH₄, a critical
336 cofactor that stabilizes the active dimeric form of eNOS and facilitates electron transfer for the
337 oxidation of L-arginine, is thought to contribute to uncoupling in which eNOS produces more O_2^-
338 than NO.²⁷ Uncoupling of eNOS fuels a cycle of excess ROS production, oxidative stress and
339 reduced NO availability. In HUVEC exposed to BPS, NO production was suppressed, while

340 supplementation of BH4 or inhibition of eNOS normalized ROS production, suggesting that
341 uncoupled eNOS contributes to BPS-induced oxidative stress in endothelial cells. Thus, reduced
342 NO bioavailability due to vascular oxidative stress may be the mechanism underlying impaired
343 endothelium-dependent vasodilation.

344

345 In addition to uncoupled eNOS, vascular ROS arise from other enzymatic sources, including
346 xanthine oxidase and the NADPH oxidases, as well as the mitochondria electron transport chain.
347 Under normal conditions, electrons are leaked from redox centres of the electron transport chain,
348 reducing molecular O₂ to the superoxide anion, O₂⁻, which is quickly converted to H₂O₂ by
349 mitochondria SOD and subsequently reduced to water by glutathione peroxidase. Mitochondria
350 are not only sources of ROS, but also targets of ROS that are prone to oxidative damage.
351 Dysfunctional mitochondria produce excess ROS that overwhelm antioxidant defenses and
352 contribute importantly to the development of atherosclerosis and other CVD.^{28,29} Our findings
353 show increased production of mitochondria-derived ROS in BPS-exposed HUVEC and restoration
354 of redox balance and NO availability in HUVEC treated with mitochondria-targeted antioxidants
355 prior to BPS exposure, suggesting that dysfunctional mitochondria contribute to the instability of
356 the redox environment of endothelial cells. Bioenergetic profiling revealed no perturbations in
357 basal or maximal mitochondrial respiration after 48 hrs of exposure, except for a decrease in
358 respiratory reserve capacity. However, using both the TMRE and JC-1 assays, we show that BPS
359 exposure results in a loss of mitochondria membrane potential, which is associated with increased
360 ROS production by mitochondria and triggers mitophagy and cell death.

361

362 The adverse health effects of bisphenols have largely been attributed to their endocrine disrupting
363 properties; however, our data reveal oxidative stress to be an important mechanism linking
364 bisphenols to cardiovascular dysfunction. It is possible that the endocrine disrupting properties of
365 BPS modulate the redox response, and this may explain the sex-dependent effects we observed in
366 the microvasculature of BPS-exposed mice. BPA and its analogues are known to bind to estrogen
367 receptors (ER), albeit with lower affinity than endogenous estrogen, and exhibit estrogenic
368 activity. In endothelial cells, ER α localizes to caveolae in the cell membrane and activates Akt-
369 dependent NO production via phosphorylation of eNOS; while the transcription of eNOS is
370 activated upon binding of cytosolic or nuclear ER α /ER β to estrogen response elements in the

371 eNOS promoter.³⁰ Acute activation of ER α leads to NO-dependent vasodilation and suppression
372 of O₂⁻ levels, including mitochondria-derived O₂⁻.³¹ The use of a HUVEC line in the current study
373 precluded investigation into the molecular basis of sex differences observed in BPS-exposed mice;
374 however, data from our lab show that acute exposure to BPS augments NO-dependent relaxation
375 in isolated aortae from female mice only. These findings suggest that the estrogenic activity of
376 BPS counteracts its ROS-promoting effects, providing a potential explanation for the sex-
377 dependent vascular effects observed in the present study where females appeared protected from
378 BPS-induced endothelial dysfunction.

