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First evidence of aryl hydrocarbon receptor as a druggable target in hypertension induced by chronic intermittent hypoxia

Short Title: AHR in intermittent hypoxia-induced hypertension

Nuno R. Coelho[1], Céline Tomkiewicz[2], M. João Correia[1], Clara Gonçalves-Dias[1], Robert Barouki[2], Sofia A. Pereira[1*]†, Xavier Coumoul[2*]†, Emília C. Monteiro[1]†

[1]CEDOC, Centro de Estudos Doenças Crónicas, Faculdade de Ciências Médicas | Nova Medical School, Universidade Nova de Lisboa, Campo Mártires da Pátria, 130, 1169-056 Lisboa, Portugal; [2]INSERM UMR-S 1124, 3TS, Environmental Toxicity, Therapeutic Targets, Cellular Signaling and Biomarkers; Université de Paris, 45 rue des Saints-Pères, 75006 Paris, France

[*] **Corresponding authors:**
Sofia A. Pereira, PhD
CEDOC - Chronic Diseases Research Center
NOVA Medical School, Universidade NOVA de Lisboa
Rua Câmara Pestana n.º 6, Lab 3.16
1150-082 Lisboa, Portugal
Tel.: +351 218 803 000 (ext. 26012)
sofia.pereira@nms.unl.pt

Xavier Coumoul, PhD
INSERM UMR-S 1124 - T3S,
Faculté des Sciences Fondamentales et Biomédicales
Université de Paris, 45 rue des Saints-Pères
75006 Paris, France; Tel: +33 1 42 86 33 59
xavier.coumoul@parisdescartes.fr

† Equal contributions: Emília C. Monteiro, Xavier Coumoul and Sofia A. Pereira

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Abstract

Background and Purpose Obstructive sleep apnea (OSA) is associated to a high prevalence of resistant arterial hypertension (HTN) justifying the research on novel targets. Chronic intermittent hypoxia (CIH) is a key feature in the development of OSA comorbidities, including HTN.

Experimental Approach We used a rat model of CIH-induced HTN to disclose the hypothesis that the aryl hydrocarbon receptor (AHR) is activated by CIH once it shares the same binding partner of HIF-1α and promotes pro-oxidant, pro-inflammatory (NF-kB) and pro-fibrotic events in common with CIH.

Key Results Upon established hypertension (21 days exposure to CIH), we observed an increase in *Cyp1a1* mRNA in kidney cortex (6-fold), kidney medulla (3-fold) and liver (3-fold), but not in other tissues. Increased renal expression of *Ahr* and markers of inflammation (*Rela*), epithelial to mesenchymal transition markers, the rate-controlling step of gluconeogenesis, *Pepck1*, and members of HIF-pathway, namely, *Hif3a* were also observed. Daily administration (14 days) of AHR antagonist, CH-223191 (5 $mg.kg^{-1}.day^{-1}$, gavage), simultaneously to CIH prevented the increase in systolic blood pressure (SBP) by 53 ± 12% and in diastolic blood pressure (DBP) by 44 ± 16%. Moreover, its administration (14 days) upon already established HTN reversed the increase in SBP by 52 ± 12%.

Conclusion and Implications CIH caused an activation of AHR signaling particularly in the kidney and its pharmacological blockade had a significant impact reverting already established HTN. This first evidence inspires innovative research opportunities for the understanding and treatment of this particular type of HTN.

Keywords: Secondary hypertension; CH-223191; AHR; kidney; CYP1A1; blood pressure;

1. Introduction

Obstructive sleep apnea (OSA) is a highly prevalent sleep-related breathing disorder [1]. It is characterized by recurrent episodes of airflow cessation or reduction, mainly due to mechanical obstruction in the upper airways. The main hallmark of OSA is chronic intermittent hypoxia (CIH) that is responsible for the vast majority of OSA-related comorbidities, namely cardio-metabolic diseases and particularly systemic hypertension (HTN) [2].

Apart from being an independent risk factor for HTN, OSA is also a major secondary cause of resistant HTN to available antihypertensive drugs, including adrenergic blockers [3, 4] and justifying the identification of novel therapeutic drug classes. Typically, OSA patients lose the nocturnal physiological reduction in blood pressure (BP) and BP remains elevated in the absence of obstructive events during wakefulness [5]. There is substantial evidence that the mechanisms behind CIH-induced HTN include carotid body mediated long-term facilitation of sympathetic activity [6]. Also, emerging evidence supports additional origins for the autonomic imbalance behind CIH-induced HTN, such as aberrant reno-renal reflexes [7] and direct central activation of sympathetic tone to cardiovascular effectors. However, the molecular pathways that mediate diurnal chronic HTN after withdrawal of the nocturnal hypoxic stimuli remain uncertain. Activation of transcription factors (triggered by sympathetic activation or not) might explain the gap between short-term hypoxic stimuli and long-term multisystem effects. Indeed, intermittent hypoxia increases hypoxia inducible factor 1α (HIF-1α) stabilization [8-11] and nuclear factor kappa B (NF-kB) activity [12, 13]. However, HTN is not a comorbidity particularly associated to other conditions characterized by systemic HIF-1α stabilization (e.g. high altitude or chronic obstructive pulmonary disease) or NF-kB activations (e.g. rheumatoid arthritis, asthma). It is

