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## **SUR1 as a new therapeutic target for pulmonary arterial hypertension**

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HAL Authorization

1 SUR1 as a new therapeutic target for pulmonary arterial hypertension

2 Short title: SUR1/Kir6.2 in PH

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32

33

34 **Abstract**

35 Mutations in *ABCC8* have been identified in pulmonary arterial hypertension (PAH). *ABCC8* encodes  
36 SUR1, a regulatory subunit of the ATP-sensitive-potassium channel Kir6.2. However, the  
37 pathophysiological role of the SUR1/Kir6.2 channel in PAH is unknown. We hypothesized that  
38 activation of SUR1 could be a novel potential target for PAH. We analysed the expression of  
39 SUR1/Kir6.2 in the lungs and pulmonary artery (PA) in human PAH or experimental pulmonary  
40 hypertension (PH). The contribution of SUR1 in human or rat PA tone was evaluated, and we measured  
41 the consequences of *in vivo* activation of SUR1 in control and PH rats. SUR1 and Kir6.2 protein  
42 expression was not reduced in the lungs or human pulmonary arterial endothelial cells and smooth  
43 muscle cells (hPAECs and hPASMCs) from PAH or experimentally induced PH. We showed that  
44 pharmacological activation of SUR1 by 3 different SUR1 activators (diazoxide, VU0071063, and  
45 NN414) leads to PA relaxation.

46 Conversely, the inhibition of SUR1/Kir6.2 channels causes PA constriction. *In vivo*, long- and short-  
47 term activation of SUR1 with diazoxide reversed monocrotaline-induced PH in rats. Additionally, *in*  
48 *vivo* diazoxide application (short protocol) reduced the severity of PH in chronic-hypoxia rats.  
49 Moreover, 3 weeks of diazoxide exposure in control rats had no cardiovascular effects. Finally, *in vivo*,  
50 activation of SUR1 with NN414 reduced monocrotaline-induced PH in rats. In PAH and experimental  
51 PH, the expression of SUR1/Kir6.2 was still presented. *In vivo* pharmacological SUR1 activation by  
52 two different molecules alleviated experimental PH, providing proof-of-concept that SUR1 activation  
53 should be considered for PAH and evaluated more thoroughly.

54

55 **Keywords:** SUR1, *ABCC8*, pulmonary arterial tone, nateglinide

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58 **Clinical relevance:** Recently identified mutations in *ABCC8* encoding SUR1 in PAH highlighted a  
59 novel potential target. We provide proof of concept that pharmacological activation of SUR1 should be

60 considered in PAH. The use of more specific SUR1 activators (NN414) in humans could be helpful for  
61 PAH therapy.

62

### 63 **Introduction**

64 Pulmonary arterial hypertension (PAH) is a rare and severe disease. At the last World Symposium on  
65 Pulmonary Hypertension (PH), PAH was defined as an elevation of the mean pulmonary artery (PA)  
66 pressure  $>20$  mmHg and by a pulmonary vascular resistance (PVR)  $>3$  Wood units at rest. It is also  
67 defined as a PA wedge pressure  $\leq 15$  mmHg(1). The prevalence of the disease is estimated to be between  
68 15 and 50 cases per million(2). Increased PVR is the consequence of remodelling of the small PA  
69 (diameter  $<500$   $\mu\text{m}$ ), which results in right ventricular (RV) hypertrophy and right heart failure and  
70 death(3).

71 PAH is a complex and multifactorial disease. There is idiopathic PAH (iPAH, where the cause is  
72 unknown), heritable PAH (hPAH, due to identifiable genetic mutations or family history), PAH induced  
73 by drugs or toxins, and PAH associated with other diseases. During the last two decades, 18 PAH-  
74 predisposing genes have been identified in hPAH: *BMPR2*, *EIF2AK4*, *ACVRL1*, *TBX4*, *GDF2*, *SOX17*,  
75 *ENG*, *KCNK3*, *ABCC8*, *ATP13A3*, *SMAD9*, *AQP1*, *CAV1*, *BMP10*, *SMAD4*, *SMAD1*, *BMP9*, and  
76 *KDR*(4, 5).

77 In 2018, Bohnen et al., using exome sequencing, discovered 12 different mutations in ATP-binding  
78 cassette subfamily C member 8 (*ABCC8*) in PAH patients(6). The *ABCC8* gene encodes the SUR1  
79 protein, a regulatory subunit of ATP-sensitive- $\text{K}^+$  channels (KATP). *ABCC8* mutation carriers are  
80 younger than those with iPAH (median age at diagnosis 14 vs. 42 years old)(6). KATP channels, initially  
81 discovered in the heart, are characterized by inhibition of  $\text{K}^+$  channels when the intracellular ATP  
82 concentration increases(7). Like all  $\text{K}^+$  channels, KATP channels are crucial for membrane excitability  
83 and the cellular metabolic state and regulate many cellular functions, including hormone secretion,  
84 vascular tone, and cardiomyocyte contractility(8). KATP channels are composed of four Kir6.x (Kir6.1  
85 and Kir6.2) channels and four sulfonylurea receptor subunits (SUR1, SUR2A, and SUR2B). SUR1 can  
86 co-assemble and regulate Kir6.1 and Kir6.2 channels(9). Additionally, some reports have suggested that

87 SUR1 interacts with and regulates transient receptor melatonin 4 (TRPM4), a nonselective cationic  
88 channel(10). Electrophysiological recordings of Cos-7 cells overexpressing mutated *ABCC8* plus Kir6.2  
89 channels demonstrated that 11 of 12 *ABCC8* mutations showed a loss of function of the KATP  
90 channel(6). *ABCC8* mutations are well known to cause neonatal diabetes and hyperinsulinism due to the  
91 dysregulation of insulin secretion(11). SUR1/Kir6.2 channels in pancreatic cells regulate the resting  
92 membrane potential and indirectly regulate insulin secretion(12). However, the role of the SUR1/Kir6.2  
93 channel in PAH physiopathology remains unknown and requires further investigation to elucidate a  
94 putative pathophysiological role of SUR1/Kir6.2 channels in the development of PAH.

95 In the present study, we hypothesized that pharmacological activation of SUR1 could reduce PH in an  
96 experimental model. We investigated the expression, localization, and function of SUR1 and Kir6.2 in  
97 iPAH and hPAH patients without *ABCC8* mutations. Moreover, this approach was performed in three  
98 different experimental models of PH. We measured the role of SUR1/Kir6.2 channels in pulmonary  
99 arterial tone using organ bath studies in isolated PA from non-PAH patients, iPAH patients and PA from  
100 control rats and the well-established monocrotaline (MCT)-induced PH rat model (MCT-PH).  
101 Additionally, we evaluated the effect of specific pharmacological activation of SUR1 on the  
102 development of experimental PH.

### 103 **Materials and methods**

104 Because of space limitations, a detailed description of the Materials and Methods is presented in the  
105 Online Supplement.

### 106 **Results**

107 **The SUR1/Kir6.2 channel is expressed in lungs and PA from PAH patients.** To assess the relative  
108 quantity of SUR1 and Kir6.2 in lung tissues, we performed Western blots using proteins from control  
109 and iPAH patients (Figure 1A). We found that the SUR1 and Kir6.2 proteins were expressed at the same  
110 levels in iPAH and control lung tissues (Figure 1A). The protein expression of SUR1 and Kir6.2 was  
111 not different between PA isolated from controls and iPAH patients (Figure 1B). We also found that  
112 SUR1 and Kir6.2 expression in the lungs of hPAH patients (*BMPR2* mutations) was the same as that in  
113 the lungs of controls (Supplemental Figure 1A-B). Surprisingly, we found that the mRNA levels of

114 *ABCC8* and *KCNJ11* (coding for Kir6.2) were increased in the lungs of iPAH and hPAH patients  
115 compared to those of controls (Supplemental Figure 1C). We found that the mRNA levels of *ABCC8*  
116 and *KCNJ11* were unchanged in PA isolated from iPAH compared to controls (Supplemental Figure  
117 1D).

118 Then, we analysed the localisation of SUR1 and Kir6.2 by immunohistochemistry in paraffin-embedded  
119 lung sections of controls and iPAH patients (Figure 1C-D). We found in both controls and iPAH patients  
120 that SUR1 and Kir6.2 staining labelled pulmonary arterial endothelial cells (PAECs; blue arrow) and  
121 PSMCs (yellow arrow) (Figure 1C-D). Positive controls of SUR1 and Kir6.2 staining were used to  
122 validate the specificity of the primary antibodies in human pancreas tissues (Supplemental Figure 2). In  
123 human lung sections, rabbit pre-immune serum incubation gave negative immunostaining as presented  
124 in Supplemental Figure 2. Coimmunostaining against SUR1 and Kir6.2 in control and PAH PA  
125 confirmed that both proteins were colocalized, as highlighted by white arrows (Figure 1E-F).

126 **The SUR1/Kir6.2 channel is expressed in human PA endothelial cells (hPAECs) and PA smooth**  
127 **muscle cells (hPSMCs).** As immunostaining is not quantitative, we used Western blot analysis to  
128 quantify the expression of SUR1 and Kir6.2 proteins in primary hPAECs and primary hPSMCs from  
129 control and iPAH patients. As illustrated in Figure 2A-D, SUR1 and Kir6.2 proteins were expressed in  
130 cultured control and iPAH hPAECs as well as hPSMCs. Compared to control-PSMCs, SUR1 protein  
131 expression was unchanged, while Kir6.2 protein expression was increased in iPAH-hPSMCs (Figure  
132 2A-B). Compared to control-hPAECs, SUR1 protein expression was unchanged, while Kir6.2 was  
133 decreased in iPAH-hPAECs (Figure 2C-D). Therefore, we evaluated the role of SUR1 in hPSMCs and  
134 hPAECs proliferation following pharmacological activation of SUR1 with diazoxide for 24 hours.  
135 Interestingly, we found that pharmacological activation of SUR1 reduced the proliferation rate of  
136 control-hPAECs and control-hPSMCs without altering the proliferation of iPAH-hPAECs and iPAH-  
137 hPSMCs (Figure 2E-F).