379

380

381 **Summary**

382 The current study demonstrates that chronic exposure to a low dose of the commonly used BPA
383 substitute, BPS, leads to sex-dependent impairments in NO-mediated vasodilation in the
384 microvasculature. Our findings substantiate epidemiological evidence of a link between bisphenol
385 exposure and vascular disease, by providing direct evidence of endothelial dysfunction in
386 pressurized microvessels; however, interpretations are limited to a single dose and single toxicant
387 exposure. Using a HUVEC cell line that preserves characteristics of primary endothelial cells, we
388 provide evidence that BPS-induced endothelial dysfunction is due to NO depletion as a result of
389 eNOS uncoupling, mitochondria dysfunction and consequent redox imbalance. Thus, our study
390 highlights a potential molecular mechanism underlying the link between bisphenol exposure and
391 vascular diseases observed in human populations.

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Table 1: Primers

Gene	Sequence	Accession
CAT	Forward GAAGAAAGCGGTCAAGAACT	NM_001752.4
	Reverse GTGAATCGCATTCTTAGGCT	
SOD3	Forward ACCCAGAGGTCTCCCTATAC	NM_003102.4
	Reverse GGTGTTTCGGTACAAATGGA	
HO-1	Forward CAGTTGCTGTAGGGCTTTAT	NM_002133.3
	Reverse CTTTCCAGAGAGAGGGACAC	
GPX1	Forward GGGCAAGGTACTACTTATCG	M21304.1
	Reverse GTTCTTGGCGTTCTCCTGAT	
NQO1	Forward GCGAGTGTTTCATAGGAGAGT	NM_001025433.2
	Reverse GATCCCTTGCAGAGAGTACA	
RPLP0	Forward GTGGTCGTGGAAGTGACAT	NM_001002.4
	Reverse ATGGTGTTCCTTGCCCATCAG	
ACTB	Reverse AGGATTCCTATGTGGGCGAC	NM_0011101.5
	Forward GGATAGCACAGCCTGGATAG	

Table notes: CAT, catalase; SOD3, superoxide dismutase 3; HO-1, heme oxygenase 1; GPX1, glutathione peroxidase 1; NQO1, NAD(P)H quinone dehydrogenase 1; RPLP0, ribosomal protein lateral stalk subunit PO; ACTB, actin beta.

Table 2: EC50

		Sex	Vehicle	BPS
Methacholine (Mch)	EC ₅₀ (M) (95% CI)	Male	6.99x10 ⁻⁹ (3.24x10 ⁻⁹ , 1.47x10 ⁻⁸)	9.49x10 ⁻⁹ (2.93x10 ⁻⁹ , 2.88x10 ⁻⁸)
Methacholine (Mch)	EC ₅₀ (M) (95% CI)	Female	5.20x10 ⁻⁸ (1.79x10 ⁻⁸ , 1.44x10 ⁻⁷)	1.97x10 ⁻⁸ (4.50x10 ⁻⁹ , 8.19x10 ⁻⁸)
Phenylephrine (Phe)	EC ₅₀ (M) (95% CI)	Male	5.10x10 ⁻⁷ (1.58x10 ⁻⁷ , 2.01x10 ⁻⁶)	4.89x10 ⁻⁷ (1.78x10 ⁻⁷ , 2.87x10 ⁻⁶)
Phenylephrine (Phe)	EC ₅₀ (M) (95% CI)	Female	3.57x10 ⁻⁷ (1.60x10 ⁻⁷ , 7.89x10 ⁻⁷)	4.71x10 ⁻⁷ (1.21x10 ⁻⁷ , 1.79x10 ⁻⁶)
KCl	Contraction (% ± SEM)	Male	40.9 ± 0.90	42.7 ± 3.51
KCl	Contraction (% ± SEM)	Female	43.0 ± 3.01	39.3 ± 3.69
Passive (Ca ²⁺) diameter	Diameter (µm± SEM)	Male	216.7 ± 12.02	239.1 ± 6.99
Passive (Ca ²⁺) diameter	Diameter (µm± SEM)	Female	226.8 ± 9.69	193.5 ± 10.89 * p = 0.036