79 believed that at least some other tissue-specific mechanisms relevant to BP control may be
80 activated by CIH.
81 Herein, we have investigated the effects of CIH on the transcriptional pathways triggered by the
82 aryl hydrocarbon receptor (AHR). AHR is a ligand-activated transcription factor that belongs to
83 the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family [14]. ARNT (also named HIF-1ß)
84 is a shared dimerization partner for AHR and other molecular pathways, such as HIF-1 pathway
85 [15]. The competition between AHR and HIF-1α regarding dimerization to ARNT [16] might be
86 relevant to consider in the context of CIH. Additionally, it is widely known that AHR and NF-kB
87 subunits interact [17], namely the heterodimerization of RelA-AHR, that is able to repress
88 CYP1A1 activation [18]. Regulation of CYP1A1 expression is presently considered a sensitive
89 marker of AHR activation [19].
90 AHR has been shown to promote cardiovascular alterations due to xenobiotic exposure in
91 humans [20, 21] and in animals [22] and during development and/or homeostasis in animal
92 models [23-26]. Polymorphisms of the AHR signaling pathway are reported to be closely
93 associated with the pathogenesis of essential HTN [27]. Additionally, genome-wide association
94 studies showed that human *CYP1A1* polymorphisms are associated with kidney glomerular
95 filtration rate [28] and increased risk of HTN [29]. This highlights the putative importance of the
96 AHR-CYP1A1 axis in the homeostasis of BP. However, to the best of our knowledge, the role of
97 the AHR pathway in OSA comorbidities has never been addressed.
98 In this study, using a rat model of CIH-HTN, we first investigated the effect of CIH on AHR
99 pathway. Then, we pharmacologically targeted this pathway with an AHR antagonist CH-
100 223191, in order to clarify how the manipulation of this molecular pathway interferes with BP

and heart rate (HR) under CIH conditions, providing new opportunities for the understanding and the treatment of this particular type of HTN.

2. Methods

2.1 Study design

Five different sets of experiments with animals were performed. The animals were allocated randomly to each subset.

A first set of animals was used to characterize the molecular pathway of AHR and other genes of interest after exposure to 21 days of CIH (n=8) after 2 days of chamber acclimatization under normoxic conditions (Nx, *i.e.* 21% O_2 + 79% N_2). Control animals (n=5) were exposed for 21 days to Nx in the same room as the CIH groups.

The second and third sets of experiments were used to investigate the effect of the AHR antagonist CH-223191 [30] on BP and HR.

Since the use of CH-223191 in rats *in vivo* is scarce, we decided to perform a first dose-exploratory experiment (n=3) to disclose the effects of 5 $mg.kg^{-1}$ once a day, by oral gavage in 1 mL of vegetable oil on BP and HR in Nx, followed by the its ability to prevent and revert HTN induced by CIH (second set, n=3, see study design scheme in Figure 5A). To the best of our knowledge there are only two studies that administered CH-223191 by gavage to rats: 8 $mg.kg^{-1}$ per day for 14 days in a model of pulmonary hypertension [31] and 5 $mg.kg^{-1}$ per day for 14 days in a model of arthritis [32].

The third set of experiments (n=5) intended to validate the reversion in HTN with CH-223191 (see study design scheme in Figure 6A).

A fourth set of experiments was used to assess the effect of the administration of CH-223191 (5 $mg.kg^{-1}$, by oral gavage, daily, for 14 days; n=3) in Nx (see study design scheme in supplementary figure 2).

The last two groups of animals were used with the purpose of assessing the effects of CIH in the body weight growth: one group was exposed to Nx for 35 days (n=6) and another group with telemeters implanted was exposed to CIH for 35 days (n=4).

2.2 Animals

Thirty nine male Wistar Crl:WI (Han) (Rattus norvegicus L), aged 8-12 weeks, with mean body weights of 249 ± 5 g, were obtained from the NOVA Medical School animal facility. Animals were housed in polycarbonate cages with wire lids (Tecniplast, Buguggiate, Varese, Italy) and maintained under standard laboratory conditions: artificial 12 h light/dark cycles (lights on from 9 am - 9 pm), at room temperature (22 ± 2.0 °C) and a relative humidity of 60 ± 10%. Rats were given standard laboratory diet (SDS diets RM1) and reverse osmosis water *ad libitum*.