138 Since diazoxide was also demonstrated to activate the SUR2A/Kir6.1 or SUR2B/Kir6.1 channel, we  
139 evaluated whether hPAECs and hPSMCs expressed these proteins. We found that SUR2A, SUR2B,  
140 and Kir6.1 were also expressed in control and iPAH conditions (data not shown). Supplementary

141 experiments are needed to decipher the role of the SUR2/Kir6.1 channel in the pulmonary vasculature  
142 and the pathophysiology of PAH.

143 Next, we evaluated the consequence of SUR1 activation on PA relaxation in control human patients  
144 (Figure 2G). PA were precontracted by applying 1  $\mu\text{mol/L}$  U46619 (Thromboxane A2 mimetic). We  
145 found that the activation of SUR1 by diazoxide induced PA relaxation (by  $16.50\% \pm 4.82$ ) compared  
146 with PA treated with DMSO ( $1.94 \pm 1.17$ ). These results demonstrate that SUR1/Kir6.2 is functionally  
147 expressed in controls PA and contributes to the regulation of PA tone.

148 **Pharmacological modulation of the SUR1/Kir6.2 channel modulates rat and human PA tone** We  
149 assessed the role of SUR1/Kir6.2 in control rats PA tone using a pharmacological activator of SUR1,  
150 diazoxide, and an inhibitor of Kir6.2, nateglinide (Figure 3). Nateglinide inhibits SUR1/Kir6.2 channels  
151 with a high affinity for SUR2A/Kir6.2 and low affinity for SUR2B/Kir6.2 channels (13). As expected,  
152 the contractile response of PA in response to increasing doses of KCl (from 10 to 90 mmol/L) was  
153 significantly shifted to the right when PA were preincubated with diazoxide, showing a delay of PA  
154 contraction compared to the PA contraction recorded under DMSO conditions (Figure 3A), indicated  
155 by the increase in Hill slope values (Figure 3B) and EC50 values (Figure 3C). The contractile responses  
156 of PA in response to increasing doses of U46619 (from 0.1 to 3  $\mu\text{mol/L}$ ) was significantly shifted to the  
157 right when PA were preincubated with diazoxide (Supplemental Figure 3A).

158 Next, we incubated PA with nateglinide before the contractile dose-response to U46619 (Figure 3D). In  
159 the presence of nateglinide, we recorded a significant decrease in the Hill slope values (Figure 3E) and  
160 a significant decrease in the EC50 values (Figure 3F). However, the contractile response of PA in  
161 response to increasing doses of KCl was not affected by the preincubation with nateglinide  
162 (Supplemental Figure 3B). These data suggest that the pharmacological inhibition of SUR1 favors PA  
163 constriction in control rat PA.

164 Then, we measured SUR1-dependent PA relaxation in rat control isolated PA (Figure 3G-H). PA were  
165 contracted with U46619 (1  $\mu\text{mol/L}$ ). After 15 min (stable contraction), we added DMSO with basal  
166 relaxation of PA for 10 min ( $9\% \pm 2.5$  of relaxation), and then we added diazoxide for 10 min. Diazoxide  
167 application produced significant PA relaxation in control rat PA ( $48\% \pm 6.3$ ) (Figure 3G-H). These data  
168 suggest that the pharmacological activation of SUR1 leads to relaxation of the control rat PA. At the

169 end of the experiments, the vitality of our PA was checked by an application of 100 mmol/L of KCl  
170 (Supplemental Figure 3C).

171 Interestingly, the relaxation produced by diazoxide was similar to the relaxation mediated by  
172 acetylcholine (Supplemental Figure 3D-F).

173 To determine the relative involvement of PAECs expressing SUR1 and PASMCs expressing SUR1 on  
174 PA relaxation, we evaluated the consequence of SUR1 activation on PA relaxation with or without  
175 endothelium. As presented in Supplemental Figure 4, in the absence of endothelium and after pre-  
176 constriction of PA with U46619, the relaxation mediated by diazoxide is reduced by 20% (Supplemental  
177 Figure 4A), suggesting that the endothelial SUR1 also contributes to PA arterial tone.

### 178 **Pharmacological activation of SUR1 produces PA relaxation in MCT-PH rats and PAH patients.**

179 We also measured the SUR1 and Kir6.2 protein levels in lung samples from MCT-PH rats (Figure 4A-  
180 B). In contrast to the human lung, we found that Kir6.2 and SUR1 expression was increased in MCT-  
181 PH rats compared to control rats. As to control PA, we found that the contractile response to increasing  
182 doses of KCl (from 10 to 90 mmol/L) in isolated PA from MCT-PH rats was significantly shifted to the  
183 right in the presence of diazoxide (Figure 4C), as indicated by increased Hill slope values (Figure 4D)  
184 and EC50 values (Figure 4E). In response to increasing doses of U46619, the PA contractile response  
185 from MCT-PH rats was significantly shifted to the left compared to control PA (Figure 4F). In PA from  
186 MCT-PH rats, in the presence of nateglinide, the contractile dose-response to U46619 was not  
187 significantly shifted to the left (Figure 4F-H).

188 We also measured the consequence of SUR1 activation on the relaxation of PA from MCT-PH rats  
189 (Figure 4I). After induction of contraction by 1  $\mu\text{mol/L}$  U46619, we added DMSO for 10 min ( $2.7\% \pm$   
190  $1.90$  relaxation) or diazoxide at 100  $\mu\text{mol/L}$ . After applying diazoxide, we measured significant PA  
191 relaxation (by  $43\% \pm 8.8$  in MCT-PH and  $48\% \pm 6.3$  in control) compared to DMSO. The diazoxide-  
192 mediated PA relaxation was similar in control and MCT-isolated PA (Figure 4I). These data show that  
193 SUR1 is functionally expressed in PA from MCT-PH rats, and pharmacological activation of  
194 SUR1/Kir6.2 is efficient for PA relaxation in MCT-PH rats.

195 Next, we evaluated the consequence of SUR1 activation on PA relaxation in iPAH patients (Figure 4J).

196 We found that diazoxide induced the same relaxation of controls PA than in iPAH-PA. These results

197 suggest that SUR1/Kir6.2 contributes to the regulation of PA tone in iPAH patients. Using supplemental  
198 myograph experiments that confirmed PA vasoconstriction in iPAH patients, we found that the  
199 contractile dose-response to KCl was strongly shifted to the left in PA from iPAH patients compared to  
200 PA isolated from controls (Supplemental Figure 4B).

201 **Expression of SUR1/Kir6.2 in the heart from MCT induced-PH rats and PAH patients** -Because  
202 SUR1 and Kir6.2 are expressed in the heart, we performed RTqPCR and Western blot experiments to  
203 measure the expression of *ABCC8/SUR1* and *KCNJ11/Kir6.2* in hearts from MCT-PH rats. The  
204 expression of SUR1 and Kir6.2 were decreased at mRNA and proteins levels in RV tissues from MCT-  
205 PH rats compared to control RV tissues (Supplemental Figure 5A-B). In the LV, we found that *Abcc8*  
206 and *Kcnj11* mRNA were unchanged from MCT-PH rats compared to controls (Supplemental Figure  
207 5C), while SUR1 and Kir6.2 protein levels were decreased in LV from MCT-PH rats compared to  
208 control LV (Supplemental Figure 5D). From RTqPCR, we found a similar expression of *KCNJ11* in the  
209 RV of PAH patients while we found a decrease in *ABCC8* in the RV of PAH patients compared to  
210 controls (Supplemental Figure 5E). In the LV, the *KCNJ11* and *ABCC8* mRNA levels were not  
211 significantly different between PAH patients and controls (Supplemental Figure 5F). However, these  
212 results do not rule out a possible regulation of functional SUR1 and Kir6.2 proteins.

213 ***In vivo* diazoxide treatment interferes with the development of PH induced by MCT and by**  
214 **chronic hypoxia exposure.** The present study found that SUR1 was still expressed in the lungs and PA  
215 from iPAH patients and MCT-PH rats. Therefore, we next evaluated the consequences of *in vivo*  
216 pharmacological activation of the SUR1 channel in MCT-PH rats (Figure 5). We administered diazoxide  
217 20 mg/kg/day *in vivo* from days 1 to 21 to MCT-PH rats (Figure 5A). We found an improvement in the  
218 RV systolic pressure (RVSP) (Figure 5B), cardiac output (CO -Figure 5C), PVR evaluated by  
219 calculating the RVSP/CO ratio (Figure 5D) and RV hypertrophy (evaluated by Fulton index values)  
220 (Figure 5E) compared to MCT-vehicle (DMSO) rats. Diazoxide treatment also reduced aberrant wall  
221 thickness of muscularized pulmonary vessels induced by MCT exposure in rats (Figure 5F). However,  
222 in MCT diazoxide rats, we found a reduction in pulmonary neomuscularization, as attested by the  
223 increased non-muscularized vessels and decreased muscularized vessels in MCT diazoxide rat's vs

224 MCT DMSO rats (Figure 5G). The LV stroke volume, systemic blood pressure measured in the carotid  
225 artery, and heart rate were unchanged between each experimental group (Supplemental Figure 6A-C).  
226 Moreover, when administered *in vivo* from days 14 to 21 to MCT-PH rats (Figure 5H), diazoxide  
227 significantly improved CO (Figure 5I) and the RVSP/CO ratio (Figure 5J) but not the RVSP (Figure  
228 5K) or Fulton index (Figure 5L) or pulmonary vessel wall thickness (Figures 5M). Importantly, we  
229 found in MCT diazoxide rats a reduction of pulmonary neomuscularization vs MCT DMSO rats (Figure  
230 5N)

231 The LV stroke volume, systemic blood pressure measured in the carotid artery, and HR were unchanged  
232 between MCT DMSO and MCT diazoxide groups (Supplemental Figure 6D-F).

233 Together, these results demonstrate that diazoxide treatment reduces the development of PH in MCT-  
234 exposed rats.

235 Next, we measured lung expression of SUR1 and Kir6.2 proteins in chronic hypoxia (CH)-exposed rats.  
236 Kir6.2 protein expression was unchanged in the lungs of CH-exposed rats compared to control rats,  
237 while SUR1 expression was decreased (Figure 5O). We evaluated the consequences of *in vivo*  
238 pharmacological activation of the SUR1 channel in CH-PH rats (Figure 5P). We administered 20  
239 mg/kg/day of diazoxide *in vivo* from days 14 to 21 to CH-PH rats, and we found only an improvement  
240 in the RVSP (Figure 5Q). The CO was similar in all three experimental groups (Figure 5R). The PVR  
241 and RV hypertrophy (evaluated by Fulton index values) were not significantly reduced (Figure 5S-T)  
242 compared to MCT-vehicle (DMSO) rats. At the pulmonary vascular level, short diazoxide treatment had  
243 no consequence on the aberrant pulmonary vessel wall thickness (Figures 5U) but reduced pulmonary  
244 vessel neomuscularization induced by CH in rats (Figure 5V).

