Increased carbon dioxide levels stimulate neutrophils to produce microparticles and activate the nucleotide-binding domain-like receptor 3 inflammasome

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Running Heading: CO₂-induced microparticles and inflammasome activation

Key Words: Mitochondrial carboxylation enzymes, NADPH oxidase, nitric oxide synthase,

S-nitrosylation, indoor air pollution, NLRP3 inflammasome, interleukin-1 β

21 **Abbreviations:** Actin free barbed ends (FBE), Apoptosis-associated Speck protein with CARD domain
22 (ASC), citrate transporter (CT), cytosolic NADP-dependent malic enzyme (ME), 2,7-dihydrodichloro-
23 fluorescein diacetate (DCF-DA), focal adhesion kinase (FAK), endoplasmic reticulum (ER), interleukin-
24 1β (IL- 1β), microparticles (MPs), inositol 1,3,5-triphosphate (IP3), knock-out (KO), *n*-octyl -
25 glucopyranoside (OG), NADPH oxidase (Nox), NADPH oxidase inhibitory peptide (Nox2ds),
26 nucleotide-binding domain-like receptor 3 (NLRP3), oxaloacetate (OAA), phosphoenolpyruvate
27 carboxykinase (PEP-CK), protein disulfide isomerase (PDI), pyruvate carboxylase (PCB), type 2 nitric
28 oxide synthase (iNOS), S-nitrosylated actin (SNO-actin), small inhibitory RNA (siRNA), thioredoxin
29 interacting protein (TXNIP), thioredoxin reductase (TrxR), vasodilator-stimulated phosphoprotein
30 (VASP).

31

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34

35 **Abstract:** We hypothesized that elevations of carbon dioxide (CO₂) commonly found in modern
36 buildings will stimulate leukocytes to produce microparticles (MPs) and activate the nucleotide-binding
37 domain-like receptor 3 (NLRP3) inflammasome due to mitochondrial oxidative stress. Human and
38 murine neutrophils generate MPs with high interleukin-1 β (IL-1 β) content when incubated *ex vivo* in
39 buffer equilibrated with 0.1 to 0.4% additional CO₂. Enhanced MPs production requires mitochondrial
40 reactive oxygen species production, which is mediated by activities of pyruvate carboxylase and
41 phosphoenolpyruvate carboxykinase. Subsequent events leading to MPs generation include perturbation
42 of inositol 1,3,5-triphosphate receptors, a transient elevation of intracellular calcium, activation of
43 protein kinase C and NADPH oxidase (Nox). Concomitant activation of type-2 nitric oxide synthase
44 yields secondary oxidants resulting in actin S-nitrosylation and enhanced filamentous actin turnover.
45 Numerous proteins are linked to short filamentous actin including vasodilator-stimulated
46 phosphoprotein, focal adhesion kinase, the membrane phospholipid translocation enzymes flippase and
47 floppase, and the critical inflammasome protein ASC (Apoptosis-associated Speck protein with CARD
48 domain). Elevations of CO₂ cause oligomerization of the inflammasome components ASC, NLRP3,
49 caspase 1, thioredoxin interacting protein, and calreticulin - a protein from endoplasmic reticulum,
50 leading to IL-1 β synthesis. An increased production rate of MPs containing elevated amounts of IL-1 β
51 persists for hours after short-term exposures to elevated CO₂.

52

53 **Introduction:** Over 60 years ago investigators reported that leukocyte O₂ consumption was increased
54 with progressive elevations of CO₂ up to 2%; presumably by hastening carboxylation of
55 phosphoenolpyruvate and pyruvate to oxaloacetate within mitochondria [1, 2]. Based on this information,
56 we hypothesized that CO₂ may trigger microparticle (MPs) production, a process stimulated by
57 oxidative stress. We previously reported that human and murine neutrophils generate MPs in response to
58 reactive species produced by NADPH oxidase (Nox) which, in conjunction with activation of type 2
59 nitric oxide synthase (iNOS), leads to S-nitrosylation of cytosolic actin, increased actin polymerization
60 and activation of phospholipid transporters [3]. Activation of Nox is central to MPs production; and
61 what is pertinent to this investigation, there are several pathways by which superoxide and hydrogen
62 peroxide from mitochondria can activate Nox [4]. Moreover, interleukin (IL)-1 β production via the
63 nucleotide-binding domain-like receptor 3 (NLRP3) inflammasome is intimately associated with oxidant
64 production [5], leading us to investigate whether this multiprotein complex is formed and IL-1 β
65 exocytosed via MPs in response to elevated CO₂. Adding to these issues, there is mounting evidence that
66 CO₂/bicarbonate may participate in complex oxidative stress pathways under physiological conditions
67 and when CO₂ is elevated [6].

68

69 Our primary interest was possible biochemical effects of CO₂ elevations on the order of 0.1 to 0.4%
70 (1000-4000 parts per million, ppm). The general population spends 80–90% of their time indoors [7].
71 Concentrations of CO₂ inside buildings depend primarily on occupant density and ventilation rates [8].
72 The World Health Organization in 1983 estimated that up to 30% of new and remodeled buildings
73 worldwide had unhealthy indoor air quality [9]. Improvements in many factors have occurred since then,
74 but not for CO₂ levels and air exchange rates [10, 11]. The CO₂ concentration may be as low as that
75 found outdoors (~380 ppm, 0.038%) but often is 5-10-fold higher than outdoors and sometimes reaches

76 over 4500 ppm [8, 12, 13]. This is alarming because breathing CO₂ at ~1000 ppm for 8 hours, or higher
77 concentrations for 2-3 hours, causes marked cognitive dysfunction [14-16], and may cause subtle
78 cardiovascular effects [14]. CO₂ is now viewed as the major factor for the gastrointestinal, respiratory
79 and cognitive disorders described as 'sick building syndrome' [8, 17-19]. There are no mechanisms
80 identified for these effects.

81

82 MPs are 0.1 to 1 µm diameter membrane vesicles shed from all vascular cells in response to various
83 stimuli [20]. MPs formation involves cytoskeletal turnover and exposure of phosphatidylserine on the
84 membrane surface as a consequence of translocation exerted by activated floppase(s) with inhibition of
85 flippase(s) activity and activation of scramblase by calcium influx [21, 22]. MPs serve as intercellular
86 messengers because they may contain cytokines or other signaling proteins, messenger RNA or
87 microRNA, and they generate free radicals [23]. MPs containing IL-1β are known to cause endothelial
88 injury [24]. A growing body of evidence indicates MPs are biological vectors involved with endothelial
89 dysfunction, inflammation, thrombosis, and contribute to the progression of macro- and micro-vascular
90 complications in numerous disorders [25].

91

92 Inflammasomes are part of a superfamily of Pattern Recognition Receptors (PRR), and NLRP3 appears
93 unique in that excess oxidants cause activation [5]. The oxidant source-varies, some find Nox isoforms
94 to be required [26], others mitochondria [27]. Recently, a group reported Nox-derived superoxide was
95 not required for NLRP3 activation, but was required for IL-1β efflux [28]. This last issue is extremely
96 important, as IL-1β is synthesized without a leader peptide so cannot utilize the conventional secretory
97 pathway (endoplasmic reticulum-Golgi-plasma membrane). Several groups have describe IL-1β

packaging into MPs [29-31], but whether inflammasome activation influences MPs production is not clear. The inflammasome protein called Apoptosis-associated Speck protein with CARD domain (ASC) can activate Rac to stimulate actin polymerization – a critical process for MPs formation, and ASC was recently shown to be closely associated with polymerizing cytoskeletal actin [32, 33]. Multimers of ASC are well known to serve as a platform for oligomerization of NLRP3 inflammasome components that are then capable of caspase 1 activation and IL-1 β synthesis [34].

Methods and Materials:

Materials: The majority of chemicals and their sources have been listed in prior publications [3]. A list of antibodies and manufacturers can be found in the on-line supplement (Supplemental Table 1), information on small inhibitory RNA (siRNA) in Supplemental Table 2, and other new reagents not used previously are listed below. Compressed air mixtures containing elevations of CO₂ were purchased from Air Products and Chemicals, Inc. (Allentown, PA). Genepin, oxylate, 3-mercaptopicolinic acid, benzenetricarboxylic acid, GF 109203X, 2-aminoethoxydiphenyl borate, were purchased from Tocris Bioscience, Ellisville, MO. Ebselen was purchase from Cayman Chemicals, Ann Arbor, MI.

Studies with human blood: All procedures were reviewed and approved by the University Committee on Studies Involving Human Beings. Subjects were healthy adults who had taken no medications for at least four days prior to phlebotomy. Blood was acquired in heparinized tubes.

118 **Animals:** All aspects of this study were reviewed and approved by the Institutional Animal Care and
119 Use Committee and with adherence to the NIH Guide for the Care and Use of Laboratory Animals.
120 C57BL/6J mice (*Mus musculus*) were purchased from Jackson Laboratories (Bar Harbor, ME), housed
121 in the university animal facility, fed a standard rodent diet Laboratory Rodent Diet 5001 (PMI Nutritional
122 Inc., Brentwood, MO) and water *ad libitum*. Colonies of iNOS and gp91phox knock-out mice were
123 maintained from breeding pairs purchased from Jackson Laboratories. Mice were anesthetized
124 [intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)] skin was prepared by
125 swabbing with Betadine and blood was obtained into heparinized syringes by aortic puncture.

126

127 **Isolation of leukocytes followed by exposure to various agents:** Heparin-anticoagulated blood (4 ml)
128 from healthy human volunteers was centrifuged through a two-layer preparation of Histopaque 1077 and
129 1119 (Sigma) at 400 g for 30 min to isolate monocytes and neutrophils. Murine cells were isolated from
130 heparinized blood of anesthetized mice as previously described [35]. At the termination of each
131 experiment leukocyte viability was determined as trypan blue exclusion using a Countess automated cell
132 counter (Invitrogen, Inc., Carlsbad, CA)

133

134 Procedures for *ex vivo* gas exposures were the same for human and murine cells, and all studies were
135 done at room temperature. Bicarbonate-based RPMI-1641 medium was equilibrated by stirring
136 overnight in an incubator environment with air + 5% or 5.4% CO₂ and at the start of an experiment cells
137 were added to achieve a concentration of 5.5×10^5 /ml. For studies performed in PBS buffer (Phosphate
138 buffered saline + 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM glucose) procedures were similar except
139 buffer was equilibrated by bubbling 0.5 ml solutions with 50 ml of air or air plus the desired elevated

140 fraction of CO₂ gas using a syringe and a 21 gauge needle in a test tube loosely covered with plastic film
141 to prevent ambient air influx. Because various manipulations involving centrifugation steps became
142 common through the project, the majority of studies were performed with PBS buffer and not RPMI
143 where loss of CO₂ and possible pH changes in samples temporarily left open to air would confound the
144 interpretation of results.