All applicable institutional and governmental regulations concerning the ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese regulation and laws on the protection of animals used for scientific purposes (Law nº 113/2013). All procedures were approved by the Ethical Committee of the NOVA Medical School (protocol nº 15/2017/CEFCM).

Rats were weighed at baseline and once a week throughout the entire study. Water and food intake were also measured once a week. At the end of the experiments, the rats were

146 anaesthetized by intraperitoneal injection with a solution of medetomidine (0.5 mg.Kg^{-1} body
147 weight; Domitor®, Pfizer Animal Health, Auckland, New Zealand) and ketamine (75 mg.Kg^{-1}
148 body weight; Imalgene 1000®, Mérial, Lyon, France), and cardiac puncture was performed
149 without thoracotomy. Death was confirmed by cervical dislocation before organs and tissue
150 collection.
151

152 **2.3 Cardiovascular parameters**

153 Indwelling radiotelemeters (HD-S10, *Data Sciences Corporation*, USA) were inserted in the
154 abdominal aorta, to allow the recording of BP and HR in freely moving rats, as previously
155 described in a recent paper by our group [33].
156 SBP, DBP and HR values were obtained during 30-s sampling periods and were recorded for 20
157 min, every day at 8am, during the light-off phase (rats' active period), with exclusion of the first
158 5 minutes in each measurement for each rat. The light-off period was chosen to mimic more
159 accurately the diurnal HTN in OSA patients. The development of stable HTN in this model
160 occurs in approximately 14-21 days of exposure to CIH [33].
161

162 **2.4 Chronic intermittent hypoxia paradigm**

163 Since the seminal work of Fletcher and colleagues [34], animal models of CIH have been
164 extensively and successfully employed to assess changes in cardiovascular outcomes arisen from
165 CIH [3]. The paradigm of CIH herein employed was already validated by our group [33] allows
166 the lowering of O_2 levels from 21% to ~5-6% in each cycle of CIH, and animals were exposed to
167 5.6 CIH cycles.h^{-1} for 10.5 h.day^{-1}. This paradigm mimics the same pattern of CIH present in
168 mild to moderate clinical OSA [33].

169 Briefly, the animals exposed to CIH experiments were maintained in a eucapnic atmosphere
170 inside medium A-chambers (Biospherix Ltd, NY, USA), three animals by chamber and oxygen
171 concentrations were controlled through an OxyCycler A420C (Biospherix Ltd, NY, USA).
172 Chambers were infused with 100% N_2 for 3.5 min to quickly reduce the O_2 concentration,
173 following by an infusion with 100% O_2 for 7 min to restore O_2 to ambient levels until the start of
174 the next CIH cycle. Rats were exposed to CIH in their sleep period (light phase), from 9.30 am to
175 8.00 pm, during 14, 21, 35 or 42 days, depending on the experiments. During the remaining
176 hours of the day, the chambers were infused with 21% of O_2.
177
178 **2.5 Markers of AHR activation and quantitative real-time PCR**
179 Liver and adipose tissue (used as comparator tissues not directly related to HTN) and kidney
180 (medulla and cortex) were homogenized in Trizol® (Life Technologies) and stored at -80 ºC.
181 After total RNA extraction, cDNA was synthesized from 1 μg of RNA, using the NZY First-
182 Strand cDNA synthesis kit (NZYTech, Lisbon, Portugal), according to the manufacturer's
183 directions. Quantitative real time polymerase chain reaction (qPCR) was performed with 20 ng
184 of cDNA, using SensiFAST™ SYBR® Hi-ROX Kit (Bioline, United Kingdom), with duplicates
185 for each experiment. Supplementary Table 1 gives the gene-specific primers used for the genes
186 analyzed. The relative amounts of mRNA were estimated using the $\Delta\Delta C_T$ method with
187 cyclophilin A (*Cypa*) as the reference gene [35]. We assessed mRNA levels of several genes
188 associated with the AHR axis. The markers analyzed included those directly related with AHR
189 pathway (*Ahr*, *Arnt*, *Cyp1a1*, *Cyp1a2*, *Cyp1b1*, and *Pepck1*); markers of fibrosis and epithelial-
190 mesenchymal transition (EMT) (collagen 1A1 - *Col1a1*, α smooth muscle actin – *aSma*,
191 fibronectin – *Fn1*, vimentin - *Vim*), epithelial markers (E-cadherin - *Ecad*) as well as markers of