245 The systemic blood pressure measured in the carotid artery and HR were unchanged between each CH  
246 DMSO and CH diazoxide group (Supplemental Figure 7A-B). We obtained similar results when  
247 diazoxide was administered *in vivo* from days 1 to 21 to CH-PH rats with an improvement of RVSP and  
248 pulmonary vessels wall thickness as well as a reduction of pulmonary vessel neomuscularization  
249 (Supplemental Figure 7C-K). Together, these results suggest that diazoxide treatment interferes with the  
250 development of PH in CH-exposed rats, mainly by regulating PA tone.

251 Additionally, we measured lung expression of SUR1 and Kir6.2 proteins in Sugen/Hypoxia (Su/Hx)-  
252 exposed rats. Kir6.2 and SUR1 protein expression were unchanged in the lungs of SU/Hx-exposed rats  
253 compared to control rats (Supplemental Figure 8).

254 Since SUR1 was also found to co-assemble with TRPM4, we analyzed the expression of TRPM4 protein  
255 in the lungs. As shown in Supplemental Figure 9, lung expression of TRPM4 was unchanged in iPAH  
256 or hPAH compared to controls (Supplemental Figure 9A) and in MCT-PH or CH-PH rats compared to  
257 control rats (Supplemental Figure 9B-C).

258 ***In vivo* diazoxide treatment is safe at the cardiovascular level in healthy rats.** In the literature, some  
259 reports have indicated that diazoxide treatment in young hyperinsulinism patients induced significant  
260 increases in mean PA pressure(14). To test the safety of diazoxide treatment on control rats, we  
261 chronically exposed control rats to 20 mg/kg/d for 3 weeks (Figure 6A). We found that *in vivo* chronic  
262 activation of SUR1 had no pulmonary vascular consequence, as indicated by similar RVSP, CO, PVR,  
263 Fulton index, and pulmonary vascular cell proliferation (EdU-incorporated cells) (Figure 6B-F).  
264 Pulmonary vessel muscularization was not statistically different; however, the percentage of  
265 muscularized vessels tended to be higher in the diazoxide-treated group, suggesting that diazoxide  
266 treatment could favor pulmonary vessel neomuscularization (Figure 6G-I). With echocardiography, we  
267 did not observe any difference in right and left heart functions (Table 1). LV thickness was reduced in  
268 diazoxide-treated animals without any consequence to LV fractional shortening and LV stroke volume.  
269 These results indicate that right and left heart function were unaffected by *in vivo* SUR1 activation by  
270 chronic exposure to diazoxide (Table 1). The systemic blood pressure measured in the carotid artery and  
271 HR were similar in DMSO- and diazoxide-exposed rats (Figure 6J and Table 1). After 2 weeks of  
272 diazoxide exposure, our data suggest the relative safety of SUR1 activation by diazoxide in the  
273 pulmonary vasculature and heart in healthy rats. However, the fact that we found insignificant increases  
274 in pulmonary vessel neomuscularization and a reduced LV thickness in diazoxide-treated rats highlights  
275 the need to monitor diazoxide side effects carefully.

276 **Additional SUR1 activators (VU0071063 or NN414) produce important PA relaxation and reduce**  
277 **the development of PH induced by MCT exposure.**

278 It was demonstrated that diazoxide has several additional effects besides acting as a SUR1/Kir6.2  
279 channel opener, including activation of SUR2, alteration of other ion channels and ATPases functions,  
280 and direct regulation of mitochondrial energetics (15–18). To avoid misinterpretation about the  
281 diazoxide effect on SUR1/Kir6.2, we performed an additional experiment with more selective activators  
282 of SUR1/Kir6.2 channel: VU0071063 and NN414. Due to their higher selectivity for SUR1/Kir6.2  
283 channels, both molecules were recently proposed by Sikimic et al. as possible new strategies for treating  
284 congenital hyperinsulinism instead of diazoxide (19).

285 With the additional SUR1 activators, we measured the consequence of SUR1 activation on the relaxation  
286 of PA from control rats. After induction of contraction by 1  $\mu\text{mol/L}$  U46619, we applied increasing  
287 concentrations (100 nmol/L to 100  $\mu\text{mol/L}$ ) of VU0071063, of NN414, of diazoxide or DMSO. As  
288 shown in Figure 7A, the PA contraction is similarly reduced by increasing VU0071063 or NN414 or  
289 diazoxide concentration, indicating that SUR1 activators mediated PA relaxation as attested by the  
290 decrease in PA contraction (Figure 7B).

291 Importantly in isolated aorta from control rat, we found a significant difference in aorta relaxation  
292 induced by SUR1 activators (diazoxide, VU0071063, or NN414) and the SUR2 activator (pinacidil). As  
293 presented in Figure 7C, we found that the SUR2 activator pinacidil produced aorta relaxation (effective  
294 dose 50,  $\text{ED}_{50} = 1.3 \mu\text{mol/L}$ ) compared to SUR1 activators (55  $\mu\text{mol/L}$ , 66  $\mu\text{mol/L}$  and 230  $\mu\text{mol/L}$  for  
295 diazoxide, VU0071063, and NN414, respectively). These results indicated that SUR1 plays a crucial  
296 role in pulmonary arterial tone (by three different molecules) compared with his role in regulating aorta  
297 tone.

298 Finally, we evaluated the consequences of *in vivo* pharmacological activation of the SUR1 channel by  
299 NN414 in MCT-PH rats (Figure 7D). We administered 1.5 mg/kg/day NN414 twice daily to MCT-PH  
300 rats from days 14 to 21. We found an improvement in the RVSP (Figure 7E), CO (Figure 7F), PVR  
301 (Figure 7G), and RV hypertrophy (Figure 7H). Similarly, with brief diazoxide treatment, the pulmonary  
302 vessel wall thickness remained unchanged in the MCT-NN414 group compared to the MCT-DMSO  
303 group (Figure 5M and 7I). In contrast, the pulmonary vessel neomuscularization was reduced by the  
304 NN414 treatment (Figure 7J). The systemic blood pressure measured in the carotid artery and HR were  
305 unchanged in each experimental group (Supplemental Figure 10A-B).

306 **Discussion**

307 Using a combination of biochemical and *ex vivo* and *in vivo* approaches, we have provided 5 significant  
308 findings regarding the expression/function of SUR1/Kir6.2 in the context of PAH. First, SUR1 and  
309 Kir6.2 are expressed in human PSMCs and PAECs. Second, SUR1/Kir6.2 expression and function  
310 (evaluated by myograph experiments) were not reduced in the lungs and PA of PAH patients (iPAH or  
311 hPAH) or three experimental models of PH (MCT-PH, CH-PH, and Sugen/hypoxia). Third, the use of  
312 three different SUR1 activators (diazoxide, VU0071063, and NN414) showed that the SUR1/Kir6.2  
313 channel modulates PA tone similarly in humans and rats in controls and PH conditions. Fourth,  
314 pharmacological activation of SUR1 with diazoxide has no consequence on pulmonary circulation in  
315 healthy rats. Finally, *in vivo* pharmacological activation of SUR1 (by diazoxide and NN414) reduces  
316 the development of PH in the MCT-PH rat model.

317 **Composition of KATP channels in the pulmonary circulation**

318 KATP channels are octameric complexes characterized by four pore-forming Kir channel (Kir6.1 or  
319 Kir6.2) subunits associated with four regulatory SUR1 or SUR2 subunits. SUR2B is ubiquitously  
320 expressed, while the alternatively spliced variant SUR2A is exclusively expressed in the heart(20).  
321 SUR1 expression is prominent in neuronal and pancreatic tissues and many other tissues, including  
322 cardiac, atria, and lung tissues(6, 21, 22). Previous work suggested that in systemic vascular smooth  
323 muscle and endothelial cells, KATP channels are predominantly constituted by SUR2 and not by SUR1.  
324 However, as Bohnen et al. (6) suggested, we show that PSMCs and PAECs express SUR1 and Kir6.2.  
325 We also found that pharmacological activation of SUR1 produces significant PA relaxation in controls  
326 and PAH patients and PA from MCT-PH and control rats. In PSMCs, KATP channels, similar to other  
327 K<sup>+</sup> channels, contribute to the maintenance of membrane potential. In addition, KATP closure should  
328 lead to an increase in intracellular Ca<sup>2+</sup> concentration, enhancing PSMCs contraction. In contrast,  
329 KATP opening increases K<sup>+</sup> efflux, leading to membrane hyperpolarization, inhibition of Ca<sup>2+</sup> influx,  
330 and PSMC relaxation. KATP may be activated by NO and endothelium-derived hyperpolarizing factor  
331 (EDHF)(23). Although KATP is understudied in PAEC, KATP channels are regulated by shear stress  
332 and are responsible for maintaining resting membrane potential in bovine PAEC(24). In this study, the  
333 use of a Kir6.2 inhibitor (nateglinide) demonstrated that Kir6.2 inhibition facilitates PA constriction,

334 similar to the role of Kir6.1 described in systemic vascular tissues(25). These results indicate that the  
335 SUR1/Kir6.2 channel contributes to the regulation of PA tone and that *ABCC8* loss-of-function  
336 mutations should initiate PAH via an alteration of PA tone and a reduction in PA relaxation. Thus far,  
337 no abnormal cardiovascular phenotype has been reported in *sur1*<sup>-/-</sup> mice, while *kir6.1*<sup>-/-</sup> or *sur2*<sup>-/-</sup> mice  
338 developed hypertension and coronary vascular disorders(26, 27).