Table notes: Half maximal effective concentration (EC₅₀) of methacholine (Mch) or phenylephrine (Phe) concentration-response curves generated in mesenteric arteries were calculated by transforming concentrations to log form, normalizing the data and fitting to nonlinear least squares fit curve. Maximal responses to a high dose of potassium chloride (KCl) were calculated relative to baseline. Passive internal diameter was recorded in pressurized mesenteric arteries under Ca²⁺-free conditions and compared by student's t-test. n = 6-15/group

487 **Figure Legends**

488 **Figure 1:** Redox balance in HUVEC exposed to BPS. Production of ROS was measured by
489 quantifying relative fluorescent intensity (RFI) by fluorescence spectroscopy in HUVEC exposed
490 to BPS for 30 min and loaded with a DCFDA probe (A). After 24 or 48 hrs of BPS exposure,
491 CellROX Deep Red stain, an indicator of oxidative stress, was quantified by fluorescence
492 spectroscopy (B). CellROX staining was also quantified using flow cytometry (C & D) and
493 expressed as mean fluorescent intensity (MFI). Images of CellROX-stained HUVEC were
494 captured on an epifluorescence-equipped microscope (E). mRNA expression analysis of genes
495 involved in antioxidant defences was performed by qPCR (F) and the activity of superoxide
496 dismutase (SOD) was determined using a SOD activity assay kit (G). Differences were compared
497 using one-way ANOVA with a Dunnett's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** p
498 < 0.0001 BPS vs. vehicle.

499 **Figure 2:** NO production and eNOS uncoupling in HUVEC exposed to BPS. Relative fluorescence
500 intensity (RFI) of DAF-FM diacetate, a fluorescent NO probe, was measured in HUVEC exposed
501 to BPS for 30 (A) or 90 min (B). Representative blots showing expression of phosphorylated and
502 total eNOS or AKT in HUVEC exposed to BPS for 48 hrs (C). The ratio of phosphorylated/total
503 protein was calculated by densitometry (D). The contribution of eNOS to ROS production was
504 determined by inhibiting eNOS with L-NAME or supplementing with BH4 prior to quantification
505 of DCFDA. DCFDA fluorescence was quantified by spectroscopy (E & F) or flow cytometry (G).
506 Comparisons were made by one-way ANOVA with a Dunnett's post hoc test (A-D, BPS vs.
507 vehicle) or Tukey's post hoc test (E-G). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

508 **Figure 3:** Mitochondria membrane polarization and mitochondria-derived ROS in HUVEC
509 exposed to BPS. Mitochondria polarization was determined by calculating the ratio of red
510 fluorescence (JC-1 aggregates) to green fluorescence (JC-1 monomers) using either fluorescent
511 spectroscopy (A) or flow cytometry (B). Mitochondria membrane potential was also determined
512 by quantifying fluorescence of the cationic dye, TMRE, by spectroscopy (C) or flow cytometry (E
513 & F). Mitochondria-derived ROS was measured in HUVEC exposed to BPS for 30 min by
514 measuring the fluorescence of MitoSOX Red by plate reader (F) or flow cytometric methods (G)

515 & H). Differences were compared by one-way ANOVA with a Dunnett's multiple comparison
516 test. * $p < 0.05$; ** $p < 0.01$; *** $p > 0.001$ BPS vs. vehicle.

517 **Figure 4:** Bioenergetic profile of HUVEC exposed to BPS. Extracellular flux analysis was
518 performed with a Seahorse Xfp Analyzer using a Mito stress test kit. After 48 hrs of exposure to
519 2.5 μM BPS, the oxygen consumption rate (OCR) (A) and extracellular acidification rate (ECAR)
520 (B) were measured under basal conditions and after injection of oligomycin, FCCP and
521 rotenone/antimycin A. Basal respiration (C), maximal respiration (D), spare respiratory capacity
522 (E), non-mitochondrial oxygen consumption (F), proton leak (G) and ATP production (H) were
523 calculated. Differences were compared with a student's t-test.