145

146 Where indicated, prior to gas exposures some murine cell suspensions were exposed in PBS buffer for
147 20 hours at room temperature to 0.08 nM small inhibitory (siRNA) following manufacturer's
148 instructions using control siRNA that does not cause specific degradation of any known cellular mRNA
149 or siRNA specific for mouse pyruvate carboxylase (PCB), mitochondrial or cytosolic
150 phosphoenolpyruvate carboxykinase (PEP-CK), citrate transporter (CT), or cytosolic malic enzyme
151 (ME). The degree of protein reduction after these treatments was assessed by Western blotting. Where
152 indicated, inhibitors were present in cell suspensions as follows: 5 μM genepin (UCP inhibitor), 100 μM
153 oxylate (PCB inhibitor), 750 μM 3-mercaptopicolinic acid (PEP-CK inhibitor), 2 mM
154 benzenetricarboxylic acid (CT inhibitor), 100 μM 2-aminoethoxydiphenyl borate (IP3 inhibitor), 5 μM
155 GF 109203X (PKC inhibitor), 200 μM MK571 (floppase inhibitor), 200 μM SCH28080 (flippase
156 inhibitor), 20 μM PT 573228 (FAK inhibitor), 30 μM quercetin-3-rutinoside (PDI inhibitor), 10 μM
157 Nox2ds (NOX inhibitor) or 10 μM of the scrambled sequence control peptide to Nox2ds. In other
158 experiments after gas exposures but prior to specific studies cell suspensions were illuminated at room
159 temperature for 5 minutes when placed 5 cm from a mercury vapor UV lamp (Blak-Ray B-100A,
160 Ultraviolet Products, Inc., San Gabriel, CA) with 38 ± 2 (n=6) mW/cm² output at 5 cm from the lamp as
161 verified using a Solarmeter Model 5.0 Sensor (Solarlight, Inc. Glenside, PA). In six trials, the solution
162 temperature at the start was $19.6 \pm 1.0^{\circ}\text{C}$ and after irradiation $20.5 \pm 1.0^{\circ}\text{C}$ (not statistically significant).

163

164 **Reactive species generation:** After gas exposures, leukocyte suspensions were prepared with 10 μ M
165 2,7-dihydrodichlorofluorescein-diacetate DCF-diacetate (DCF-DA) and fluorescence was monitored
166 (492 nm excitation, 530 nm emission) as described previously [3]. Because DCF can auto-catalyze its
167 own oxidation [36], in all studies DCF-DA was added immediately prior to performing the fluorescence
168 assay. That is, whether assays were performed immediately or 2 hours after CO₂ exposure.

169

170 **Intracellular pH:** Studies were performed with isolated neutrophils loaded with 10 μ M SNARF-AM,
171 washed and resuspended in RPMI-1641 buffer that had been equilibrated with air + 5 % CO₂ or air +
172 5.4% CO₂ . Cells were first incubated in 10 μ M carboxy-seminaphthorhodafluor (SNARF)-AM at 37°C
173 for 30 minutes, then washed once and re-suspend to 2.5×10^6 /ml in the same buffer. Comparable studies
174 were also performed with neutrophils in phosphate buffer (PBS + 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5
175 mM glucose) where washed cells load with 10 μ M SNARF-AM in 0.1 ml samples were bubbled with a
176 desired gas following the standard protocol as for MPs generation. With either RPMI or phosphate
177 buffer, after incubation times of up to 2 hours, samples were inserted into the flow cytometer where
178 10,000 events were collected over ~ 2 seconds. Ratio-metric analysis was done with excitation by a blue
179 laser (488 nm) and emissions detection in the B2 channel (max 565-605 nm) and B3 channel (max 655-
180 730). Intracellular pH was evaluated as a ratio of median fluorescence of B2/B3. A calibration curve was
181 generated by suspending cells in high K⁺ buffer (30 mM NaCl, 120 mM KCl, 1 mM CaCl₂, 0.5 mM
182 MgSO₄, 5 mM glucose, 10 mM HEPES, 10 mM PIPES and proportional 1 mM Na₂HPO₄/ NaH₂PO₄ to
183 establish pH 6.0, 6.5, 7.0, 7.5, 8.0) + 10 μ M nigericin for 5 minutes before running assays.

184

185 **Malate concentration:** Isolated neutrophil malate concentration was assessed using a commercial kit
186 (Sigma, Cat number MAK067, St. Louis, MO) according to the manufacturer's instructions. The
187 concentration was calculated using 155 fl as the neutrophil volume [37].

188

189 **Actin polymerization in permeabilized cells:** Neutrophils suspensions were permeabilized using 0.2% n-
190 octyl-b-glucopyranoside (OG) then exposed to air +/- CO₂ as outlined above and actin polymerization
191 assayed exactly as described in [3].

192

193 **NOS activity assay in permeabilized neutrophils:** Neutrophils suspensions were permeabilized using
194 0.2% n-octyl-b-glucopyranoside (OG) then exposed to air +/- CO₂ and NOS activity assessed exactly as
195 described in [3].

196

197 **Calcium assay:** Neutrophil suspensions were incubated at 37°C for 30 minutes with 6 µM Fura Red-AM
198 and 3 µM Fluo-3-AM, washed and re-suspend to 1 x 10⁷/ml in PBS containing 1 mM CaCl₂, 1.5 mM
199 MgCl₂ and 5.5 mM glucose, and then equilibrated to air or air + 0.4% CO₂ for up to 2 hours prior to
200 analysis in a flow cytometer. Ratio-metric analysis followed methods exactly as described by Assinger,
201 Volf and Schmid [38].

202

203 **Cytoskeletal protein associations based on Triton solubility:** After CO₂ exposures, 0.5 mM dithiobis
204 (succinimidyl propionate) (DTSP) was added to neutrophil suspensions to cross-link sulfhydryl-
205 containing proteins within a proximity of ~ 12 Å following published procedures [35]. Cell lysates were

206 partitioned into Triton-soluble G-actin and short F-actin and Triton-insoluble protein fractions and
207 subjected to electrophoresis in gradient 4 - 15 % SDS-PAGE gels followed by Western blotting as
208 described in [35].

209

210 ***Cell extract preparation and biotin-switch assay:*** After gas exposures, neutrophil suspensions were
211 transferred to HEN buffer (250 mM Hepes pH 7.7, 1 mM EDTA, 0.1 mM neocuproine), lysed and
212 subjected to the biotin-switch assay as previously described [35].

213

214 ***Flow cytometry:*** MPs were analyzed as described in previous publications [39, 40]. In brief, flow
215 cytometry was performed with an 8-color, triple laser MACSQuant® Analyzer (Miltenyi Biotec Corp.,
216 Auburn, CA) using MACSQuantify™ software version 2.5 to analyze data. MACSQuant was calibrated
217 every other day with calibration beads ((Miltenyi Biotec Corp., Auburn, CA). Forward and side scatter
218 were set at logarithmic gain. Photomultiplier tube voltage and triggers were optimized to detect sub-
219 micron particles. Micro-beads of 3 different diameters 0.3 µm (Sigma, Inc., St. Louis, MO), 1.0 µm and
220 3.0 µm (Spherotech, Inc., Lake Forest, IL) were used for initial settings and before each experiment as
221 an internal control. Samples were suspended in Annexin binding buffer solution (1:10 v/v in distilled
222 water, (BD Pharmingen, San Jose, CA), and antibodies as listed in Supplemental Table 2, with positive
223 staining determined following the fluorescence-minus-one control test. All reagents and solutions used
224 for MPs analysis were sterile and filtered (0.1 µm filter). MPs were defined as annexin V-positive
225 particles with diameters of 0.3 to 1 µm diameter. In this way, smaller particles that may include
226 exosomes (<0.1 µm diameter, an alternative way IL-1β can be packaged for exocytosis [41]) and non-

227 vesicle protein aggregates were excluded. The concentration of MPs in sample tubes was determined by
228 MACSQuant® Analyzer according to exact volume of solution from which MPs were analyzed.

229

230 ***IL-1 β measurements and NLRP3 inflammasome immunoprecipitation:*** Human and mouse-specific
231 IL-1 β ELISA Kits (eBioscience, San Diego, CA) were used to evaluate concentration of IL-1 β in MPs
232 samples following the manufacturer's instructions. MPs were first prepared the same as for flow
233 cytometry analysis, but then centrifuged at 100,000g for 60 min and the MPs pellet lysed with 0.3 ml
234 lysis buffer (20 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA
235 and 0.1% SDS (pH 7.5) with protease inhibitors cocktail (Sigma)). The protein content of the sample
236 was measured, diluted to 5 mg/ml, and 20 μ g protein used in the IL-1 β ELISA kits.

237

238 Immunoprecipitations to determine NLRP3 inflammasome oligomerization were performed on murine
239 neutrophils incubated in buffer equilibrated with air and air + 0.2 or 0.4% CO₂, where proteins were
240 crosslinked with DTSP (as described above for actin-associated protein associations) prior to cell lysis.
241 Lysates containing 250 μ g protein were pre-cleared and then incubated with 5 μ g anti-ASC antibodies
242 on a shaker overnight at 4°C, then 30 μ l 20% (w/v) protein G-Sepharose (pre-blocked with 2 % BSA)
243 was added and incubated for 1.5 hours at 4°C. Samples were processed, electrophoresed and analyzed by
244 Western blotting.

245

246 ***Statistical analysis:*** Results are expressed as the mean $\pm$ SE for three or more independent experiments.
247 Data were compare by analysis of variance (ANOVA) using SigmaStat (Jandel Scientific, San Jose, CA)
248 and Newman-Keuls post-hoc test. The level of statistical significance was defined as $p < 0.05$.