339 SUR1 subunits are mostly co-assembled with Kir6.2; however, in the adult mouse hippocampus,  
340 endogenous SUR1 may interact with Kir6.1, suggesting that they form functional SUR1/Kir6.1- KATP  
341 channels(28, 29). As Kir6.2 expression, we found that Kir6.1 expression was unchanged in the lungs of  
342 iPAH patients or experimental PH models. In addition, we found that both SUR1 and Kir6.2 are  
343 expressed in hPAECs and hPASCs from control and iPAH patients. Interestingly, we found that  
344 pharmacological activation of SUR1 reduced the proliferation rate of hPAECs from control and iPAH,  
345 while SUR1 activation did not affect iPAH-PASCs proliferation. These results indicated that the role  
346 of SUR1 in pulmonary arteries is mainly related to the regulation of pulmonary arterial tone. Moreover,  
347 myograph experiments performed with or without endothelium suggest that the endothelial SUR1  
348 function contributes to almost 20 % of the PA relaxation mediated by diazoxide.

349 SUR1 was also reported to interact with and regulate TRPM4 channels(30). TRPM4 is known to be  
350 expressed in PASCs(31), and we found that TRPM4 expression in the lungs of iPAH and hPAH  
351 patients and several experimental models of PH was unchanged. However, we could reasonably propose  
352 that SUR1-TRPM4 assembly occurs in the pulmonary vasculature and that *ABCC8* loss-of-function  
353 mutations could also modify TRPM4 function in pulmonary vascular cells.

#### 354 **The function of SUR1/Kir6.2 in the right ventricle**

355 SUR1 and Kir6.2 are expressed in the human heart(32). However, there is no strong evidence regarding  
356 the contribution of SUR1/Kir6.2 in the regulation of KATP in human ventricular myocytes. In mice,  
357 SUR1 is not an essential component of the ventricular KATP channel but is necessary for the atrial  
358 KATP channel. The recombinant SUR2A/Kir6.2 channels were shown to contribute to the ventricular  
359 KATP channel(33). - Mice overexpressing SUR1 develop arrhythmias, including atrial fibrillation and  
360 sudden death(34), while hearts from *sur1*<sup>-/-</sup> mice have an improved functional recovery after  
361 ischemia(35). Kir6.2 subunit contributes to the depression of cardiac excitability and contractility

362 induced by the KATP channels opener(36). *Kir6.2*<sup>-/-</sup> mice have significant cardiac metabolic  
363 abnormalities, such as increased glycogen storage and decreased glycolysis(37). In mice, *kir6.2*  
364 deficiency reduces cardiac recovery after exercise and causes arrhythmias and sudden death after  
365 sympathetic overactivation(38). However, *kir6.2* lack does not alter cardiac function under basal aerobic  
366 conditions(36). Here we found that the mRNA level of *ABCC8* is reduced in RV tissues from PAH  
367 patients and RV from MCT-PH rats. In contrast, *Kcnj11* is decreased only in the RV from MCT-PH rat,  
368 contributing to abnormal PH-RV cardiomyocytes' excitability, contractility, and metabolism(39, 40). In  
369 LV tissues, we found unchanged mRNA expression (*Abcc8* and *Kcnj11*), while protein expression is  
370 reduced in LV from MCT rats. Therefore, we can hypothesize that transcriptional/translational  
371 mechanisms negatively regulate SUR1 and Kir6.2 protein levels. Due to the limited quantities of human  
372 cardiac tissues, unfortunately we cannot perform Western blot analysis on human hearts. Despite the  
373 existence of *sur1* or *kir6.2* deficient mice, no study has assessed the consequence of *sur1* or *kir6.2*  
374 deletion in pulmonary vascular or RV levels. Future studies are needed to investigate the pulmonary  
375 phenotype of *sur1*<sup>-/-</sup> or *kir6.2*<sup>-/-</sup> mice at baseline and after exposure to PH-inducers.

### 376 **Unchanged SUR1 expression in PAH pulmonary circulation contrary to other outward K<sup>+</sup>** 377 **channels: a good therapeutic target?**

378 Even though *ABCC8* loss-of-function mutations have been identified in some PAH patients(6), we found  
379 unchanged expression of SUR1 and Kir6.2 in lungs from PAH patients, which is unusual for K<sup>+</sup> channels  
380 in PAH. Indeed, most voltage-gated K<sup>+</sup> channels, including Kv1.1, Kv1.2, Kv1.5, Kv2.1, Kv4.3, and  
381 Kv9.3, have decreased expression in PAH lungs(23). Moreover, 16 different loss-of-function mutations  
382 in *KCNK3* genes have also been identified in PAH patients(23, 41), and we demonstrated that *KCNK3*  
383 (or TASK-1) loss of expression/function is a hallmark of PAH and experimental PH(42). These different  
384 observations highlight the great difficulty of pharmacologically activating K<sup>+</sup> channels that are no longer  
385 expressed in PAH (Kv channels or *KCNK3*), which means that the activation of SUR1 is a great  
386 therapeutic opportunity for PAH since we found that SUR1 is correctly expressed in PAH.

### 387 **SUR1 activation by diazoxide**

388 *ABCC8* loss-of-function mutations cause congenital hyperinsulinism(43), and these patients are  
389 currently treated with diazoxide. In this study, we investigate the consequences of the pharmacological

390 SUR1 activation by diazoxide. Diazoxide is the only U.S. Food and Drug Administration–approved  
391 drug to treat hyperinsulinaemic hypoglycaemia(44). Acute SUR1 activation by diazoxide application is  
392 described to strongly reduce PAPm in some PAH patients(45, 46). Here, we show in human and  
393 experimental PH models that acute SUR1 activation produced substantial PA relaxation.  
394 Moreover, we found that long-term *in vivo* SUR1 activation treatment reduced the development of PH  
395 with reduced RVSP, suggesting that SUR1 activation treatment mainly acts on the regulation of PA tone  
396 via an improvement in PA relaxation. Surprisingly, we found no significant modification of systemic  
397 blood pressures in animals treated with the SUR1 activators (diazoxide or NN414), while SUR1  
398 activation reduced RVSP in MCT-PH rats mainly by inducing pulmonary arterial relaxation while his  
399 role in the regulation of aorta tone is smaller. As we found that aorta tone is more sensitivity to SUR2  
400 activation than SUR1 activation, our results indicate that SUR2 highly contribute to the regulation of  
401 aorta tone while SUR1 play a key role in the regulation of PA tone, and thus probably explain why  
402 SUR1 activation did not affect systemic blood pressure.

#### 403 **Limitations of the use of diazoxide**

404 Until diazoxide is proven to be safe in PAH, diazoxide is not recommended as a treatment for PAH.  
405 Several reports have indicated that hypoglycaemic infants develop PH secondary to diazoxide  
406 treatment(47, 48), and the discontinuation of diazoxide treatment leads to the resolution of PH. The fact  
407 that diazoxide was found to induce PH in hyperglycaemic infants remains a limitation of the use of  
408 diazoxide in PAH. Recently, Ohnishi et al. suggested a dose threshold for PH caused by diazoxide  
409 treatment and that diazoxide toxicity is influenced mainly by the dose rather than the duration of  
410 administration. Their study indicated that PH-induced by diazoxide would possibly develop higher than  
411 10.4 mg/(kg/day)(49). Based on the analysis, lower doses of diazoxide, less than 10.4 mg/kg/day, should  
412 be used in hyperglycaemic infants. The mechanisms underlying diazoxide-induced PH are unknown,  
413 and additional studies are needed to explain why diazoxide administration induces PH in a small portion  
414 of hyperglycaemic infants (with around 7% of incidence) (47, 48). Adebisi et al. suggested that  
415 diazoxide-induced mitochondrial depolarization independent of SUR1 could have side effects(50).  
416 In contrast, several studies suggested a cardioprotective effect of diazoxide- via an action on the  
417 mitochondrial respiratory chain(51). However, in the present study, we demonstrated diazoxide-induced

418 PA relaxation in PA from PAH patients who did not respond to acute NO administration. Our *in vivo*  
419 results indicate that chronic SUR1 activation improved PH symptoms, suggesting that this strategy  
420 might be helpful for patients with PAH. The fact that we found that diazoxide treatment in control rats  
421 favors pulmonary vessel neomuscularization could partly explain the deleterious effect of diazoxide.  
422 Diazoxide-induced PH is mimicked by people with Cantu syndrome, who have SUR2 gain-of-function  
423 mutations(52). Currently, our study does not support recommending the administration of diazoxide to  
424 PAH patients. Of note, SUR1 and SUR2 proteins are both expressed in the pulmonary vascular cells.  
425 Because diazoxide was found to activate the reconstituted SUR2/Kir6.1(53), we cannot exclude that a  
426 part of diazoxide effects could be due to SUR2 channels activation. Because of the reported diazoxide  
427 side effects, we used two additional SUR1 activators; VU0071063 and NN414. Due to their superior  
428 selectivity for SUR1/Kir6.2 channels, both molecules were recently proposed as possible new strategies  
429 for treating congenital hyperinsulinism by targeting SUR1(19). VU0071063 is selective for  
430 SUR1/Kir6.2 and not for SUR2A/Kir6.2 and SUR2A/Kir6.1 channels, and VU0071063 opens  
431 SUR1/Kir6.2 channels with greater potency than diazoxide(54). NN414 is 100-fold more potent  
432 selective agonist than diazoxide SUR1/Kir6.2 channels(55). *In vitro* and *in vivo* studies demonstrated  
433 the potential of NN414 in the treatment of disorders resulting from excessive insulin release(56–58).  
434 However, VU0071063 seems to act directly on mitochondria, independently of SUR1. Thus, we focused  
435 on NN414 *in vivo*. However, we found that VU0071063 and NN414 produced similar PA relaxation to  
436 diazoxide and that NN414 treatment reduces MCT-PH phenotypes.

437 The SUR1/Kir6.2 and SUR2/Kir6.2 channels display differences in sulfonylurea sensitivity to  
438 diazoxide(59). So, further additional studies are needed to decipher the contribution of SUR2/Kir6.1  
439 channels in PAH pathobiology. Moreover, we found that SUR1/Kir6.2 is expressed in RV and LV, and  
440 since diazoxide treatment may potentially induce cardioprotection (18), we cannot exclude that a part  
441 of diazoxide effects could be due to diazoxide-mediated cardioprotection. Our results suggest that  
442 pharmacological activation of SUR1 could be of value in PAH and suggest that NN414 molecules  
443 should be further assessed as a PAH treatment.