192 inflammation (interleukin 1b - *Il1b* and interleukin 6 - *Il6*) and endoplasmic reticulum stress
193 (*Grp78)*.
194 We have also quantified the transcription factors involved in CIH responses and related with
195 AHR, namely NF-kB subunits, *Rela* and *p49/p100*, and members of HIF family, such as *Hif1a,*
196 *Epas1* (HIF-2α)*, and Hif3a,* and its associated target gene vascular endothelial growth factor A
197 (*Vegfa)*. Additionally, markers of the renin-angiotensin system, namely renin (*Ren)*, angiotensin
198 II receptor type 1 (*At1*) and angiotensin-converting enzyme (*Ace1*) were also analyzed due to
199 their relevance in BP control.
200
201 **2.6 Statistical analyses**
202 GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA, USA) was used to perform
203 all the statistical analysis. Data are presented as the mean ± standard deviation or mean ±
204 standard error of the mean (SEM). Differences were considered significant at $p<0.05$ (***
205 $p<0.001$, ** $p<0.01$; * $p<0.05$) and obtained using one-way ANOVA or Mann–Whitney's U-test
206 whenever applicable.
207
208 **3. Results**
209 **3.1 CIH activates AHR signaling particularly in kidney tissue**
210 An overexpression of the *Cyp1a1* gene (indicator of AHR activation) was observed primarily in
211 the kidney cortex, but also in the kidney medulla and liver (Figure 1A). Among other target
212 genes of AHR, neither *Cyp1b1* nor *Cyp1a2* were modified by CIH in any of the four analyzed
213 tissues. *Ahr* mRNA expression was also increased, except in visceral adipose tissue (VAT)
214 (Figure 1B). The binding partner of both AHR and HIF family pathways, *Arnt*, also known as

HIF-1β, was not upregulated by CIH in the kidney but exhibited increased expression in liver (2-fold,). Overall, despite no exogenous exposure to any classical AHR ligands, we showed that CIH leads to activation of the AHR signaling pathway in several tissues (kidney, liver) important for xenobiotic metabolism and the development of HTN.

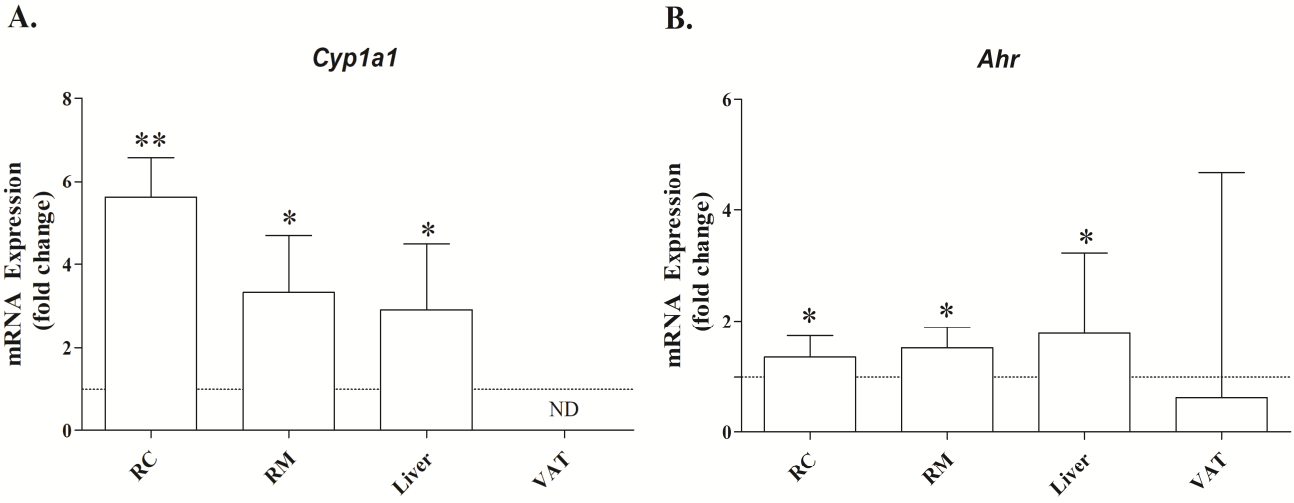


Figure 1. Chronic intermittent hypoxia (CIH) activates aryl hydrocarbon receptor (A*hr*) and *Cyp1a1* mRNA expression, particularly in renal cortex (RC), but also in renal medulla (RM) and liver. *Cyp1a1* mRNA was not expressed in visceral adipose tissue (VAT). Relative changes in transcriptional levels between samples from CIH-exposed (21 days, n=8) and normoxic animals (n= 5) were obtained using comparative Ct method ($2^{-\Delta\Delta Ct}$). Data are presented as mean fold change ± SD; * $p<0.05$, ** $p<0.01$; Mann-Whitney test. ***Cyp1a1:*** cytochrome P450 family 1 subfamily A member 1; **ND**: not detectable.