524 **Figure 5:** The contribution of mitochondria to oxidative stress and NO availability after BPS
525 exposure. HUVEC were treated with the mitochondria-targeted antioxidants, MitoTempol or
526 SS31, for 1 hr prior to BPS exposure. NO production was measured by loading cells with a DAF-
527 FM diacetate probe (A & B) and ROS production was measured using a DCFDA probe (C & D).
528 Fluorescence was quantified by spectroscopy and flow cytometry. Differences were compared
529 using one-way ANOVA with Tukey's post hoc test.

530 **Figure 6:** Endothelium-dependent vasodilation in microvessels isolated from male and female
531 mice exposed to BPS. Concentration-dependent contractile responses to phenylephrine (Phe) were
532 measured in pressurized mesenteric arteries from female (A) and male (C) C57BL/J6 mice exposed
533 to vehicle or 250 nM BPS via drinking water for ~ 9 weeks. Responses to cumulative
534 concentrations of the endothelium-dependent vasodilator, methacholine (Mch), were calculated in
535 pressurized Phe pre-contracted arteries isolated from female (B) or male (D) mice. In a subset of
536 male vessels, concentration-dependent dilatory responses to Mch were assessed in the presence of
537 the eNOS inhibitor, L-NAME (D). The % change in diameter was normalized to passive diameter
538 under Ca^{2+} -free conditions, concentrations were log-transformed, fitted to a non-linear least
539 squares curve, and compared by two-way ANOVA. The p value denotes significance in the
540 interaction between concentration of Mch and treatment group. E_{max} of Mch responses in the
541 presence or absence of L-NAME were compared by two-way ANOVA (Figure E). Area under the
542 curve (AUC) was calculated and the AUC of dilatory responses in the presence of L-NAME

543 subtracted from the Mch curve without L-NAME to determine the NO-sensitive component of
544 vasodilation in vehicle (63%) and BPS-treated males (27%). n = 6-15 mice/group.

545 **Supplementary Figure 1:** Representative tracings from pressure myography. After equilibration
546 of vessels at 60 mmHg and testing of viability, mesenteric arteries from control (A) or BPS-
547 exposed males (B) were pre-contracted with Phe and dilated with cumulative concentrations of
548 Mch. After washing, contractile responses to cumulative doses of Phe were assessed. Internal
549 diameter was recorded and normalized to passive diameter in Ca²⁺-free conditions.

550 **Supplementary Figure 2:** Glucose tolerance in mice exposed to BPS postnatally. Glucose
551 tolerance tests were performed in fasted male (A) or female (B) C57BL/J6 mice after exposure to
552 250 nM BPS via drinking water. Glucose was measured with a glucometer at baseline and after
553 injection of glucose (IP). n = 9-14 mice/group.