444

445 **Conclusions**

446 In conclusion, we show evidence that the SUR1/Kir6.2 channel contributes to PA tone in humans and  
447 rats. SUR1/Kir6.2 channel expression and contribution to PA tone are maintained in PA from iPAH and  
448 experimental PH. Moreover, we show that *in vivo* SUR1 activation by two different activators (diazoxide  
449 and NN414) is effective at restoring/ameliorating PH in two experimental PH models. Despite the  
450 limitations of diazoxide and reported side effects in hyperglycaemic infants, the present results provide  
451 proof of concept that pharmacological activation of SUR1 should be considered in PAH. Using a more  
452 potent SUR1 activator (NN414), we confirmed that SUR1 could be considered as a pharmacologic target  
453 in PAH. Further evaluation of NN414 is needed to assess safety and efficacy in PAH.

454

455

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470

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651 2000;275:717–720.  
652

653 **Table 1: Evaluation of RV and LV function by echocardiography measured in diazoxide- or DMSO-**  
654 **treated rats.** HR: Heart rate, RVET: RV ejection time, PAAT: PA acceleration time, VTI: velocity-time  
655 **integral, RV EDd: RV end-diastolic diameter, RV Eds: RV end-systolic diameter, RV FS: RV fractional**  
656 **shortening, LV EDd: LV end-diastolic diameter, LV Eds: LV end-systolic diameter, LV FS: LV fractional**  
657 **shortening. ns: non-significant. Two-tailed unpaired Student's t-tests assessed the difference between**  
658 **two groups.**

	<b>DMSO n=6</b>	<b>diazoxide n=6</b>
<b>HR (bpm)</b>	402.6 ± 16.6	397.5 ± 7.6
<b>RVET (ms)</b>	76.7 ± 2.4	78.2 ± 1.6
<b>PAAT (ms)</b>	32.5 ± 0.7	34.8 ± 0.9
<b>VTI Pulmonary Artery (mm)</b>	6.98 ± 0.3	6.48 ± 0.39
<b>RV EDd (mm)</b>	3.4 ± 0.3702	4.27 ± 0.14
<b>RV EDs(mm)</b>	1.85 ± 0.27	2.4 ± 0.29
<b>RV thickness (mm)</b>	1.1 ± 0.06	1 ± 0.09
<b>RV FS %</b>	45.4 ± 7	43.8 ± 7.2
<b>VTI aorta (mm)</b>	6.7 ± 0.2	6.7 ± 0.63
<b>LV EDd (mm)</b>	7.1 ± 0.27	7.2 ± 0.19
<b>LV EDs(mm)</b>	3.68 ± 0.35	4 ± 0.2
<b>LV thickness (mm)</b>	1.3 ± 0.05	1.15 ± 0.065 *
<b>LV FS %</b>	48.1 ± 5	44.3 ± 3.2

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670 **Figure legends**

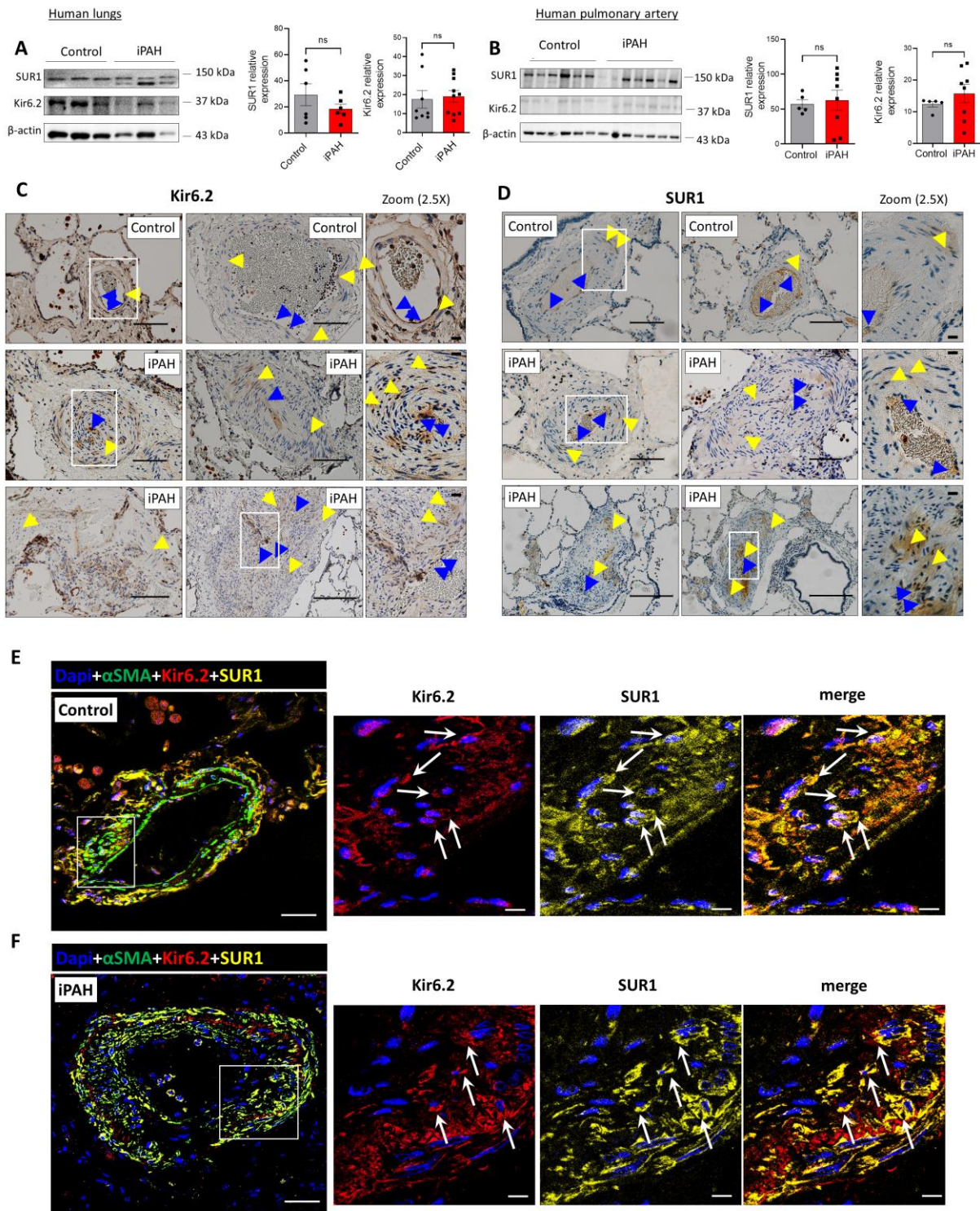


Figure 1

671

672 *Figure 1: Expression of SUR1 and Kir6.2 proteins in PAECs and PSMCs in control and iPAH*

673 *patients.* (A) Representative Western blots of SUR1 and Kir6.2 in lungs from control and iPAH patients

674 and quantification of SUR1 and Kir6.1 expression in lungs from control and iPAH patients (n=9–12).

675 (B) Representative Western blots of SUR1 and Kir6.2 in isolated PA from control and iPAH patients  
676 and quantification of SUR1 and Kir6.1 expression in PA from control and iPAH patients (n=5–7). Ns:  
677 non-significant vs. control (C-D) SUR1 or Kir6.2 immunostaining in paraffin-embedded lung sections  
678 from control and iPAH patients; PAEC (blue arrow) and PASMC (yellow arrow) staining is visible.  
679 Scale Bar 100  $\mu$ m and 30  $\mu$ m. (E-F) Co-immunostaining against Kir6.2, SUR1, and  $\alpha$ SMA (smooth  
680 muscle marker) confirmed the colocalization (white arrow) of Kir6.2 and SUR1 in pulmonary vascular  
681 cells in control and iPAH patients. Scale Bar 50 and 10  $\mu$ m. ns: non-significant. Two-tailed unpaired  
682 Student's t-tests assessed the difference between two groups.  
683

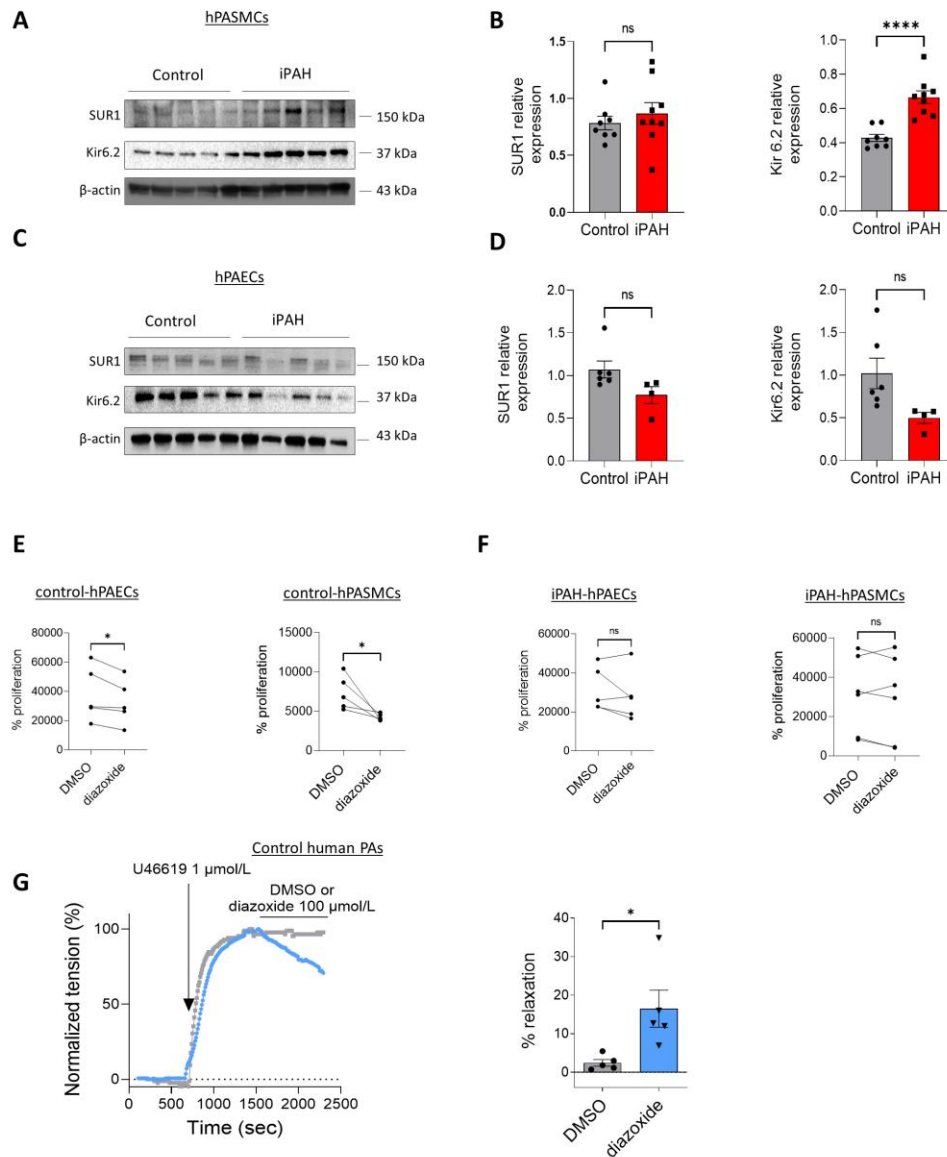


Figure 2

684

685 **Figure 2: The SUR1/Kir6.2 expression in human PA endothelial cells (hPAECs) and PA smooth**  
 686 **muscle cells (hPASMCs).** (A) Representative Western blots of SUR1 and Kir6.2 in hPASMCs from  
 687 control and PAH patients (B) Quantification of SUR1 and Kir6.2 expression in hPASMCs from control  
 688 and iPAH patients (n=5). (C) Representative Western blots of SUR1 and Kir6.2 in hPAECs from control