249

250 **Results:**

251 CO₂-induced MPs and elevated IL-1 β content: Neutrophils and monocytes were isolated from human
252 and mouse blood, then incubated *ex vivo* in buffer equilibrated with various CO₂ concentrations. Initial
253 studies used neutrophils incubated for 2 hours in RPMI medium, which has a bicarbonate concentration
254 of 23.8 mM. Control cells, incubated in buffer equilibrated with air + 5.0% CO₂ to maintain pH at $7.3 \pm$
255 0.1 (SE, n=7) generated virtually no MPs; 0.05 ± 0.02 (SE, n=4) MPs/human neutrophil and 0.03 ± 0.03
256 (n=7) MPs/murine neutrophil. Buffer equilibrated with air + 5.4% CO₂ had pH 7.3 ± 0.2 (n=7, no
257 significant difference (NS) versus 5% CO₂) and human neutrophils generated 2.4 ± 0.4 (p<0.05)
258 MPs/cell. Murine neutrophils generated 2.5 ± 0.2 MPs/ cell (p<0.05). Monocytes did not generate MPs
259 when exposed to elevated CO₂. For example, human monocytes incubated in air + 5% CO₂ produced
260 0.03 ± 0.02 MPs (n=4)/cell and in buffer equilibrated with 5.4% CO₂ generated 0.04 ± 0.03 MPs/cell
261 (NS). Murine monocytes responded in the same fashion.

262

263 To assess CO₂ dose-response, single cell stocks were prepared and aliquots divided among phosphate
264 buffered solutions equilibrated with a range of CO₂ concentrations. The MPs production rates of
265 suspensions equilibrated with air versus air + 0.4% CO₂ were analogous to studies with RPMI medium
266 incubated with air + 5 % versus 5.4 % CO₂. Neutrophil data are shown in Figure 1A, where values
267 indicate MPs produced per neutrophil after 2 hour incubations. Results were similar for human cells and
268 murine cells, and monocytes did not generate more MPs with elevated CO₂ (not shown). Changes in
269 MPs levels were not detected within 30 minutes of incubation, but MPs elevations could be detected if
270 cells were left for at least one 1 hour, and production peaked by 2 hours.

271

272 The same rate and magnitude of MPs production occurred when cells were only exposed to elevated
273 CO₂ for 30 minutes. For example, human cells incubated in buffer equilibrated with air + 0.4% CO₂ for
274 30 minutes, then centrifuged and incubated for 1.5 hours in buffer equilibrated with fresh air generated
275 2.5 ± 0.4 MPs/neutrophil ($p < 0.05$, $n = 4$), comparable to the rate shown in Figure 1A. Centrifugation
276 alone did not cause MPs production. If human neutrophils were exposed to just air-equilibrated buffer
277 for 30 minutes, centrifuged and resuspended in fresh air-equilibrated buffer for 1.5 hours they generated
278 0.04 ± 0.04 MPs/neutrophil (mean $\pm$ SE, $n = 4$), again comparable to the control cells in Figure 1A.
279 Murine cells responded similarly and exposure to only air-equilibrated buffer with centrifugation at 30
280 minutes resulted in production of 0.01 ± 0.01 MPs/neutrophil, whereas exposure for 30 minutes in
281 buffer equilibrated with air + 0.4% CO₂, centrifugation and incubation for 1.5 hours in air-equilibrated
282 buffer generated 2.8 ± 0.1 ($p < 0.05$, $n = 4$) MPs/neutrophil. If cells were incubated for up to 4 hours, no
283 more MPs were produced compared to values seen with 2 hour incubations.

284

285 MPs generation could not be attributed to alterations of neutrophil viability. Viability of human
286 neutrophils after 2 hours in air-saturated buffer was $81 \pm 8\%$, $n = 5$ and after exposure to air + 0.4 % CO₂
287 $80 \pm 9\%$, $n = 5$; viability of monocyte samples was also greater than 83% (not shown). Viability did not
288 differ between air-exposed murine neutrophils ($86 \pm 4\%$, $n = 20$) and those exposed for 2 hours to air +
289 0.4% CO₂ ($84 \pm 6\%$, $n = 20$). Studies were not conducted for times longer than 4 hours as viability of
290 neutrophil samples incubated with CO₂ decreased.

291

292 The concentrations of IL-1 β in MPs from neutrophils paralleled the rates of MPs production, as shown
293 in Figure 1B. Similar elevations of IL-1 β within MPs occurred when cells were exposed to elevations of
294 CO₂ for only 30 minutes, then left for 1.5 hours in air-equilibrated buffer. These data will be discussed
295 in greater detail below.

296

297 Intracellular pH measurements: The majority of studies were carried out in phosphate-buffered saline,
298 where the buffer pH was unchanged during exposures to air + up to 2 % CO₂ (pH 7.3 $\pm$ 0.1 pre-
299 exposure and 7.3 $\pm$ 0.1 post-exposure, n=6). The cytoplasmic pH values of neutrophils incubated in
300 buffer equilibrated with the various gas combinations were also unchanged based on flow cytometry
301 ratio-metric measurements using cells incubated with 10 μ M carboxy-SNARF-1-AM (Table 1, and
302 Supplemental Figure 1). Similarly, cells incubated in RPMI equilibrated with air + 5 % CO₂ versus air +
303 5.4% CO₂ exhibited no statistically significant differences in intracellular pH (data not shown).

304

305 Assessment of mitochondrial and cytosolic enzymes involved with CO₂-mediated MPs production:

306 Mechanisms for MPs generation were investigated using murine neutrophils, as – contrary to human
307 cells – they are sufficiently robust to maintain viability during overnight incubations with small
308 inhibitory RNA (siRNA) to deplete specific proteins. As mentioned in Introduction, our hypothesis was
309 shaped by work suggesting that elevations of CO₂ act by influencing enzymes involved with
310 carboxylation reactions [1, 2]. Figure 2 is a schematic that illustrates the enzymes investigated and
311 intracellular locations. Studies involving enzyme depletion and inhibition with chemical agents were
312 performed with cells in phosphate buffer equilibrated with air versus air + 0.4% CO₂. Supplemental
313 Table 3 shows that CO₂ does not stimulate MPs production if cells were depleted of mitochondrial

314 pyruvate carboxylase (PCB, siRNA reduced cell content by $75.6 \pm 3.5\%$, n=4), mitochondrial or
315 cytosolic phosphoenolpyruvate carboxykinase (PEP-CK, content reduced by, respectively, $77.4 \pm 4.4\%$,
316 n=4 and $72.2 \pm 2.4\%$, n=4), the citrate transporter (CT, content reduced by $91.1 \pm 0.9\%$, n=4) which is
317 required to shuttle mitochondrial citrate (produced from oxaloacetate) to the cytosol for further
318 metabolism, and cytosolic NADP-dependent malic enzyme (ME, reduced by $78.8 \pm 2.1\%$, n=4). Similar
319 results were seen when neutrophils were incubated with chemical inhibitors to these enzymes including
320 oxylate (PCB inhibitor), 3-mercaptopicolinic acid (PEP-CK inhibitor), and benzenetricarboxylic acid
321 (CT inhibitor). Enhanced MPs production also did not occur with exposures to buffer equilibrated with
322 air + 0.4% CO₂ using neutrophils from knock-out (KO) mice lacking iNOS or gp91phox component of
323 the NADPH oxidase. Similarly, cells incubated with 1400W (iNOS inhibitor) or the specific Nox
324 inhibitor, Nox2ds (but not scrambled sequence control peptide), failed to generate MPs with elevated
325 CO₂ (Supplemental Table 3).

326

327 Neutrophil malate concentration: We next investigated a possible mechanism for the biphasic neutrophil
328 MPs production response to CO₂ shown in Figure 1A. As pH was unchanged with these exposures, we
329 looked to the results from the siRNA studies summarized in Supplemental Table 3 for an explanation.
330 Our focus centered on malic enzyme (ME) because oxidative decarboxylation of malate to pyruvate by
331 cytosolic ME (but not mitochondrial ME) is inhibited by malate, and to a lesser degree by oxaloacetate
332 (OAA) [42]. Whereas the OAA concentration in cells (~9 $\mu\text{mol/L}$ cell volume) is well below the K_i for
333 ME (0.62 mM), the normal mammalian cell malate concentration is on the order of 0.3 mmol/L cell
334 volume and ME inhibition occurs at 1 mM or more [42, 43]. As shown in Table 2, malate concentration
335 did not change significantly among cells incubated in air-equilibrated buffer, versus buffer equilibrated

336 with air + 0.1 or 0.4% CO₂. However, the concentration increased over 10-fold when murine neutrophils
337 were incubated in buffer equilibrated with air + 1 or 2 % CO₂.

338

339 CO₂ triggers hydroethidine oxidation in neutrophils: Fluorescence of MitoSOX Red increased linearly
340 at 2.5 fold greater rate when murine neutrophils were exposed to buffer equilibrated with air + 0.4%
341 CO₂ versus only air (Figure 3A). As with MPs generation, the rate of MitoSOX Red fluorescence
342 exhibited a biphasic response to elevations of CO₂, and the rates of MitoSOX Red fluorescence were not
343 increased by exposures to air + 1 or 2 % CO₂ (Figure 3A, Supplemental Table 4). MitoSOX Red
344 selectivity for superoxide and for mitochondrial activity is not absolute [44], so studies showing no
345 enhanced fluorescence using cells depleted of PCB or mitochondrial PEP-CK by siRNA incubations
346 (Supplemental Table 3, 2nd column) provide additional support that measurements relate to
347 mitochondrial activity. Studies were also done with cells depleted of mitochondrial uncoupling protein 2
348 (UCP2, siRNA reduced cell content by $87.4 \pm 6.3\%$, n=4), as well as cells incubated with genepin, a
349 UCP2 inhibitor. These interventions abrogated the CO₂-induced increases in MitoSOX Red fluorescence
350 and MPs production (Supplemental Table 3).

351

352 Changes in MitoSOX Red fluorescence by CO₂ were not observed with murine monocytes. When cells
353 (5.5×10^5 cells/ml) were exposed to air-saturated buffer the fluorescence rate was 0.13 ± 0.08 (n=4)
354 units/minute and with exposure to buffer equilibrated with air + 0.4% CO₂ the rate was 0.16 ± 0.06
355 (n=4, NS).