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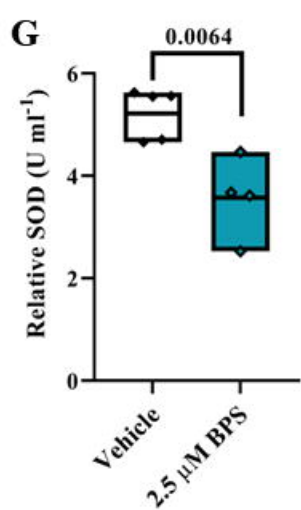
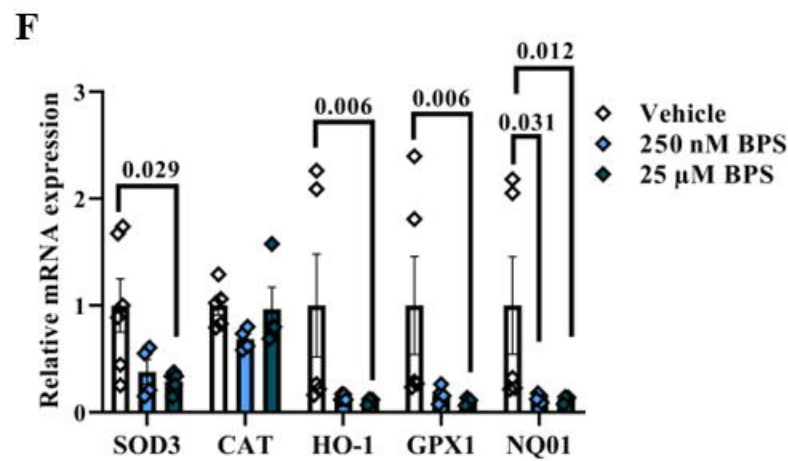
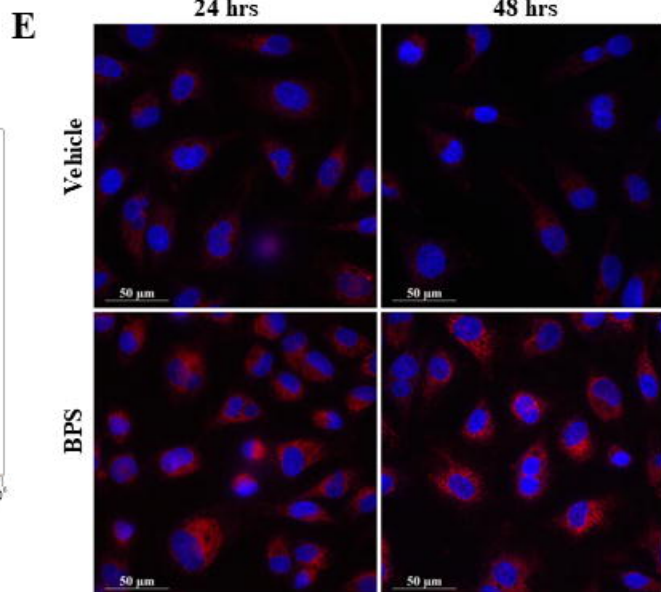
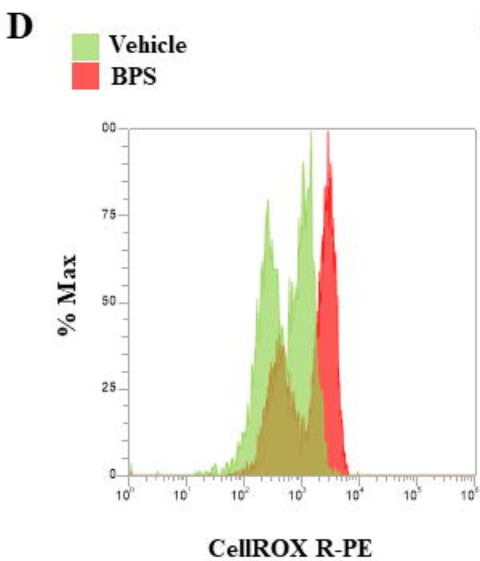
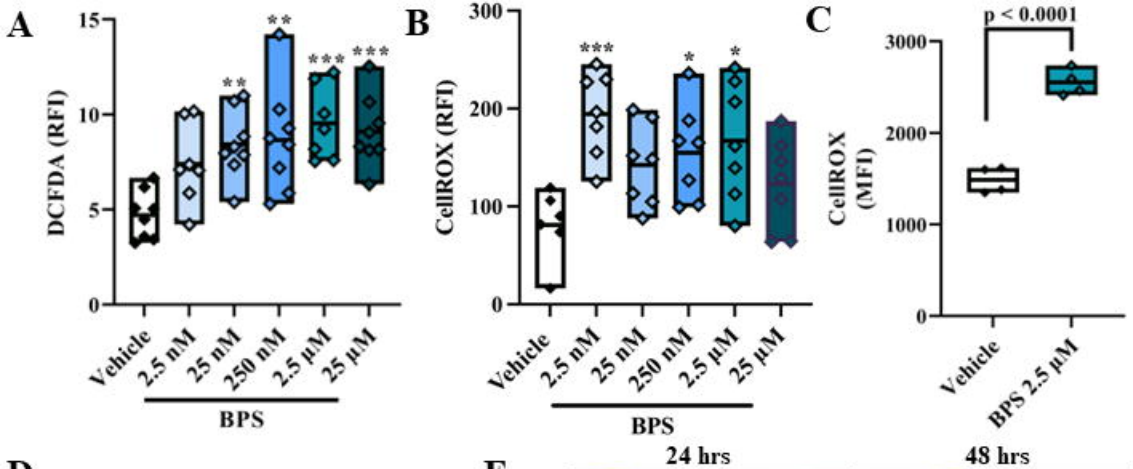