689 and iPAH patients (D) Quantification of SUR1 and Kir6.2 expression in hPAECs from control and iPAH  
690 patients (n=4–6). (E) Analysis by BrdU assay of the proliferation rate of hPAECs and hPASMCs from  
691 control patients treated with diazoxide (100  $\mu\text{mol/L}$ ) or DMSO. (n = 6-7 patients). (F) Analysis by BrdU  
692 assay of the proliferation rate of hPAECs and hPASMCs from iPAH patients treated with diazoxide  
693 (100  $\mu\text{mol/L}$ ) or DMSO. (n = 6-7 patients). (G) Human PA control precontracted with U46619 (1  
694  $\mu\text{mol/L}$ ) and diazoxide (100  $\mu\text{mol/L}$ ) vs. the same volume of DMSO produced PA relaxation in control.  
695 Corresponding graph showing the percent relaxation 10 min after diazoxide application (n=5 controls).  
696 ns: non-significant. \* $p < 0.05$  \*\*\* $p < 0.0001$ . Two-tailed unpaired Student's t-tests assessed the  
697 difference between two groups.

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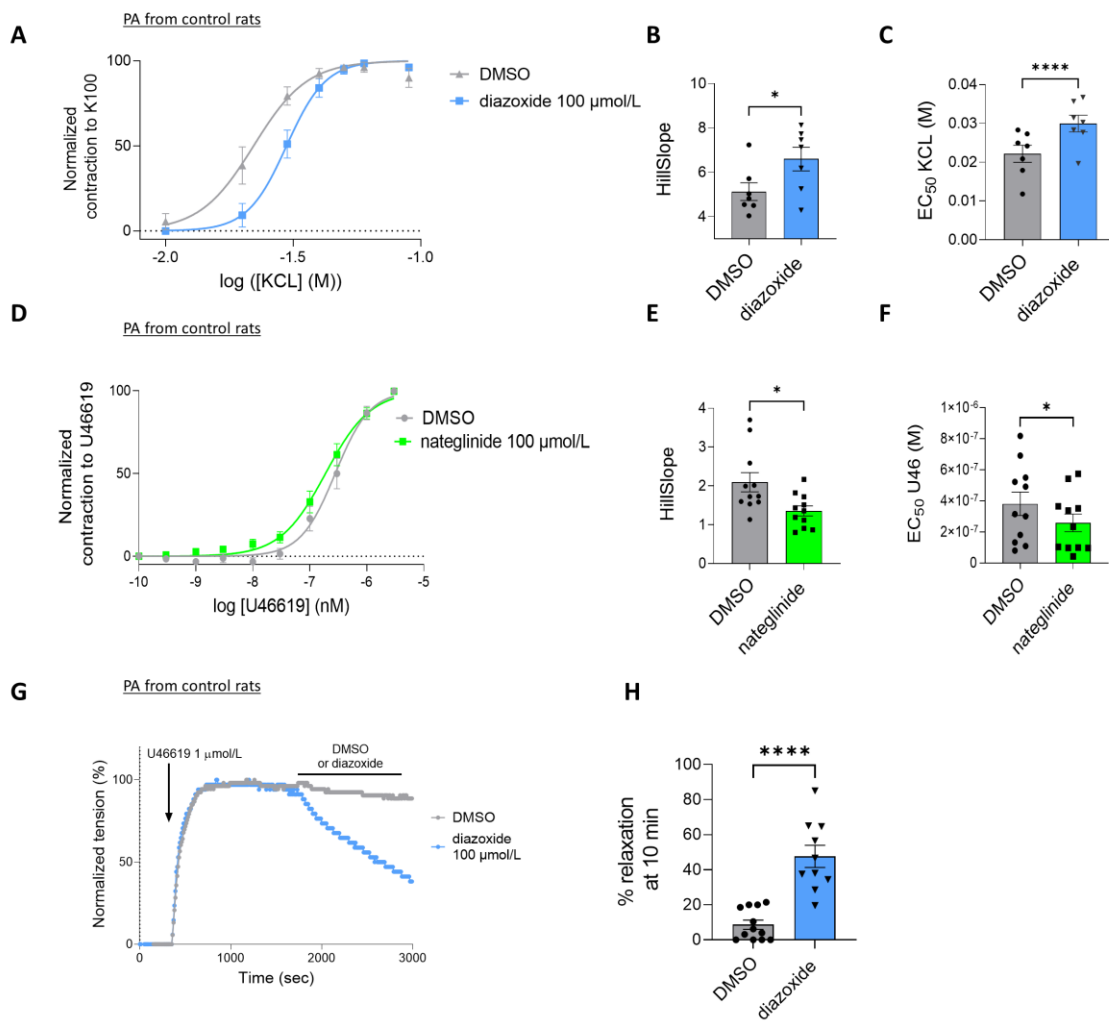


Figure 3

699

700 **Figure 3: Contribution of the SUR1/Kir6.2 channel to the regulation of pulmonary arterial tone in**  
 701 **control rats and human controls.** (A) Dose-response curve (normalized to K100) established by  
 702 applying increasing concentrations of KCl to isolated PA from control rats in the presence of DMSO or  
 703 diazoxide (SUR1 activator, at 100 µmol/L). Corresponding quantification of Hill slope (B) and EC<sub>50</sub>