356

357 To gain further insight into neutrophil reactive species generation, cells were loaded with the redox
358 active agent 2,7-dihydrodichloro-fluorescein diacetate (DCF-DA) and fluorescence monitored (Figure
359 3B). An enhanced rate was found after cells had been exposed for 30 minutes to elevated CO₂ and,
360 interestingly, the rate was significantly greater after cells were left in the buffer equilibrated with air +
361 0.4% CO₂ for 2 hours. While DCF is known to react with a relatively wide variety of reactive species,
362 we interpret the increase in fluorescence as requiring Nox activation because an enhanced rate of
363 fluorescence did not occur when cells were co-incubated with the specific Nox inhibitor, Nox2ds, or
364 with neutrophils from gp91phox KO mice (Supplemental Table 3, 3rd column). The process appears to
365 involve an interaction between Nox and mitochondria because no enhanced DCF fluorescence occurred
366 when cells were depletion of UCP2, PCB, PEP-CK, CT, or ME. Nox activation can increase
367 mitochondrial oxidant production, to allow a full circle of effects [45], which is supported by finding
368 that MitoSOX fluorescence was not enhanced by CO₂ in cells incubated with Nox2ds and with cells
369 from gp91phox KO mice (Supplemental Table 3). Similarly, the antioxidant ebselen inhibited both CO₂-
370 mediated MPs production and CO₂-mediated increases of MitoSOX Red and DCF fluorescence
371 (Supplemental Table 3). As with MitoSOX Red fluorescence, rates of DCF fluorescence were not
372 increased by exposures to air + 1 or 2 % CO₂ (Figure 3B, Supplemental Table 4).

373

374 Because DCF fluorescence rates were different after 30 minute versus after 2 hour CO₂ incubations
375 (Figure 3B) additional studies were performed. When cells were exposed to buffer equilibrated with air
376 + 0.4% CO₂ for just 30 minutes, centrifuged and resuspended in buffer equilibrated with only fresh air
377 for the remainder of 2 hours DCF fluorescence rate was the same as when cells were exposed to elevated
378 CO₂ continuously for 2 hours (Figure 3C). Centrifugation itself had no significant impact on the
379 response to CO₂ because when cells were merely centrifuged and then resuspended in the same CO₂

380 saturated buffer, the rate of DCF fluorescence was the same as when cells were exposed to CO₂ but
381 otherwise left unmanipulated for 2 hours.

382

383 Other cells were exposed to UV light for 5 minutes after the 30 minute incubation with air + 0.4% CO₂,
384 and then left undisturbed for the remainder of 2 hours in the CO₂-equilibrated buffer before DCF
385 fluorescence was assessed (Figure 3C). This was done because of evidence that S-nitrosylation is
386 involved with the CO₂ effect (see below). UV light will photo-reverse cysteine S-nitrosylation [3], and
387 UV exposure abrogated the effect of CO₂. For sake of clarity, only the one control data set with cells
388 exposed to air-saturated buffer for 2 hours is shown in Figure 3C, where in this series of studies the rate
389 of fluorescence change was 1.30 ± 0.30 (SE, n=3) fluorescence units/min. Other air-exposed control
390 groups involving centrifugation and resuspension in the same buffer, or with new air-saturated buffer,
391 and cells exposed to UV for 5 minutes, exhibited rates that were statistically insignificantly different
392 from the standard control. Rates were, respectively (n=3 for all groups), 1.63 ± 0.17 , 1.33 ± 0.33 , and
393 1.22 ± 0.30 .

394

395 Actin S-nitrosylation in CO₂-exposed cells: MPs were not generated by CO₂-exposed neutrophils from
396 iNOS and gp91phox KO mice (Supplemental Table 3), which suggests that reactive species produced by
397 interactions between nitric oxide and superoxide are required, and may be rendered even more complex
398 due to alternative reactive species generated by interactions with CO₂ and/or bicarbonate [6, 46]. Many
399 of the more highly reactive species can cause protein S-nitrosylation. S-nitrosylation of murine
400 neutrophil proteins was surveyed by the biotin switch assay, which covalently adds a disulfide-linked
401 biotin to the labile S-nitrosylation (SNO-) sites on proteins. A Western blot probing for biotin-containing

402 proteins in cells incubated using buffer equilibrated with air/control and air + 0.4% CO₂ is shown in
403 Figure 4. In separate trials, the prominent band at ~ 43 kDa was cut from nitrocellulose paper, subjected
404 to amino acid sequencing and identified as actin. The mean ratio for the band densities of SNO-actin/ β -
405 actin in air-exposed samples was 0.65 ± 0.14 (n=4) whereas for CO₂-exposed cells it was 3.95 ± 0.12
406 (p<0.05). If the biotin-switch analysis was performed on cell lysates treated with N-[6-(biotinamido)
407 hexyl]-3'-(2'-pyridyldithio) propionamide-biotin or with ascorbate (but not both), or with 1mM HgCl₂,
408 the bands were not visualized. When cells were exposed for 5 minutes to UV light after air/CO₂
409 incubation and prior to performing the biotin switch assay, the mean ratio for the band densities of SNO-
410 actin/ β -actin in air-exposed samples was 0.61 ± 0.10 (n=3) and for the CO₂-exposed cells 0.84 ± 0.21
411 (NS). As shown in Supplemental Table 3, UV exposure abrogated CO₂-induced MPs production.

412

413 Actin Polymerization: Actin polymerization plays a central role in MPs formation, and actin turnover is
414 enhanced by SNO-actin formation [3, 35]. CO₂-mediated MPs production was inhibited by cytochalasin
415 D (Supplemental Table 3), indicating a requirement for actin turnover. Actin polymerization was
416 assessed as free barbed ends formation (FBEs) in murine neutrophils permeabilized so they would take
417 up pyrene-actin (Figure 5). An enhanced rate of actin polymerization occurred when cells were assayed
418 after incubation in buffer equilibrated with air + 0.4% CO₂, which was blocked by co-incubations with
419 inhibitors of Nox (Nox2ds) and iNOS (1400W), and by exposure to UV light. The intimate association
420 among actin dynamics, reactive species production and MPs formation is also demonstrated by the
421 inhibitory effect of cytochalasin D on MitoSOX Red and DCF fluorescence responses to CO₂
422 (Supplemental Table 3).

423

424 Cytoskeletal protein associations based on Triton solubility: We were interested in evaluating
425 associations of cytoplasmic actin with proteins known to play a role in turnover dynamics. After 30
426 minute exposures in buffer equilibrated with air or air + 0.4% CO₂, neutrophils were incubated with the
427 membrane permeable protein cross-linker DTSP and then lysed. Cell lysates were separated into Triton
428 soluble G-actin, short filamentous (sF-) actin, and Triton-insoluble F-actin fractions and subjected to
429 Western blotting. These were analyzed looking for differences in protein band densities relative to actin.
430 Data shown in Figure 6 indicate that in the sF-actin fraction of CO₂ exposed cells there were marked
431 increases in associations between actin and iNOS; focal adhesion kinase (FAK) and vasodilator
432 stimulated phosphoprotein (VASP), scaffold proteins involved with actin polymerization; thioredoxin
433 reductase (TrxR) and protein disulfide isomerase (PDI), redox-related proteins linked to cytoskeletal
434 responses and Nox activation; ASC, involved with actin polymerization and NLRP3 inflammasome
435 activation; and flippase and floppase, enzymes involved with phospholipid turnover. There were
436 reciprocal decreases in these protein associations with actin in other cytoskeletal fractions, and all these
437 protein linkage responses were abrogated by exposure to UV light (Figure 6 and Supplemental Table 5).

438

439 Increased NOS activity due to CO₂: Absence of MPs production by cells incubated with 1400W, and by
440 cells from iNOS KO mice, as well as evidence for S-nitrosylation of actin, all point to activation of
441 iNOS by CO₂. Therefore, activity was assessed in murine neutrophils as conversion of [³H] L- arginine
442 to citrulline. As shown in Figure 7, citrulline production was increased in cells exposed to buffer
443 equilibrated with air + 0.4% CO₂ and the effect abrogated with UV. These results are consistent with
444 prior studies showing that iNOS activity increases due to cytoskeletal perturbations related to formation
445 of SNO-actin [3]. Results with iNOS KO mice and 1400 W in Supplemental Table 3 also show that
446 iNOS plays a role in increased rates of MitoSOX and DCF fluorescence in responses to CO₂.

447

448 *Ca²⁺ concentration and MPs generation:* Intracellular Ca²⁺ concentration in murine neutrophils
449 incubated for 2 hours with air-equilibrated buffer did not change significantly, but incubation in buffer
450 equilibrated with air + 0.4% CO₂ caused a transient increase at between 5 and 10 minutes of incubation
451 (Supplemental Figure 2). No differences versus air-exposed controls were found with incubations in the
452 presence of air + 0.4% CO₂ for longer intervals of time beyond 10 minutes.

453

454 Given apparent roles for Nox and iNOS in CO₂ responses, we also assayed intracellular Ca²⁺
455 concentrations in neutrophils from KO mice. Cytosolic Ca²⁺ in unstimulated, freshly isolated cells from
456 gp91 phox KO mice was 77.0 ± 2.5 nM (n=7, NS versus wild type) and transient elevations were not
457 found when KO cells were incubated in buffer equilibrated with air + 0.4% CO₂. For example, the
458 concentration after incubation for 5 minutes was 90.1 ± 3.8 nM, and at 10 minutes 88.3 ± 2.8 nM (n=4,
459 NS versus freshly isolated cells). We found that intracellular Ca²⁺ was statistically significantly elevated
460 in freshly isolated neutrophils from iNOS KO mice compared to wild type cells (100.6 ± 4.5 nM, n=8,
461 p<0.05 versus 83.3 ± 1.5 nM, n=16) but no significant elevations were observed in association with
462 incubations in buffer equilibrated to air + 0.4% CO₂ (e.g. after 5 minutes the concentration was $109.6 \pm$
463 3.3 nM, and at 10 minutes 107.7 ± 4.3 nM, n=8, NS versus freshly isolated cells).

464

465 *Role for inositol-1,3,5-triphosphate (IP3) receptors and PKC in CO₂ responses:* Mitochondrial oxidants
466 can sensitize IP3 receptors via their thiol groups to initiate endoplasmic reticulum (ER) Ca²⁺
467 mobilization [47]. When cells were incubated with siRNA to deplete the type 2 IP3 receptor (cell
468 content was reduced by $94.4 \pm 2.7\%$, n=4) or if cells were incubated with 100 μ M 2-

469 aminoethoxydiphenyl borate, an IP3 inhibitor, MPs were not generated by CO₂ exposure, nor did we see
470 elevated fluorescence with MitoSOX Red and DCF-DA (Supplemental Table 3).