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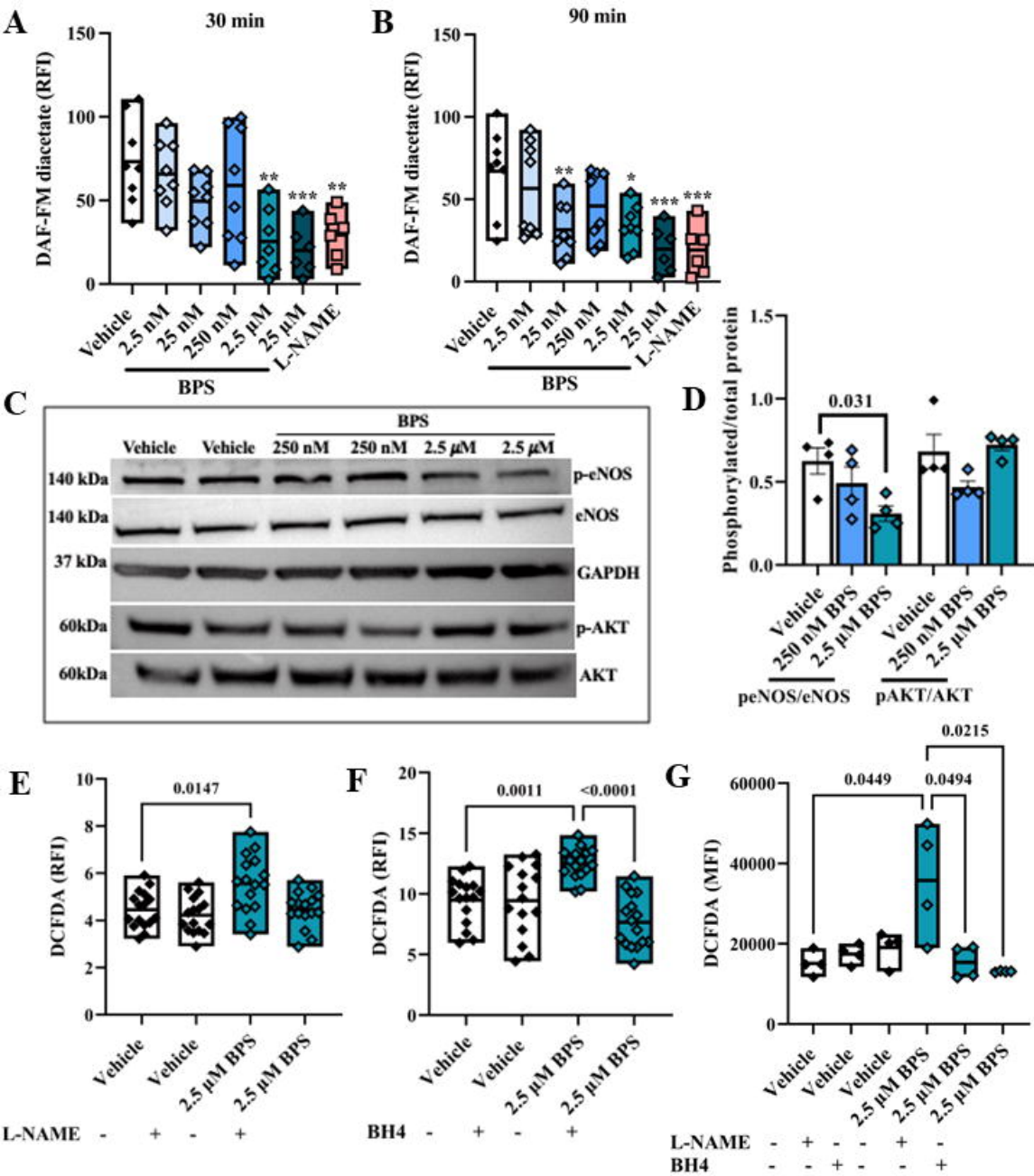
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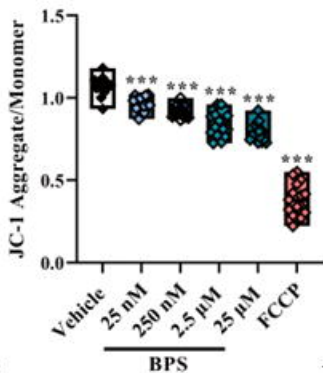
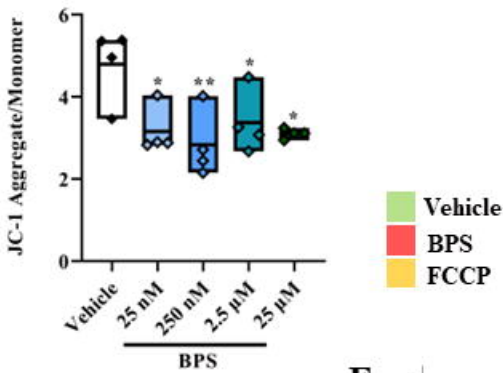