3.2 CIH activates several members of HIF pathway, particularly in kidney

We assessed the effects of CIH on the expression of HIF family (1, 2 and 3 α) and vascular endothelial growth factor (*Vegfa*), a classical transcriptional target of HIF-1α (Figure 2).

There were no changes in *Hif1a* mRNA expression in liver and visceral adipose tissue, but it was increased in both kidney cortex and medulla (Figure 2A). Accordingly, the same pattern was observed for *Vegfa* (Figure 2B). CIH also altered the expression HIF-2α (*Epas1*), particularly in renal medulla (Figure 2C). The effect of CIH in *Hif3a* mRNA expression showed a similar pattern to the one observed with HIF-1α: no effect in liver and adipose tissue and increases in the kidney (Figure 2D), thus suggesting the activation of HIF pathways upon CIH exposure, particularly in the kidney.

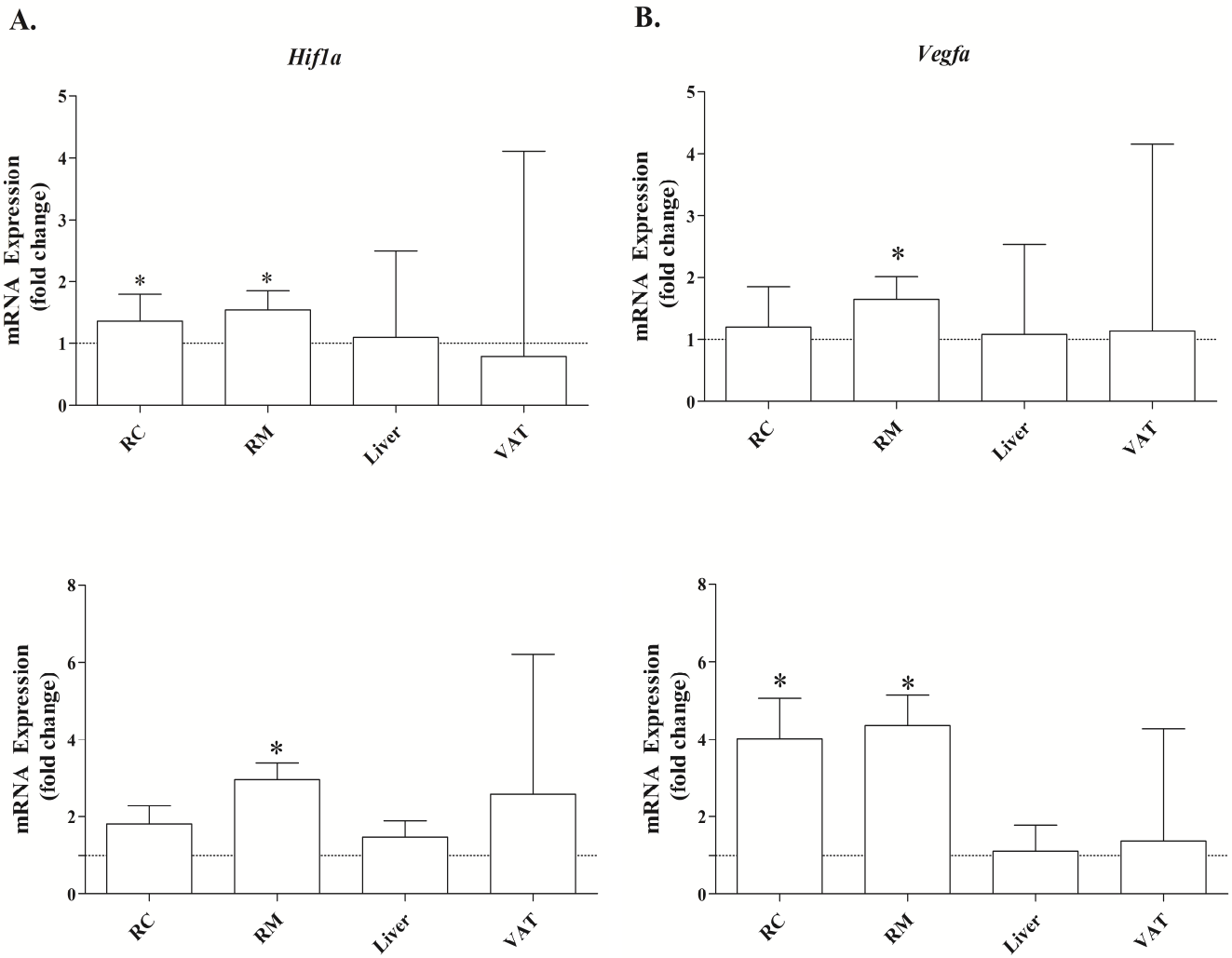


Figure 2. Chronic intermittent hypoxia (CIH) activates mRNA expression of hypoxia-inducible factors (HIF) and its target gene (*Vegfa*), in the kidney, but not in the liver and visceral adipose tissue (VAT). Relative changes in transcriptional levels between samples from CIH-exposed (21 days, n=8) and normoxic animals (n=5) were obtained using comparative Ct method ($2^{-\Delta\Delta Ct}$). Data are expressed as mean fold change ± SD; * $p<0.05$; Mann-Whitney test. ***Epas1***: endothelial PAS domain protein 1; **RC**: renal cortex; **RM**: renal medulla; ***Vegfa***: vascular endothelium growth factor A.