704 (C) values (n=7 rats). (D) Dose-response curve (normalized to K100) established by applying increasing  
705 concentrations of U46619 (thromboxane A2 mimetic) to isolated PA from control rats in the presence  
706 of DMSO or nateglinide (Kir6.2 channel inhibitor, at 100  $\mu\text{mol/L}$ ). Corresponding quantification of  
707 Hillslope (E) and EC50 (F) values (n=11 rats). (G) Relaxant response to 100  $\mu\text{mol/L}$  diazoxide in PA  
708 from control rats precontracted with U46619 (1  $\mu\text{mol/L}$ ). (H) Corresponding graph showing the percent  
709 relaxation to DMSO or diazoxide at 100  $\mu\text{mol/L}$ . (n=10–12 rats). ns: non-significant.  $*p<0.05$   
710  $****p<0.0001$ . Two-tailed unpaired Student's t-tests assessed the difference between two groups.  
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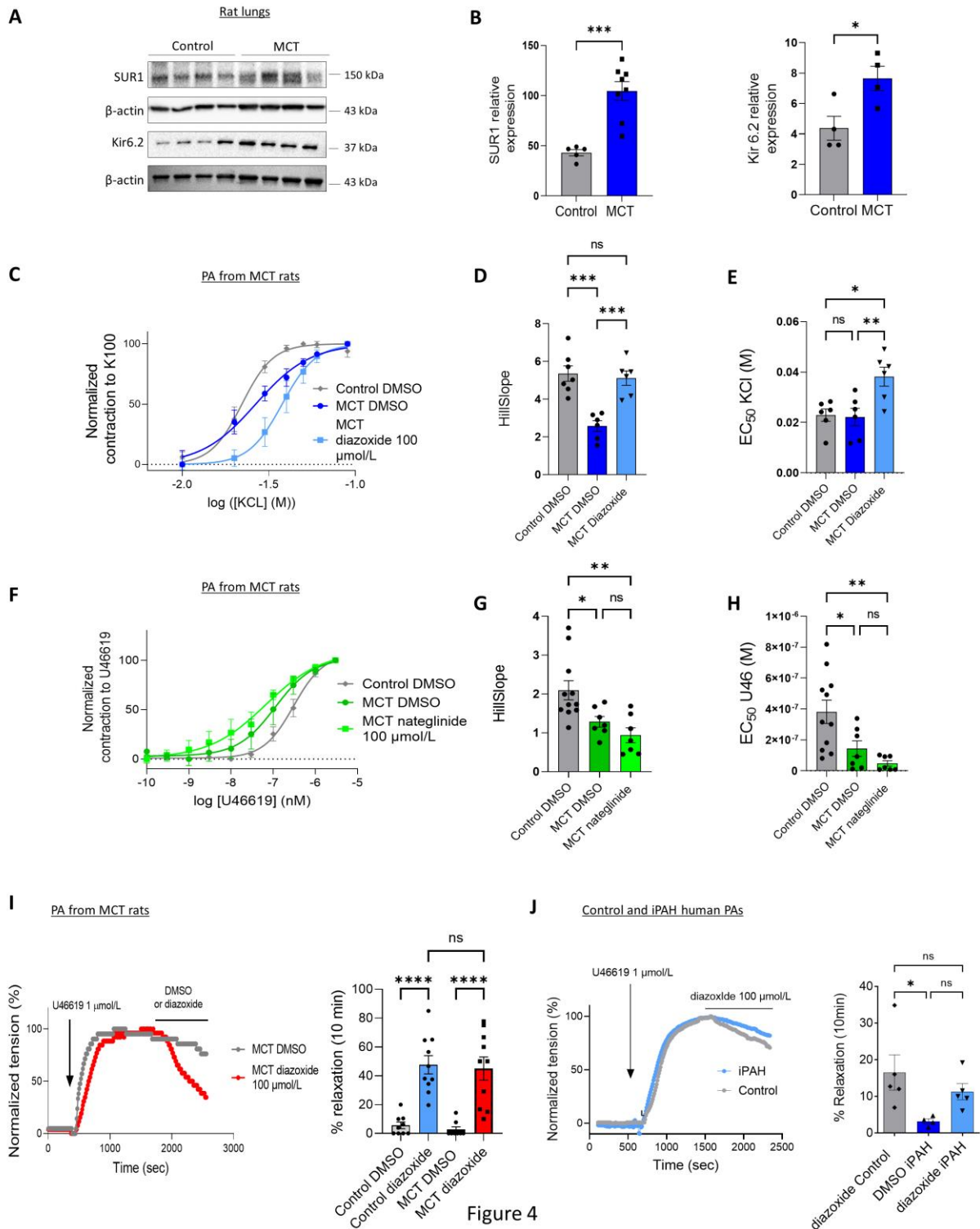
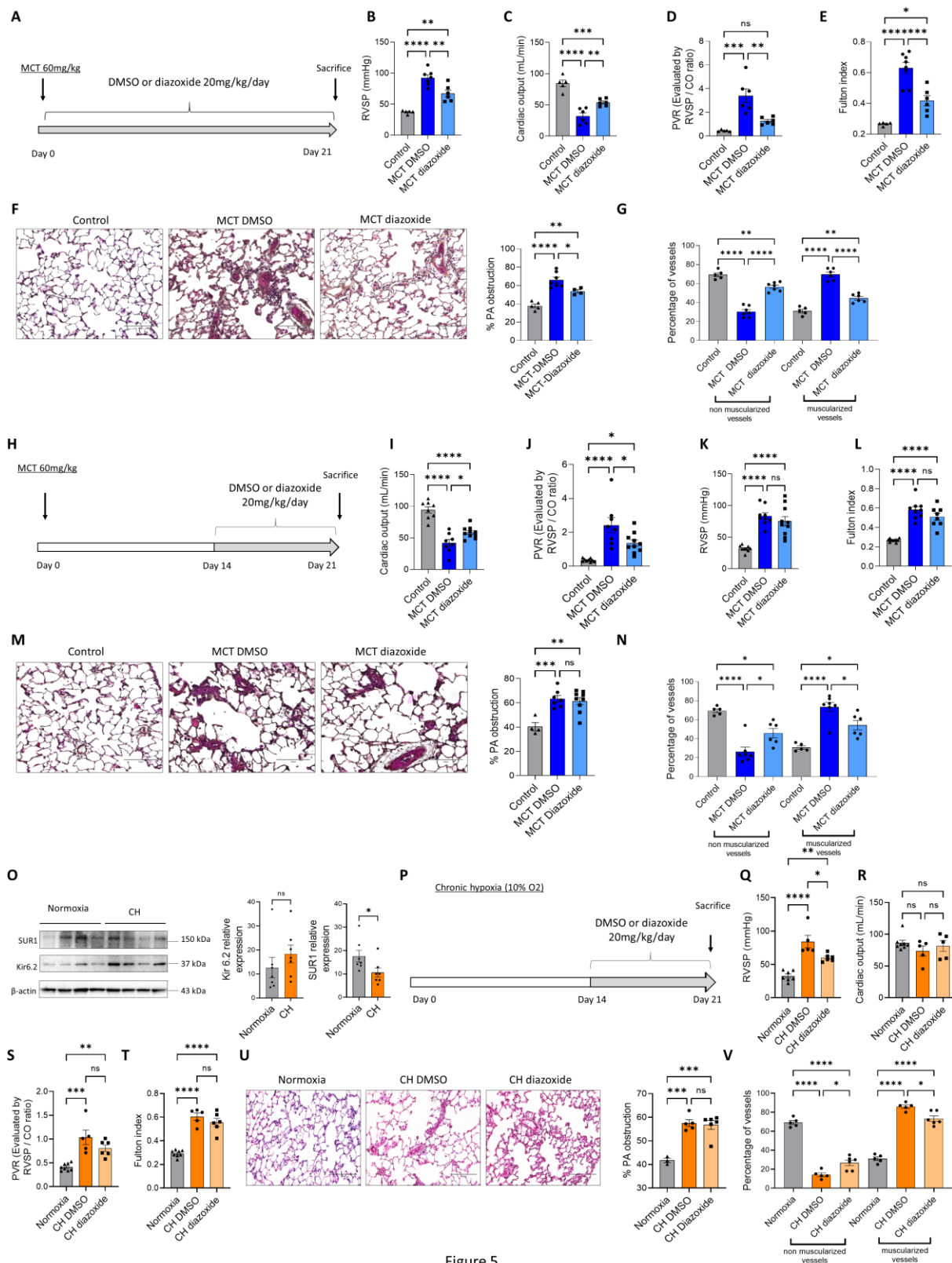


Figure 4

712

713 **Figure 4: Contribution of the SUR1/Kir6.2 channel to the regulation of pulmonary PA tone in MCT-**  
 714 **PH rats and human iPAH patients.** (A) Representative Western blots of SUR1 and Kir6.2 in lungs  
 715 from control and monocrotaline (MCT)-exposed rats (3 weeks). (B) Quantification of SUR1 and Kir6.2  
 716 expression in lungs from control and MCT- rats (n=5–8). (C) Dose-response curve (normalized to K100)

717 established by applying increasing concentrations of KCl to isolated PA from control and MCT-PH rats  
718 in the presence of diazoxide at 100  $\mu\text{mol/L}$ . Corresponding quantification of Hillslope (D) and EC50  
719 (E) values (n=6 rats). (F) Dose-response curve (normalized to K100) established by applying increasing  
720 concentrations of U46619 (thromboxane A2 mimetic) to PA isolated from control and MCT-PH rats in  
721 the presence of nateglinide (Kir6.2 channel inhibitor, at 100  $\mu\text{mol/L}$ ). Corresponding quantification of  
722 Hillslope (G) and EC50 (H) values (n=7 rats). (I) Graph showing the percent relaxation to diazoxide at  
723 100  $\mu\text{mol/L}$  in MCT-PH rats (n=8–10 rats) and corresponding graph showing the percent relaxation to  
724 DMSO or diazoxide in control and MCT rats. (n=10–12 rats). (J) Human PA control and iPAH PA  
725 precontracted with U46619 (1  $\mu\text{mol/L}$ ) and showing diazoxide (100  $\mu\text{mol/L}$ ) relaxation in control and  
726 iPAH patients and corresponding graph showing the percent relaxation 10 min after diazoxide  
727 application vs. DMSO (n=5 controls vs. 6 iPAH). ns: non-significant. \* $P < 0.05$  \*\* $p < 0.01$  \*\*\* $p < 0.001$ ,  
728 \*\*\*\* $p < 0.0001$ . Two-tailed unpaired Student's t-tests assessed the difference between two groups.  
729 Differences between three groups were assessed with one-way ANOVA completed by Tukey's multiple  
730 comparisons test for post hoc analyses.  
731



732

Figure 5

733 **Figure 5: In vivo diazoxide treatment interferes with MCT-PH and CH-PH development.** (A) In vivo  
 734 experimental design. Diazoxide (20 mg/kg/day for 3 weeks) was administered long-term during MCT  
 735 exposure by intraperitoneal injection. (B) RV systolic pressure (RVSP; mmHg; n=5–7 different rats per

736 condition) (C), Cardiac output (CO; mL/min) (n=5–6 different rats per condition). (D) Pulmonary  
737 vascular resistance (PVR, evaluated by the RVSP/CO ratio) (n=5–6 different rats per condition). (E)  
738 Fulton index (RV/LV+septum) (n=5–8 different rats per condition). (F) Pulmonary vessel occlusion (%)  
739 analysed by hematoxylin-eosin Safran staining (HES) (n = 4–7 rats per condition). (G) Percentage of  
740 non-muscularized and muscularized vessels (100 vessel per rat, (n=4-7 rats) as measured by  
741 immunostaining against  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and Von Willebrand Factor (VWF). (H) *In vivo*  
742 experimental design. Diazoxide (20 mg/kg/day for day 14 to day 21) was administered short-term during  
743 MCT exposure by intraperitoneal injection. (I) RVSP (mmHg; n=9–11 rats per condition). (J) Cardiac  
744 output (CO; mL/min) (n=8–10 rats per condition). (K) PVR, evaluated by calculating the RVSP/CO  
745 ratio (n=8–11 rats per condition). (L) Fulton index (n=8–9 rats per condition). (M) Pulmonary vessel  
746 occlusion (%) analysed by hematoxylin-eosin Safran staining (HES) (n = 4–9 rats per condition). (N)  
747 Percentage of non-muscularized and muscularized vessels (100 vessel per rat, (n=5-7 rats) as measured  
748 by immunostaining against  $\alpha$ SMA and VWF. (O) Representative Western blots of SUR1 and Kir6.2 in  
749 lungs from control and chronic hypoxia (CH)-exposed rats (3 weeks). Quantification of SUR1 and  
750 Kir6.1 expression in lungs from control and CH rats (n=4–5). (P) *In vivo* experimental design. Diazoxide  
751 (20 mg/kg/day for day 14 to day 21) was administered short-term during CH exposure by intraperitoneal  
752 injection. (Q) RVSP (mmHg; n=5–7 rats per condition). (R) CO (mL/min) (n=5–6 rats per condition).  
753 (S) PVR (n=5–6 rats per condition). (T) Fulton index (n=5–8 rats per condition). (U) Pulmonary vessel  
754 occlusion (%) analysed by hematoxylin-eosin Safran staining (HES) (n = 3–6 rats per condition). (V)  
755 Percentage of non-muscularized and muscularized vessels (100 vessel per rat, (n=5-7 rats) as measured  
756 by immunostaining against  $\alpha$ SMA and VWF. ns: non-significant \*p<0.05 \*\*p<0.01; \*\*\*p<0.001  
757 \*\*\*\*p<0.0001. Differences between three groups were assessed with one-way ANOVA completed by  
758 Tukey's multiple comparisons test for post hoc analyses.