471

472 Mobilized Ca²⁺ from IP3 receptors can activate protein kinase C isoforms, that may then activate Nox
473 [48]. Consistent with this notion, incubating cells with 5 μM GF 109203X, a PKC inhibitor, abrogated
474 CO₂-mediated MPs production as well as increased rates of fluorescence with MitoSOX Red and DCF-
475 DA (Supplemental Table 3).

476

477 CO₂ causes NLRP3 inflammasome activation: Activation of the NLRP3 inflammasome by oxidants
478 requires associations among its component parts [5]. Immunoprecipitation studies using an antibody to
479 ASC were performed on murine neutrophils incubated with buffer equilibrated with air and air + 0.2 or
480 0.4% CO₂. Neutrophils exposed to elevated CO₂ for 2 hours exhibit greater oligomerization among ASC
481 and NLRP3, caspase 1, thioredoxin interacting protein (TXNIP, which participates in inflammasome
482 activation) and calreticulin (Figure 8). Quiescent NLRP3 co-localizes with the ER protein calreticulin,
483 and linkage is upregulated with ER stress and inflammasome activation [27, 49]. There was a modest
484 elevation of proteins linked to ASC after just 30 minutes incubation in buffer equilibrated with air +
485 0.4% CO₂. However, cells exposed to air + 0.4% CO₂ for 30 minutes, then UV but left in the CO₂
486 equilibrated buffer for the remainder of 2 hours did not show significantly elevated ASC
487 oligomerization.

488

489 MPs contained similar amounts of IL-1β whether exposed continuously for 2 hours (as shown in Figure
490 1B), as when exposed to CO₂ for just 30 minutes and then re-suspended in the same buffer or in fresh,

491 air-equilibrated buffer for the remainder of 2 hours (Table 3). If neutrophils were incubated for 30
492 minutes in buffer saturated with air + 0.4% CO₂, exposed to UV for 5 minutes, and then left undisturbed
493 for the remainder of 2 hours the IL-1 β content of harvested MPs was comparable to the content in MPs
494 generated by cells exposed only to air (Table 3). Just as the antioxidant ebselen inhibited CO₂-mediated
495 MPs generation (Supplemental Table 3), it also inhibited IL-1 β production. MPs from neutrophils
496 exposed for 2 hours to air + 0.4% CO₂ had just 1.8 ± 0.6 (n=3) pg/1 x 10⁶ MPs, comparable to that
497 found in MPs from air-exposed, control cells (Table 3).

498

499

500 **Discussion:**

501 Elevations of CO₂ cause human and murine neutrophils to increase production of MPs. Results using
502 siRNA depletion and small chemical inhibitors indicate that CO₂ elevations modulate cell physiology
503 via mitochondrial and cytosolic enzymes involved with carboxylation reactions. Analogous results are
504 observed in our studies whether cells were suspended in bicarbonate-based RPMI or phosphate buffer.
505 This demonstrates that it is the transient increase of intracellular CO₂ and/or bicarbonate rather than the
506 background buffer that triggers MPs production. The schematic (Figure 2) depicts the intracellular
507 pathways we reason to be involved. However, the balance of substrates and products is likely to be
508 substantially more complex than shown. Moreover, because the kinetics are likely to vary with
509 metabolic state of the neutrophils, the impact of elevated CO₂ may differ among cells even within
510 individual experiments. For example, PCB mediated formation of oxaloacetate is quite low unless acetyl
511 CoA is present. Its K_m for bicarbonate is 400 mM with no acetyl CoA, 16 mM with 2 μM acetyl CoA
512 [50]. PEP-CK fixes CO₂. The K_m for CO₂ of cytosolic PEP-CK is 435 μM [51], and the value for the
513 mitochondrial enzyme is said to be similar [52].

514

515 We included studies examining a possible role for ME with MPs production because its activity would
516 increase NADPH production which can be utilized by a variety of enzymes including Nox. The
517 inhibitory action of malate on the cytosolic form of ME may explain the biphasic dose-response for CO₂
518 on enhancement of oxidant production, MPs formation and IL-1β synthesis. However, we have not ruled
519 out alternative mechanisms. For example, just as we and others [1,2] find biphasic effects with CO₂ in
520 whole cell studies, complex responses to elevated CO₂ are observed with isolated mitochondria and
521 sub-mitochondrial particles. CO₂ is reported to increase ATP hydrolysis and substrate uptake
522 implicating an uncoupling effect when using ~1.9 % CO₂ or more [53]; whereas others have reported

523 subtle increases of ADP:O ratios suggested as reflecting an improved phosphorylation efficiency, but an
524 overall reduction of O₂ uptake with studies from 5 to 15% CO₂ [54]. Respiratory inhibition at the
525 succinate-cytochrome c reductase step by high CO₂ was implicated in several reports [54, 55].
526 Additionally, pyruvate figures prominently in the scheme shown in Figure 2. Pyruvate is oxidized by
527 H₂O₂ and peroxynitrite, peroxynitrite-mediated decarboxylation yields a CO₂ radical that could
528 participate in neutrophil oxidative stress, and bicarbonate can inhibit reactions involving peroxynitrite
529 [46]. Our point is that over the range of CO₂ levels used in our studies there are likely to be competing
530 reactions that either enhance or impede the roles of mitochondria and various reactive species for
531 generating MPs and inflammasome activation.

532

533 We believe that events triggered by CO₂ start with altered mitochondrial oxidant production. The
534 graphical abstract illustrates a mechanism we envision based on all data generated in this project. The
535 first several arrows on the right-ward cycle between mitochondria and endoplasmic reticulum (ER) is
536 somewhat more complicated than depicted, however. Contrary to the inhibitory effects of UCP2
537 depletion/inhibition we see (Supplementary Table 3), one might expect UCP2 depletion would increase
538 MitoSOX Red fluorescence. UCP2 diminishes oxidant generation and mitochondrial permeability
539 transition pore opening [56]. UCPs influence cell respiration by partially dissipating the proton
540 electrochemical gradient across the mitochondrial membrane [57]. However, UCP2 is also a component
541 of the mitochondrial Ca²⁺ uniporter and preferentially contributes to mitochondrial Ca²⁺ uptake in
542 response to intracellular Ca²⁺ mobilization from the ER [58]. UCP2 and 3 are critical for cytosolic Ca²⁺
543 modulation, enabling mitochondria to transmit Ca²⁺ for utilization from areas of high to low
544 concentration within the cell [58]. The inhibitory effect of UCP2 depletion on MPs production suggests
545 this pathway is in operation. Others have demonstrated that Ca²⁺ elevations or ‘spikes’ are transient [59].

546 Given we are looking at a cellular pool versus individual cell measurements, the relatively broad span of
547 Ca^{2+} elevation we find between 5 and 10 minutes post- CO_2 exposure (Supplementary Figure 2) suggests
548 an averaging effect.

549

550 It is notable that the cycle shown to the left in the graphical abstract involving Nox and iNOS is virtually
551 the same as we have found in previous work with oxidative stress triggered by high inert gas pressures
552 [3, 60]. Nox activity can impinge on mitochondria and enhance oxidant production, which can create a
553 cyclic process [45]. This is supported by finding that MitoSOX fluorescence was not enhanced by CO_2
554 in cells from gp91phox KO mice, and finding no enhanced DCF fluorescence when cells were depletion
555 of PCB, PEP-CK, CT, or ME. Further, a positive feed-back mechanism may explain why DCF
556 fluorescence rate increases in cells between 30 minutes and 2 hours of CO_2 exposure (Figure 3B).
557 Others have reported neutrophil Nox activation is decreased by elevated CO_2 (at 10%) due to
558 intracellular acidification [61]. The nominal elevations of CO_2 in our study did not change intracellular
559 pH, hence results do not contradict prior findings.

560

561 Results require some added discussion pertaining to the temporal relationship among initial CO_2
562 exposure, generation of oxidants and subsequent MPs production. Cells produce MPs when they are
563 removed from CO_2 -containing buffer after just 30 minutes exposure and placed in air-equilibrated buffer
564 for the remainder of 2 hours, but UV light at 30 minutes stops the progression even though cells remain
565 in buffer saturated with air + 0.4% CO_2 for an additional 1.5 hours. We hypothesize the reason relates to
566 the transient elevation of Ca^{2+} seen with CO_2 -exposed cells (Supplementary Figure 2). It is well known
567 that elevations of intracellular Ca^{2+} are required for activation of enzymes needed to form MPs [20]. If

568 SNO-actin is eradicated at 30 minutes of CO₂ exposure by UV, which is after the transient rise in
569 intracellular Ca²⁺, the cascade leading to MPs production would be aborted.

570

571 The results show that cytoskeletal interactions and actin turnover are required for MPs generation, which
572 is consistent with reports by us and others showing that activation of Nox and iNOS occur in association
573 with cytoskeletal reorganization [3, 62]. Initial actin polymerization is slow but can be accelerated by
574 the presence of short actin filaments [63]. VASP has been documented as a major component in actin
575 dynamics, and has been shown to both bundle filaments and nucleate actin polymerization [64]. VASP
576 exhibits higher affinity for S-nitrosylated actin than un-modified actin, and we speculate this is why S-
577 nitrosylation accelerates MPs production [3, 65]. Additionally, FAK has been reported to induce Nox
578 activation [66], and we have previously shown that by linking iNOS to filamentous actin FAK increases
579 iNOS activity and accelerates MPs formation by neutrophils [3, 65]. Our cross linking studies
580 demonstrate that CO₂ increases short filamentous actin association with all of these regulatory proteins
581 (iNOS, VASP, FAK as well as Rac), PDI (which is known to facilitate Nox activation [67]), TrxR which
582 participates in SNO-actin dynamics in a complex manner [65, 68], and phospholipid transporters
583 required for MPs generation (flippase, floppase). Overlapping roles for phospholipid transporters and
584 reactive species with the dynamics of cytoskeletal turnover are recognized, but whether
585 flippase/floppase activity is modified due to cytoskeletal linkage is unknown [21, 22].