3.3 CIH impacts NF-kB inflammatory pathway and endoplasmic reticulum stress in kidney

The mRNA levels of several inflammatory markers were evaluated, namely NF-kB subunits, *Rela* (also known as p65) and *p49/p100* and the interleukins *Il1b* and *Il6* (Figure 3). Only *Rela*, which belongs to the classical pathway of NF-kB activation, was overexpressed in the kidney cortex (Figure 3A). In contrast, increased mRNA levels of *p49/p100* were observed in VAT (Figure 3B). Overall, our results suggest that pathways participating inflammation caused by CIH are tissue-specific. No changes for interleukins were observed in kidney and the *Il6* was only increased in VAT (Figure 3C and 3D). Endoplasmic reticulum (ER) stress can be a protective mechanism in early phases of CIH exposure, but over time it can turn into a deleterious mechanism leading to cell death [36]. With this in mind, we have quantified the endoplasmic tissue stress-related GRP78 (*Grp78* gene) mRNA, and found that its expression was upregulated in the kidney and liver tissues (Figure 3E).

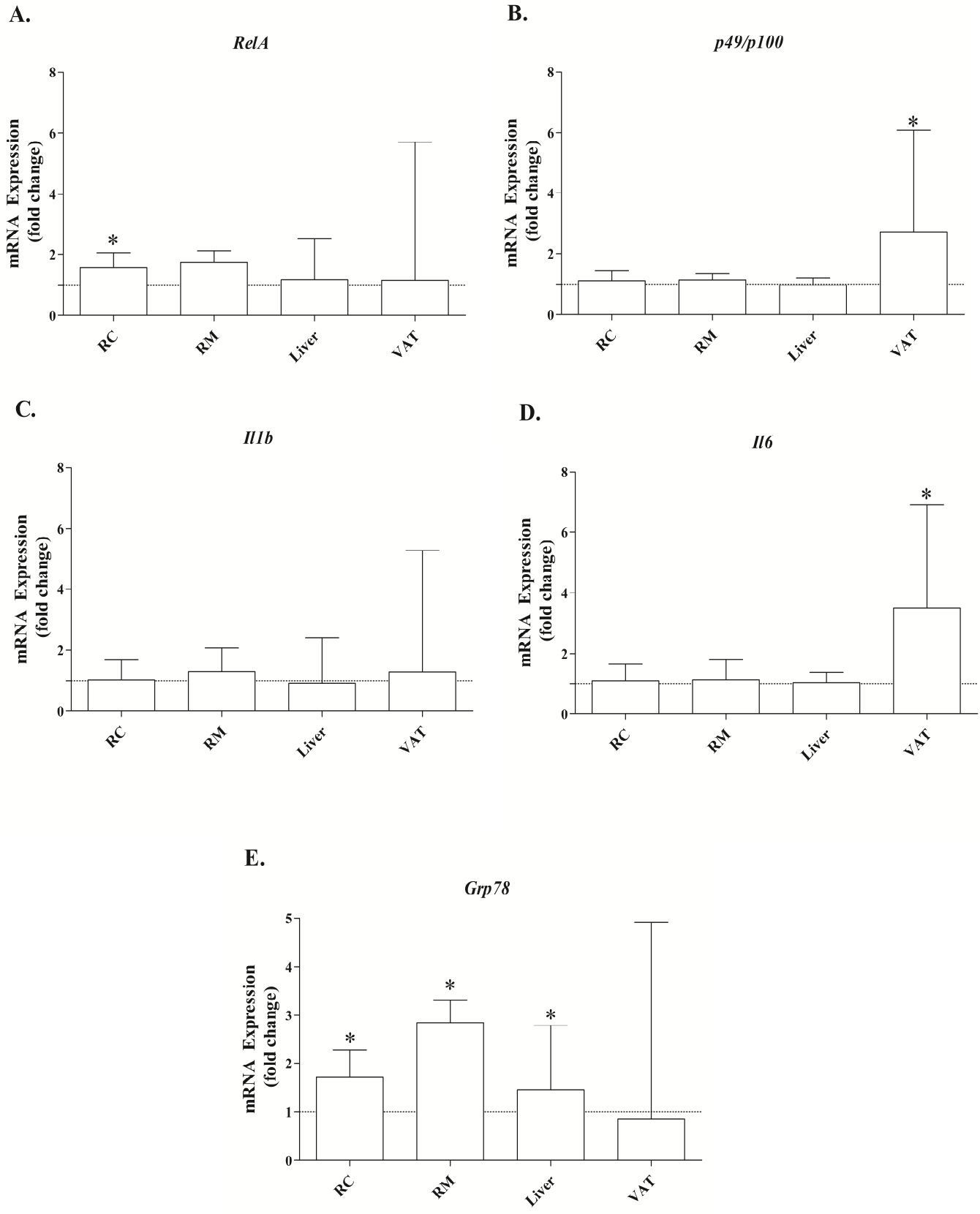

A.
RelA
mRNA Expression (fold change)
RC
RM
Liver
VAT
B.
p49/p100
C.
Il1b
D.
Il6
E.
Grp78

Figure 3. Chronic intermittent hypoxia (CIH) activates mRNA expression of NF-kB related genes (*Rela*, *p49/p100*, *IL1b*, *IL6*) and endoplasmic reticular stress marker (*Grp78*), in a tissue-dependent manner. Relative changes in transcriptional levels between samples from CIH-exposed (21 days, n=8) and normoxic animals (n= 5) were obtained using