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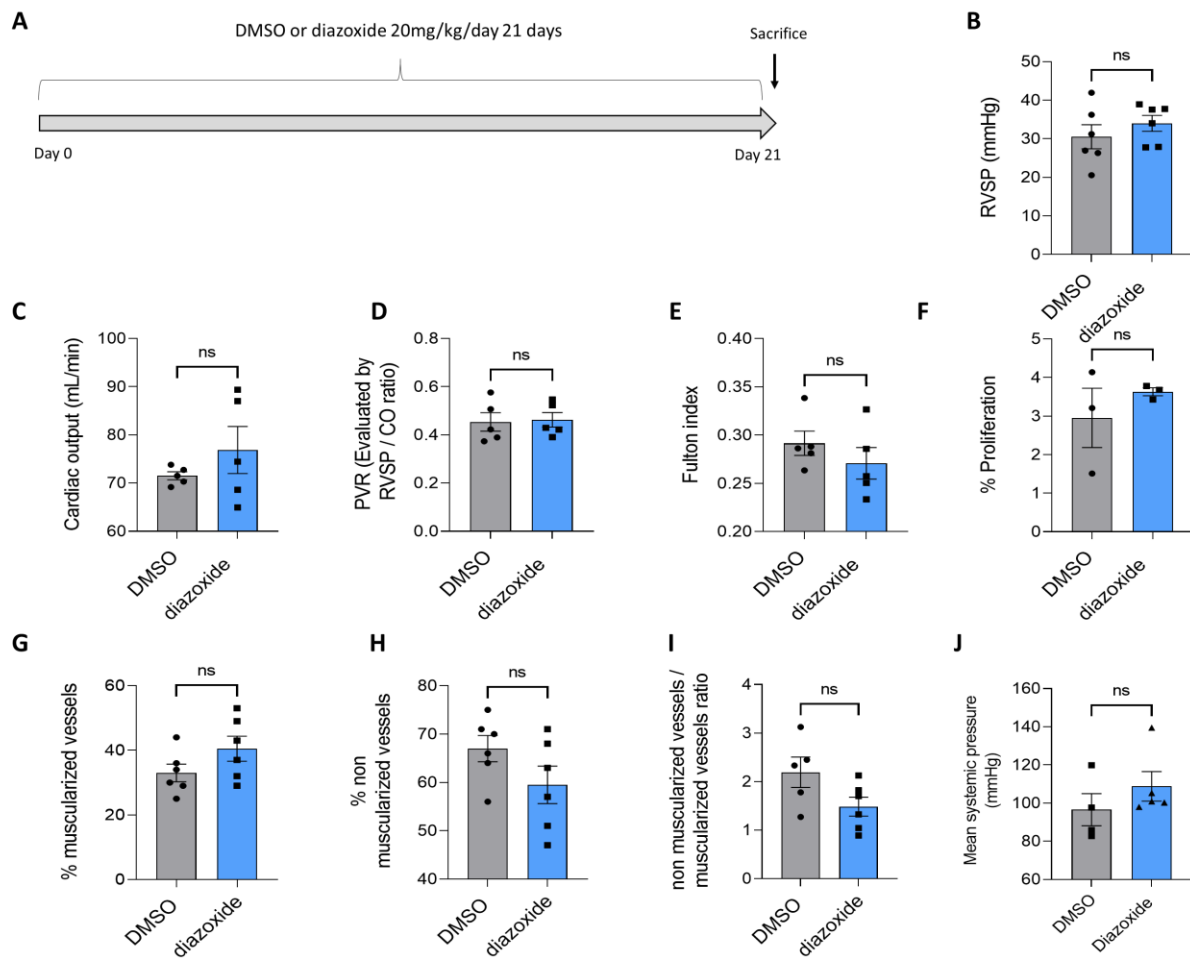


Figure 6

760

761 **Figure 6: In vivo diazoxide treatment in healthy control rats.** (A) *In vivo* experimental design.

762 Diazoxide (20 mg/kg/day for 3 weeks) was administered long-term to healthy control rats by

763 intraperitoneal injection. (B) RVSP (mmHg; n=6 rats per condition). (C) CO (mL/min) (n=5 rats per

764 condition). (D) PVR (n=5 rats per condition) (E) and Fulton index (n=5 rats per condition). (F)

765 Quantification of the percentage of lung proliferating cells after immunofluorescent staining and  
766 confocal imaging of frozen rat lung sections labelled with Click-iT 5-ethynyl-2'-deoxyuridine (EdU)  
767 (n=3 rats). (G-H) Percentage of non-muscularized and muscularized vessels (100 vessel per rat, n=6  
768 rats) as measured by immunostaining against  $\alpha$ SMA and VWF. (I) Non-muscularized/muscularized  
769 ratio vessels in DMSO- and diazoxide-exposed rats (n=6 rats). (J) Mean systemic blood pressure  
770 measured in the carotid artery from DMSO- and diazoxide-exposed rats (n=6 rats). ns: non-significant.  
771 Two-tailed unpaired Student's t-tests assessed the difference between two groups.  
772

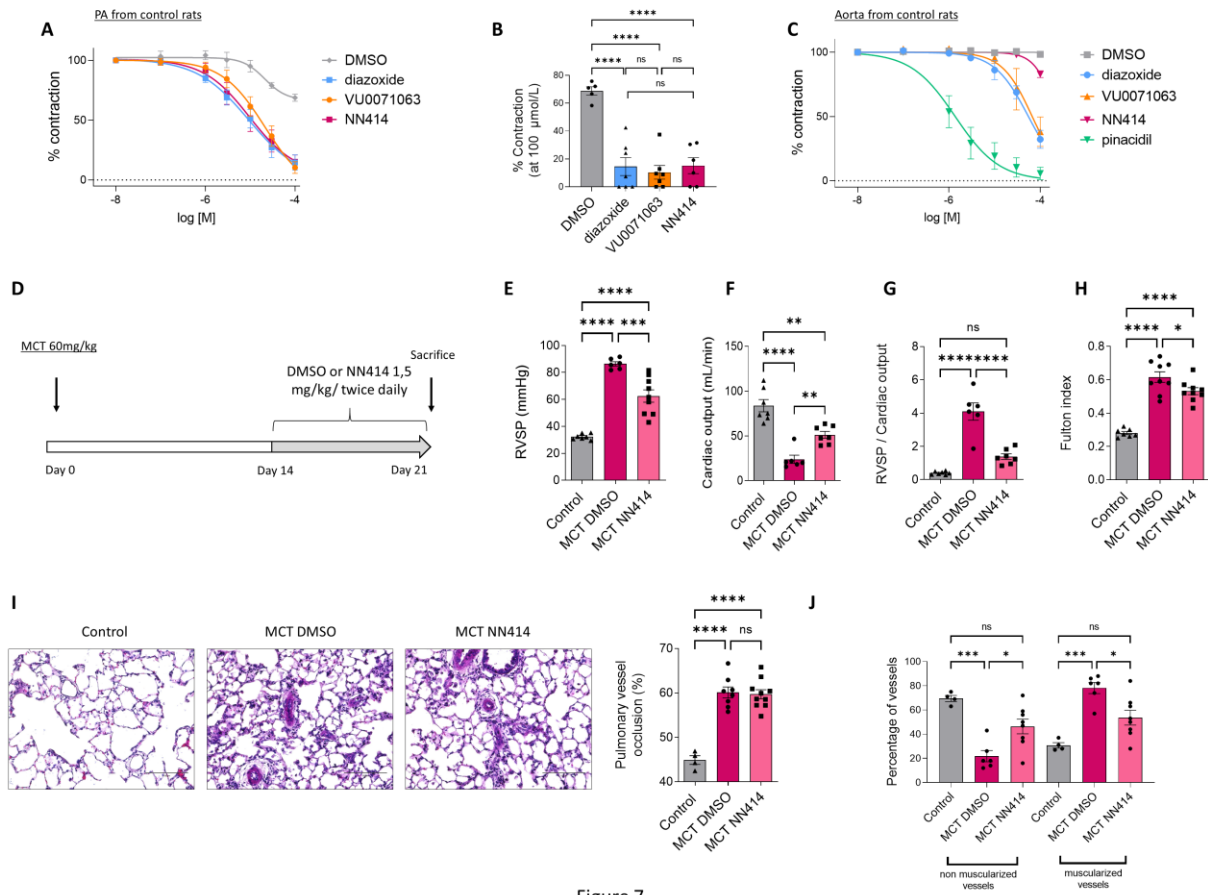


Figure 7

773

774 *Figure 7: Additional SUR1 activators (VU0071063 or NN414) mediated PA relaxation and*

775 *pharmacological activation of SUR1 with NN414 reduced PH-induced MCT. (A) Dose-response to*

776 *diazoxide (blue trace), NN414 (pink trace), VU0071063 (orange trace), and DMSO (grey trace) on*

777 precontracted control rat PA by 1  $\mu\text{mol/L}$  of U46619. (B) Graphics representing the percentage of  
778 contraction at 100  $\mu\text{mol/L}$  of PA treated with the different Sur1 activators or DMSO. (C) Dose-response  
779 to diazoxide (blue trace), NN414 (pink trace), VU0071063 (Orange trace), pinacidil (green trace), and  
780 DMSO (grey trace) on precontracted control rat aorta by 1  $\mu\text{mol/L}$  of U46619. (D) *In vivo* experimental  
781 design. NN414 (1.5 mg/kg/day twice daily for day 14 to day 21) was administered short-term during  
782 MCT exposure by intraperitoneal injection. (E) RVSP (mm Hg; n=6–9 rats per condition). (F) CO  
783 (mL/min) (n=6–7 rats per condition). (G) PVR (n=6–7 rats per condition). (H) Fulton index (n=7–10  
784 rats per condition). (I) Pulmonary vessel occlusion (%) analyzed by hematoxylin-eosin Safran staining  
785 (HES) (n = 4–10 rats per condition. (J) Percentage of non-muscularized and muscularized vessels (100  
786 vessel per rat, (n=5-7 rats) as measured by immunostaining against  $\alpha\text{SMA}$  and VWF. ns: nonsignificant  
787 \* $p < 0.05$  \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  \*\*\*\* $p < 0.0001$ . Differences between three groups were assessed with  
788 one-way ANOVA completed by Tukey's multiple comparisons test for post hoc analyses.

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