586

587 We found that MPs produced by neutrophils exposed to elevations of CO₂ have increased content of IL-
588 1 β . To our knowledge, this is the first report of inflammasome activation with a gaseous stimulus.

589 Production and exocytosis of IL-1 β traditionally requires receptor ligation to trigger a priming signal for

590 upregulated synthesis of NLRP3 and pro-IL-1 β followed by an activation signal prompting
591 inflammasome complex assembly [5]. Clearly, CO₂ triggering is more facile than expected. The
592 inhibitory effect of UV light indicates that protein S-nitrosylation is required for both NLRP3
593 inflammasome activation and MPs formation in response to CO₂. This offers substantial insight into the
594 recognized role for oxidants in MPs formation and NLRP3 inflammasome activation [5]. Moreover,
595 linkage between MPs formation and NLRP3 inflammasome activation is established because depleting
596 cells of ASC using siRNA also abrogates CO₂-mediated elevations in MitoSOX Red and DCF
597 fluorescence. This overlap is probably aided by ASC-mediated Rac activation, offering another means
598 for accelerated actin polymerization [32, 33].

599

600 As discussed in Introduction, there are many situations where people are exposed to elevations of CO₂.
601 Whether the effects we have observed have physiological relevance requires *in vivo* testing, which is
602 underway. Because MPs can cause vascular injuries and neuropathological effects [16, 69], our findings
603 could offer a novel mechanism for adverse effects related to elevations of indoor air CO₂.

604

605 Finally, it remains unclear why monocytes respond differently to CO₂ than do neutrophils. MitoSOX
606 Red and DCF fluorescence increases were not observed with monocytes incubated with elevated CO₂ so
607 there are clearly inherent differences in their metabolic responses. As CO₂/bicarbonate based radical
608 production can be enhanced by myeloperoxidase, this may play a role [6]. Differences may also be
609 influenced by the tight receptor-dependent control over Nox activation in some monocytes [70]. The
610 early studies that showed enhanced O₂ consumption by elevated CO₂ used mixed cell preparations

611 including all leukocytes from peripheral blood or bone marrow [1, 2]. Further work is needed to
612 elucidate reasons for different responses among leukocytes.

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797

798 **Figure legends:**

799 **Figure 1: MPs production per human (labeled with H) and murine (labeled with M) neutrophil**
800 **and MPs IL-1 β content.** (A) MPs were counted in suspensions of neutrophils (5.5×10^5 /ml in PBS
801 containing 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM glucose) in buffer equilibrated with air only or air
802 + elevated CO₂ from 0.1 to 2 % for 2 hours. (B) IL-1 β was measured in MPs isolated after the 2 hour
803 neutrophil exposure. Data are mean $\pm$ SE, *p<0.05 versus air-equilibrated buffer values; n=4 to 7 for
804 studies with human cells and 4 to 20 for mouse cells.

805

806 **Figure 2. Schematic of enzymes and pathways manipulated by siRNA and small chemical**
807 **inhibitors.** The figure exhibits mitochondrial and cytosolic pathways by arrows with substrates and
808 products in standard print. Light grey backgrounds identify enzymes of interest that were inhibited [shown
809 by large (X)] using siRNA and chemical inhibitors that are highlighted by boxes. Data on inhibitory
810 effects are listed in Supplementary Table 3. Enzymes included mitochondrial pyruvate carboxylase

811 (PCB), mitochondrial and cytosolic phosphoenolpyruvate carboxykinase (PEP-CK), citrate transporter
812 (CT) and cytosolic NADP-dependent malic enzyme (ME). Other abbreviations include acetyl CoA (Acl-
813 CoA), TCA (tricarboxylic acid cycle), and OAA (oxaloacetate).

814

815 **Figure 3. Reactive species production by neutrophils exposed to elevated CO₂.** Neutrophils (5.5 x
816 10⁵/ml PBS containing 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM glucose) were incubated with buffer
817 equilibrated with air or air plus elevated CO₂ for up to 2 hours. In (A) cells were first incubated with 5
818 μM MitoSOX red, then washed and incubated with buffer as shown. In (B) cells were incubated with the
819 buffer shown for 30 minutes or 2 hours, then 10 μM DCF-DA added and fluorescence monitored. Note
820 that fluorescence rates with cells incubated for just 30 minutes in the presence of air and air + 1.0 % CO₂
821 were the same as for incubations lasting 2 hours, but data were not included for sake of figure clarity. In
822 (C) cells were incubated with the buffer shown for 30 minutes, centrifuged (200 g for 10 minutes) and
823 the cell pellets resuspended in either the same buffer (CO₂-spin same buffer) or combined with fresh,
824 air-saturated buffer (CO₂-spin-fresh buffer) and incubated for the remainder of 2 hours before 10 μM
825 DCF-DA was added and fluorescence monitored. Other cells were exposed to air-saturated (Air) or air +
826 0.4% CO₂-saturated buffer for 2 hours (CO₂), or after 30 minutes incubation exposed to UV light for 5
827 minutes and then left undisturbed in the dark for the remainder of 2 hours (CO₂ + UV). Values are mean
828 ± SE, n=5 for each point.

829

830 **Figure 4. S-nitrosylated protein.** Lysates of murine neutrophils incubated with buffer equilibrated with
831 either air or air + 0.4% CO₂ for 30 minutes (representative samples of each) were prepared according to
832 the biotin switch assay and the entire blot is shown. Lower blot shows cytosolic actin in each
833 homogenate to control for protein loading.

834

835 **Figure 5. Actin polymerization rate.** Permeabilized neutrophil suspensions were incubated with buffer
836 equilibrated with either air or air + 0.4% CO₂ for 30 minutes, and where indicated samples were exposed
837 to UV light for the last 5 minutes. Table shows data for cells +/- scrambled sequence Nox2ds peptide
838 (control), Nox2ds, 1400W and UV. Actin polymerization was monitored for 5 minutes after adding 1
839 μ M pyrene-labeled rabbit skeletal muscle actin. Data are mean fluorescence/min $\pm$ SE, n=3, all groups.

840

841 **Figure 6. Protein associations in the Triton-soluble short F-actin fraction.** Murine neutrophils were
842 incubated with buffer equilibrated with either air or air + 0.4% CO₂ for 30 minutes, and where indicated
843 samples were exposed to UV light for the last 5 minutes, prior to addition of DTSP to cross-link
844 proteins. Samples were then fractionated based on Triton solubility (see Methods) and subjected to
845 Western blotting. This is a representative blot among four replicate experiments. After Western blotting,
846 protein band densities were quantified and normalized to the β actin band in each lysate. The ratio of
847 each protein relative to actin was compared to that calculated for the air-exposed, control neutrophils in
848 each experiment. Therefore, data in the figure show the fold-increase in band density normalized to the
849 ratio observed in control cells for the short F-actin fraction. Data are mean + SE (n=4), *p<0.05 versus
850 air-exposed cells.

851

852 **Figure 7. Nitric oxide synthase activity.** Data show [³H] citrulline production when [³H] arginine was
853 added to 2.5×10^5 murine neutrophils that were incubated with buffer equilibrated with either air or air +
854 0.4% CO₂ for 30 minutes and where indicated samples were exposed to UV light for the last 5 minutes
855 prior to lysis and assay. Values are mean $\pm$ SE (n=4 for each data point).

856

857 **Figure 8. Proteins associated with ASC.** Murine neutrophils incubated with buffer equilibrated with
858 either air or air + 0.2% or 0.4% CO₂ for 0.5 or 2 hours. Where indicated samples were exposed to UV
859 light for 5 minutes after incubation for 25 minutes, then left in buffer equilibrated with 0.4% CO₂ for the
860 remainder of 2 hours prior to lysis and immunoprecipitation using anti-ASC antibodies. A representative
861 blot is shown along with a summary of band densities for individual proteins relative to the ASC band
862 on 4 separate blots expressed as mean $\pm$ SE, *p<0.05 versus air-exposed control.

863

864 **Graphical Abstract: Hypothetical biochemical mechanism for CO₂-mediated MPs production:**

865 Data suggest that the initial event triggering MPs generation by elevated concentrations of CO₂ and/or
866 H₂CO₃ is carboxylation reactions that enhance mitochondrial production of superoxide (O₂⁻), H₂O₂ and
867 potentially, a varied array of CO₂-derived oxidants. These act on endoplasmic reticulum inositol 1,3,5-
868 triphosphate (IP3) receptors to trigger an elevation of intracellular calcium, followed by activation of
869 protein kinase C isoforms that increase NADPH oxidase (Nox) activity. Nox-derived O₂⁻ feeds back to
870 cause further mitochondrial oxidant production and also reacts with nitric oxide (NO). CO₂ and/or
871 H₂CO₃ are likely to interact at this step to produce agents capable of protein S-nitrosylation (SNO-)
872 leading to formation of cytosolic SNO-actin. Actin turnover is enhanced by linkage of vasodilator
873 stimulated phosphoprotein (VASP) to SNO-actin and accelerated polymerization hastens linkage of Rac
874 1/2 and focal adhesion kinase (FAK) that are depicted in the figure with dotted lines. FAK links type-2
875 nitric oxide synthase (iNOS) with actin, enhancing its activity and thus contributing to the auto-catalytic
876 nature of the process. There is also linkage of floppase and flippase to cytosolic actin, which may impact
877 enzyme activity, and which are required for the membrane phospholipid changes ultimately required for
878 MPs formation. Additionally, Apoptosis-associated Speck protein with CARD domain (ASC) links to

879 SNO-actin and secondary association of NLRP3, caspase 1 and calreticulin occur with production of
880 active IL-1 β .

881

882

883 **Table 1: Intracellular pH after 30 minute gas exposures:** Ratio-metric assessment of intracellular pH
884 using murine neutrophils loaded with 10 μ M SNARF-AM and then incubated with buffer equilibrated
885 with either air or air + the indicated concentration of CO₂ for 2 hours. Data are mean $\pm$ SE, n=7 for each
886 group, values are not statistically significantly different.

887

888

889

CO ₂ addition	Intracellular pH
Air only	7.38 $\pm$ 0.11
+0.2%	7.39 $\pm$ 0.03
+0.4%	7.40 $\pm$ 0.08
+2 %	7.39 $\pm$ 0.08

890

891 **Table 2: Intracellular malate concentration:** Murine neutrophils were exposed to buffer equilibrated
892 with air plus the CO₂ concentration shown for 2 hours, then lysed and processed as outlined in Methods.