comparative Ct method ($2^{-\Delta\Delta Ct}$). Data are presented as mean fold change ± SD; * $p<0.05$; Mann-Whitney test. ***Il1b*****:** interleukin 1 beta; ***Il6:*** interleukin 6; ***Grp78:*** glucose regulated protein 78; ***p49/p100:*** nuclear factor kappa B subunit 2; ***Rela:*** v-rel avian reticuloendotheliosis viral oncogene homolog A; **RC**: renal cortex; **RM**: renal medulla; **VAT**: visceral adipose tissue.

3.4 CIH impacts renin-angiotensin system in kidney

Higher expressions of renin (*Ren*) in the renal medulla (3.2-fold change) and of AT1 receptor (*At1*) in the renal cortex (2-fold change) were observed upon CIH exposure (supplementary figure 1). In contrast, no changes of *Ace1* (angiotensin I-converting enzyme type I) in kidney tissue were found. These results are in line with the experiments described by others [37, 38], when analyzing kidney tissue as a whole, validating our CIH paradigm.

3.5 CIH impacts epithelial to mesenchymal transition process in kidney

Increased expression of vimentin and fibronectin (mesenchymal markers) is traditionally observed during EMT while E-cadherin (epithelial marker) expression is decreased. While *Vimentin* (Figure 4A) and *Fn1* (Figure 4B) were upregulated in kidney tissue, the mRNA expression of *Ecad* (Figure 4C) and of the fibrotic markers *Col1a1* (Figure 4D) and *aSma* (Figure 4E) *s*howed no difference in kidney tissue upon CIH exposure. Overall, this suggests the acquisition of mesenchymal characteristics by kidney tissue in response to 21 days of CIH.