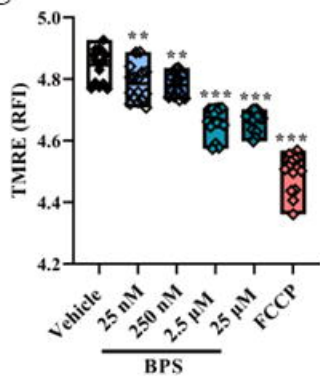
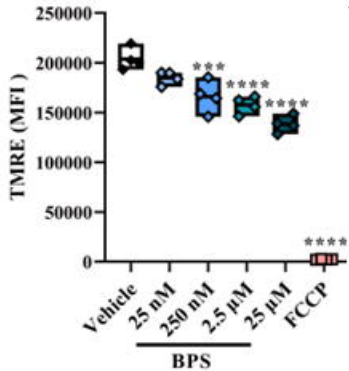
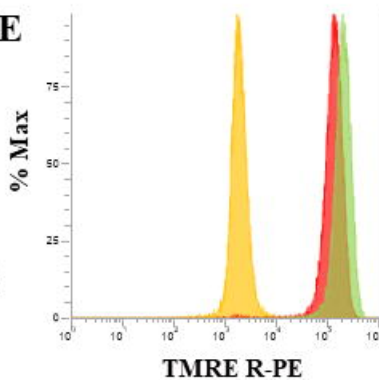
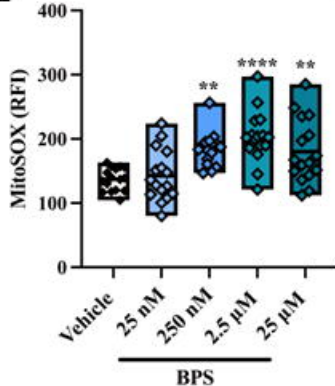
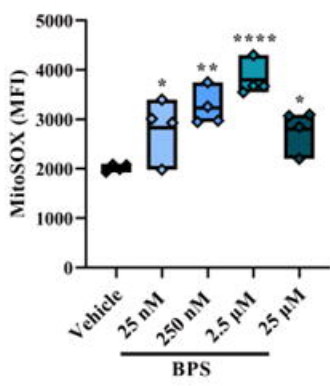
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