893 Values are mean $\pm$ SE,

894 n is as shown; * p

895 < 0.05 versus

896 Control; + p < 0.05

897 versus all other

898 samples, ANOVA.

899

900

CO ₂ addition	Malate mmol /L cell volume
Air only	0.26 $\pm$ 0.11 (5)
+0.1%	0.32 $\pm$ 0.12 (5)
+0.4%	0.47 $\pm$ 0.09 (5)
+1%	2.07 $\pm$ 0.29 (4) *
+2 %	5.66 $\pm$ 0.21 (4) +

901

902

903

904 **Table 3. IL-1 β content of MPs generated by neutrophils incubated with buffer equilibrated with**
905 **air or air + 0.4% CO₂ for 2 hours.**

Buffer/exposure	PBS-only	+ UV	Spin-same	Spin-fresh
Air	1.5 $\pm$ 0.3 (8)	1.6 $\pm$ 0.4 (3)	2.0 $\pm$ 0.6 (3)	2.2 $\pm$ 0.4 (3)
Air + 0.4% CO ₂	30.2 $\pm$ 2.6 (8) *	2.2 $\pm$ 1.2 (4)	32.2 $\pm$ 3.7 (3)*	31.4 $\pm$ 3.2 (3)*

906

907 Data show IL-1 β as pg/1 x 10⁶ MPs. Neutrophils (5.5 x 10⁵/ml in PBS containing 1 mM CaCl₂, 1.5 mM
908 MgCl₂ and 5.5 mM glucose) were incubated in buffer equilibrated with air or air + 0.4% CO₂. In the first
909 column (PBS-only) cells were incubated undisturbed for 2 hours. In the next column they were
910 incubated for 30 minutes then exposed to UV light for 5 minutes, and then left undisturbed in the dark
911 for the remainder of 2 hours. In the last two columns cells were incubated for 30 minutes, centrifuged
912 (200 g for 10 minutes) and the cell pellets re-suspended in either the same buffer (spin-same) or
913 combined with fresh, air-saturated buffer (spin-fresh) and incubated for the remainder of 2 hours. Cells
914 were then centrifuged and supernatant processed to isolate MPs and assay intra-MPs IL-1 β as described
915 in Methods. Data are mean $\pm$ SE (n) is as shown, * reflects values significantly different from 2 hour air-
916 exposed MPs (p<0.05, ANOVA).

917

918

919 **On line Supplement Figure 1. Intracellular measurement of pH.** Forward and side scatter for
920 representative samples of murine neutrophils incubated with buffer equilibrated with either air or air +
921 0.4% CO₂ for air and air + 0.4% CO₂ exposed cell samples loaded with 10 µM SNARF-AM are shown
922 in the first set of panels, fluorescence emission for the B2 channel (max 565-605 nm) and B3 channel
923 (max 655-730) in the next panels. There were no notable differences caused by CO₂.

924

925 **On line Supplemental Figure 2. Intracellular calcium.** Neutrophils were incubated at 37°C for 30
926 minutes with 6 µM Fura Red-AM and 3 µM Fluo-3-AM, washed and re-suspend to 1 x 10⁷/ml in PBS
927 containing 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM glucose, and then incubated with buffer
928 equilibrated with either air or air + 0.4% CO₂ for up to 20 minutes prior to analysis in a flow cytometer.
929 Values are mean ± SE, n=number of samples from different mice, *p<0.05 versus baseline measurement
930 using cells immediately after isolation.

931

932 **Supplemental Table 1. List of antibodies, manufacturer sources and catalogue numbers:**

Antibody	Manufacturer	Catalogue No.
CT	Santa Cruz Biotechnology, CA.	sc-134878
Flippase	“	sc-374094
Floppase	“	sc-18835
ME1	“	sc-365891
PCB	“	sc-373937
PEPCK-M	“	sc-271029
PEPCK-C	“	sc-271029
RAC	“	sc-514583
TrxR	“	sc-28321
UCP2	“	sc-390189
FAK	BD Biosciences, San Jose, CA.	610087
PDI	“	610946
VASP	“	610448
ASC	US Biological/ThermoFisher, Pittsburgh, PA	207062

Actin	Sigma-Aldrich, St. Louis, MO	A 2066
NLRP3	R&D System, Minneapolis, MN	MAB7578
TXNIP	ThermoFisher, Pittsburgh, PA	40-3700

933

934

935 **Supplemental Table 2:** Small inhibitory RNA (siRNA) sequences from Santa Cruz Biotechnology.

Control	UUCUCCGAACGUGUCACGU	
Pyruvate carboxylase: Pool of siRNA duplexes	Sense: (A) CCUUAUACUCGUCUCUUUtt (B) GCACUAUCCGAAAGGUUCAtt (C) GUAGUUGCUCACAUAUUCAtt	Antisense (A) AAAGAGACGAGUAUUAAGGtt (B) UGAACCUUUCGGAUAGUGCtt (C) UGAAUAUGUGAGCAACUACtt
Cytosolic phosphoenolpyruvate carboxykinase: Pool of siRNA duplexes	Sense: (A) CCAUGUAUGUCAUCCCAUUtt (B) GCAACUUAAGGGCUAUCAAtt (C) CAGAAGGAGUACCAUUGAtt	Antisense: (A) AAUGGGAUGACAUACAUGGtt (B) UUGAUAGCCCUUAAGUUGCtt (C) UCAAUGGGUACUCCUUCUGtt
Mitochondrial phosphoenolpyruvate carboxykinase: Pool of siRNA duplexes	Sense: (A) GCAAGACGGUGAUUGUAACtt (B) GGAUCUGCCGAAGAUUAGAtt (C) CACUGUGCAGCUAAAGAUAtt	Antisense: (A) GUUACAAUCACCGUCUUGCtt (B) UCUAUUCUUCGGCAGAUCCtt (C) UAUCUUUAGCUGCACAGUGtt
Citrate transporter: Pool of siRNA duplexes.	Sense: (A) CCAAGCCAUCCGAUUCUUUtt (B) GUGAAUAUGUGCUCACUUAtt	Antisense: (A) AAAGAAUCGGAUGGCUUGGtt (B) UAAGUGAGCACAUAUUCACtt
Cytosolic Malic enzyme: Pool of siRNA duplexes.	Sense: (A) GCAACAAUCCUACGUGUUtt (B) CUGAGAUACUCGGGUGUUUtt (C) GUGACUAAACGGUUUCUUCAtt	Antisense: (A) AACACGUAGGAAUUGUUGCtt (B) AAACACCCGAGUAUCUCAGtt (C) UGAAGAAACCGUUAGUCACtt
Uncoupling protein 2; Pool of siRNA duplexes.	Sense: (A) CCUAUGACCUCAUCAAGAtt (B) GGACAGUUUACUACCAACAAtt (C) GAAGUUACAGGGAUCUCAUtt	Antisense: (A) UCUUUGAUGAGGUCAUAGGtt (B) UGUUGGUAGUAAACUGUCCtt (C) AUGAGAUCUCCUGUAACUUCtt
Type 2 inositol triphosphate receptor. Pool of siRNA duplexes.	Sense: (A) GUGUGCCCUAUGAAUCGAUtt (B) GUGAAAUACAGCAACGUUAtt	Antisense: (A) AUCGAUUCAUAGGGCACACtt (B) UAACGUUGCUGUAUUUCACtt

936

937 **Supplemental Table 3. Impact of various agents on 0.4% CO₂-mediated enhancement of**
938 **microparticle (MPs) production, MitoSox Red and DCF-DA fluorescence.**

939

Agent	MPs/PMN		MitoSox (fluorescence/min)		DCF (fluorescence/min)	
	Air	+0.4% CO ₂	Air	+ 0.4% CO ₂	Air	+ 0.4% CO ₂
PBS	0.03±0.02 (20)	2.31 ± 0.12 (20) *	13.3 ± 0.1 (8)	33.3 ± 0.1 (8) *	1.6±0.2 (8)	9.3 ± 0.4 (8) *
NOS KO	0.08±0.03 (5)	0.04±0.01 (5)	13.3 ± 0.2 (4)	13.7 ± 0.1 (4)	1.6±0.3 (4)	1.7 ± 0.1 (4)
1400W	0.02±0.01 (5)	0.07±0.06 (5)	12.9 ± 0.1 (4)	13.3 ± 0.2 (4)	1.6±0.2 (4)	1.3±0.2 (4)
gp91phox KO	0.04 ± 0.02 (5)	0.03±0.01 (5)	13.3 ± 0.1 (4)	13.6 ± 0.1 (4)	1.5±0.2 (4)	1.7±0.3 (4)
Nox2ds	0.03 ± 0.02 (5)	0.43 ± 0.39 (5)	13.3 ± 0.1 (4)	13.9 ± 0.27 (4)	1.7±0.3 (4)	1.8±0.4 (4)
ScrmB- Nox2	0.03 ± 0.02 (5)	2.31 ± 0.02 (5)*	11.3 ± 0.2 (8)	36.3 ± 0.3 (8) *	1.4±0.3 (8)	8.9 ± 0.5 (8) *
Control si	0.04 ± 0.03 (5)	2.36 ± 0.16 (5)*	12.1 ± 0.1 (8)	30.6 ± 0.1 (8) *	1.3±0.2 (8)	9.9 ± 0.8 (8) *
PCBsi	0.03 ± 0.01 (5)	0.11 ± 0.07 (5)	13.3 ± 0.1 (4)	13.1 ± 0.1 (4)	1.6±0.3 (4)	2.1 ± 0.2 (4)
Oxalate	0.04 ±	0.07 ±	13.8 ± 0.1	13.5 ± 0.1	1.70±0.3	1.7 ± 0.1

	0.02 (5)	0.03 (5)	(4)	(4)	(4)	(4)
m-PEP-CKsi	0.03 ± 0.01 (5)	0.05 ± 0.01 (5)	12.9 ± 0.1 (4)	13.1 ± 0.1 (4)	1.7±0.3 (4)	1.8 ± 0.2 (4)
c-PEP-CKsi	0.03 ± 0.02 (5)	0.15 ± 0.02 (5)	13.2 ± 0.1 (4)	13.1 ± 0.1 (4)	1.8±0.2 (4)	1.9 ± 0.3 (4)
MPA	0.05 ± 0.02 (5)	0.06 ± 0.042 (5)	13.3 ± 0.1 (4)	13.9 ± 0.1 (4)	1.7±0.1 (4)	1.7 ± 0.1 (4)
CTsi	0.08 ± 0.02 (5)	0.11 ± 0.01 (5)	13.3 ± 0.1 (4)	13.5 ± 0.1 (4)	2.2±0.2 (4)	1.9 ± 0.2 (4)
BTA	0.06 ± 0.01 (5)	0.07 ± 0.01 (5)	12.9 ± 0.1 (4)	13.1 ± 0.2 (4)	1.7±0.2 (4)	1.8 ± 0.1 (4)
MEsi	0.08 ± 0.06 (5)	0.11 ± 0.02 (5)	12.9 ± 0.2 (4)	13.1 ± 0.2 (4)	1.8±0.1 (4)	1.8 ± 0.2 (4)
UCP2si	0.12 ± 0.04 (5)	0.21 ± 0.02 (5)	13.1 ± 0.2 (4)	13.8 ± 0.1 (4)	1.6±0.4 (4)	1.7 ± 0.3 (4)
Genepin	0.05 ± 0.06 (5)	0.09 ± 0.03 (5)	12.9 ± 0.1 (4)	14.1 ± 0.3 (4)	2.2±0.3 (4)	2.1 ± 0.2 (4)
IP3si	0.06 ± 0.03 (5)	0.11 ± 0.02 (5)	13.4 ± 0.2 (4)	13.4 ± 0.2 (4)	1.3±0.2 (4)	1.3 ± 0.4 (4)
APB	0.08 ± 0.04 (5)	0.11 ± 0.02 (5)	13.2 ± 0.1 (4)	13.4 ± 0.1 (4)	1.3±0.2 (4)	1.3 ± 0.4 (4)
GF	0.07 ±	0.11 ±	13.7 ± 0.1	13.8 ± 0.1	1.4±0.2	1.1 ± 0.3

109203X	0.03 (5)	0.02 (5)	(4)	(4)	(4)	(4)
UV	0.09 ± 0.04 (8)	0.07 ± 0.06 (8)	12.7 ± 0.2 (4)	13.8 ± 0.1 (4)	1.1 ± 0.1 (7)	1.2 ± 0.2 (7)
Ebselen	0.04 ± 0.02 (3)	0.04 ± 0.01 (3)	12.9 ± 0.1 (3)	13.2 ± 0.2 (3)	1.3 ± 0.1 (3)	1.6 ± 0.4 (3)
Cyto D	0.05 ± 0.03 (5)	0.17 ± 0.09 (5)	12.0 ± 0.2 (4)	12.8 ± 0.2 (4)	1.2 ± 0.1 (4)	1.3 ± 0.2 (4)
ASCsi	0.05 ± 0.03 (4)	0.09 ± 0.05 (4)	11.9 ± 0.1 (4)	12.8 ± 0.2 (4)	1.3 ± 0.1 (4)	1.5 ± 0.2 (4)

940 Isolated murine neutrophils (5.5×10^5 /ml PBS containing 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM
941 glucose) were incubated with buffer equilibrated with either air or air + 0.4% CO₂ for 2 hours MPs/PMN
942 reflects increases in MPs in suspensions. MitoSox reflects fluorescence from cells incubated with 5 μ M
943 MitoSox red for 10 minutes, wash and then incubated in buffer for up to 2 hours. DCF fluorescence was
944 assessed when 10 μ M DCF-DA was added to cell suspensions at the end of buffer incubations. All
945 values are mean $\pm$ SE (n=number of independent trials), *p<0.05 versus air value. Abbreviations and
946 manipulations are as follows: KO, neutrophils from iNOS or gp91phox knock-out mice; 1400W,
947 incubation with 1 mM 1400W during air/CO₂ exposure; Nox2ds incubation with 10 μ M Nox-2ds during
948 the air/CO₂ exposure; Scrm-b-Nox2ds, incubations performed with 10 μ M control, scrambled sequence
949 peptide to Nox-2ds; Cont-si cells incubated with control, scrambled sequence small inhibitory RNA for
950 20 hours prior to the experiment; PCBsi, cells incubated with small inhibitory RNA specific to pyruvate
951 carboxylase for 20 hours prior to the experiment; oxylate, incubation with 100 μ M oxylate, an inhibitor
952 of PCB; during air/CO₂ exposures; m-PEP-CKsi, cells incubated with small inhibitory RNA specific to

953 the mitochondrial form of phosphoenolpyruvate carboxykinase for 20 hours prior to the experiment; c-
954 PEP-CKsi, cells incubated with small inhibitory RNA specific to the cytosolic form of
955 phosphoenolpyruvate carboxykinase for 20 hours prior to the experiment; MPA, incubation with 750
956 μM 3-mercaptopicolinic acid, an inhibitor of PEP-CK; during air/ CO_2 exposures; CTsi, cells incubated
957 with small inhibitory RNA specific to the citrate transporter for 20 hours prior to the experiment; BTA,
958 incubation with 2 mM benzenetricarboxylic acid, an inhibitor of CT, during air/ CO_2 exposures; MEsi,
959 cells incubated with small inhibitory RNA specific to the NADP-dependent malic enzyme for 20 hours
960 prior to the experiment; UCP2si, cells incubated with small inhibitory RNA specific to uncoupling
961 protein 2 for 20 hours prior to the experiment; genepin, incubation with 5 μM genipin, a UCP inhibitor,
962 during air/ CO_2 exposures; IP3si, cells incubated with small inhibitory RNA specific to the inositol 1,4,5-
963 trisphosphate receptor type 2 for 20 hours prior to the experiment; APB, incubation with 100 μM 2-
964 aminoethoxydiphenyl borate, an IP3 receptor inhibitor, during air/ CO_2 exposures; GF 109203X,
965 incubation with 5 μM of the protein kinase C inhibitor during air/ CO_2 exposures; ebselen, incubation
966 with 1 mM of the antioxidant during air/ CO_2 exposures; UV, cells exposed to air/ CO_2 for 30 minutes,
967 then to UV light for 5 minutes and incubated for the remainder of 2 hours prior to assays; Cyto D,
968 incubation with 5 μM cytochalasin D during the air/ CO_2 exposure, ASCsi cells incubated with small
969 inhibitory RNA specific to ASC for 20 hours prior to the experiment.

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972 **Supplemental Table 4: CO₂ dose-response for neutrophil-mediated MitoSOX Red and DCF**
973 **fluorescence.** Neutrophils (5.5 x 10⁵/ml PBS containing 1 mM CaCl₂, 1.5 mM MgCl₂ and 5.5 mM
974 glucose) were incubated for 2 hours in buffer equilibrated with air plus the CO₂ concentrations shown.
975 Values are fluorescence of cells loaded with 5 μM MitoSOX red or 10 μM DCF-DA as units/min (mean

CO ₂ addition	MitoSOX Red (units/min)	DCF-DA (units/min)
Air only	13.3 ± 0.09	1.56 ± 0.18
+0.1%	20.3 ± 0.12 *	3.67 ± 0.33 *
+0.2%	21.6 ± 0.29 *	5.67 ± 0.23 *
+0.4%	33.2 ± 0.08 *	9.31 ± 0.41 *

976 ± SE, n=5 or more for each point, * p<0.05 vs control).

977

978

+1%	13.1 ± 0.24	1.17 ± 0.44
+2 %	13.7 ± 0.22	1.63 ± 0.24

979

980

981 **Supplemental Table 5. Protein associations assessed by DTSP cross-linking in G-actin and Triton-**
982 **insoluble actin fractions.** Neutrophils were incubated buffer equilibrated with air or air + 0.4% CO₂ for
983 30 minutes. Where indicated, samples were exposed to UV light for 5 minutes before DTSP addition to
984 crosslink proteins (see Methods).

G-actin	VASP/ actin	FAK/ actin	iNOS/ actin	PDI/ actin	ASC/ actin	Rac/ actin	Flippase/ actin	Floppase/ actin
+0.4% CO₂	0.36 ± 0.08*	0.51 ± 0.14*	0.43 ± 0.12*	0.51 ± 0.12*	0.49 ± 0.09*	0.50 ± 0.14*	0.41 ± 0.16*	0.36 ± 0.04*
Air + UV	0.96 ± 0.08	0.89 ± 0.06	0.86 ± 0.02	0.92 ± 0.06	0.95 ± 0.10	0.96 ± 0.09	0.96 ± 0.04	0.96 ± 0.08
0.4% CO₂ +UV	1.02 ± 0.18	0.95 ± 0.21	0.88 ± 0.13	1.00 ± 0.15	0.96 ± 0.26	0.98 ± 0.06	0.93 ± 0.20	0.77 ± 0.10

985

Triton- insoluble actin	VASP/ actin	FAK/ actin	iNOS/ actin	PDI/ actin	ACS/ actin	Rac/ actin	Flippase/ actin	Floppase/ actin
+ 0.4% CO₂	0.27 ± 0.02*	0.40 ± 0.14*	0.33 ± 0.08*	0.51 ± 0.12*	0.34 ±0.09*	0.37 ± 0.21*	0.25 ± 0.09*	0.41 ± 0.10*
Air +	0.92 ±	0.89 ±	0.89 ±	0.85 ±	0.92 ±	0.98 ±	0.87 ±	1.07 ±

UV	0.08	0.06	0.06	0.03	0.04	0.05	0.03	0.05
0.4% CO₂ +UV	0.89 $\pm$	0.89 $\pm$	0.73 $\pm$	0.79 $\pm$	0.85 $\pm$	0.87 $\pm$	0.68 $\pm$	0.80 $\pm$
	0.01	0.01	0.04	0.01	0.02	0.06	0.05	0.02

986

987 Values are ratios calculated based on the band densities of the identified protein relative to the actin
988 band density in each sample. In each table the values are normalized to air-exposed control protein band
989 ratios of individual experiments, thus values greater than 1.0 reflect increases in protein associations
990 with actin versus air-exposed control samples, whereas values less than 1.0 indicate lower protein
991 associations. Data are mean $\pm$ SE (n=4 for all samples), * reflects values significantly different from
992 normalized air-exposed control values (p<0.05, ANOVA).

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