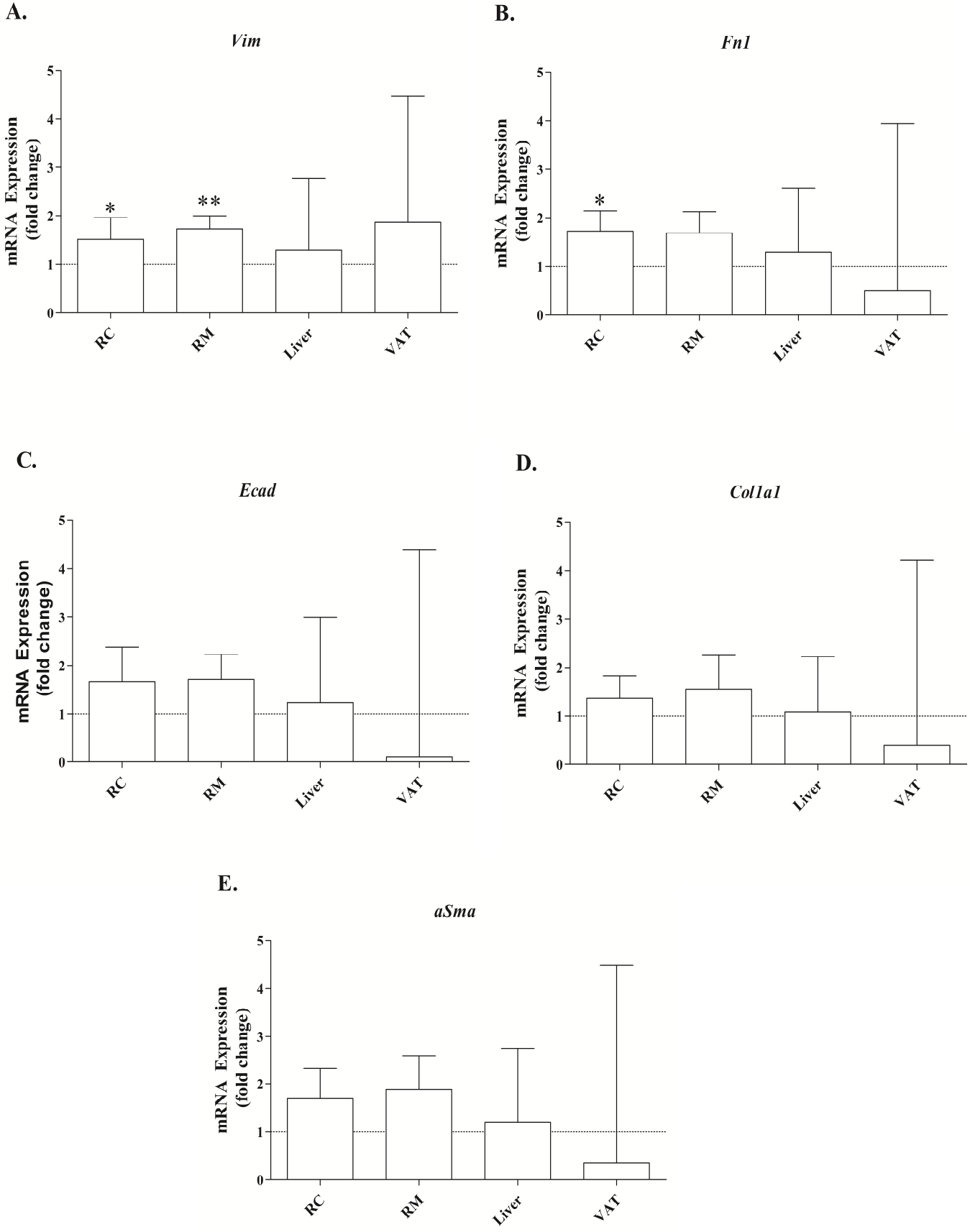

A.
Vim
mRNA Expression (fold change)
RC
RM
Liver
VAT
B.
Fn1
C.
Ecad
D.
Col1a1
E.
aSma

Figure 4. Chronic intermittent hypoxia (CIH) activates a mesenchymal phenotype in the kidney, but not in the liver and visceral adipose tissue (VAT). **(A-B)** Mesenchymal, **(C)** epithelial and **(D-E)** fibrosis markers were analyzed. Relative changes in transcriptional levels between samples from CIH-exposed (21 days, n=8) and normoxic animals (n= 5) were obtained using comparative Ct method ($2^{-\Delta\Delta Ct}$). Data are presented as mean fold change ± SD; * $p<0.05$; Mann-Whitney test. ***aSma:*** alpha smooth muscle actin; ***Col1a1:*** collagen type I alpha 1; ***Fn1***: fibronectin 1; ***Ecad:*** E-cadherin; **RC**: Renal cortex; **RM**: Renal medulla; ***Vim***: vimentin.

3.6 AHR antagonist CH-223191 prevented CIH-induced increase in blood pressure

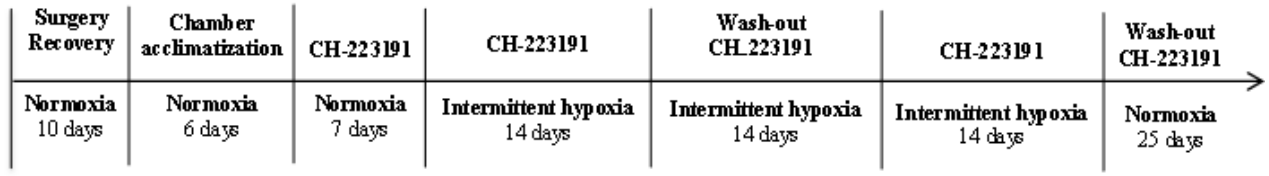
The molecular results concerning the likely activation of the AHR pathway after 21 days of CIH exposure (first set of experiments) directed the work towards the *in vivo* use of an AHR antagonist to counteract the effects of CIH (second set of experiments). Since the use of CH-223191 in rats *in vivo* is scarce, we decided to perform a first dose-exploratory experiment to clarify the effect of this compound on BP and HR and its potential to impact the HTN induced by CIH. The n=3 is justified considering that this was a first preliminary dose-exploratory experiment and that the administration of CH-22319, particularly in rats is limited by its prohibitive costs as previously reported [31].

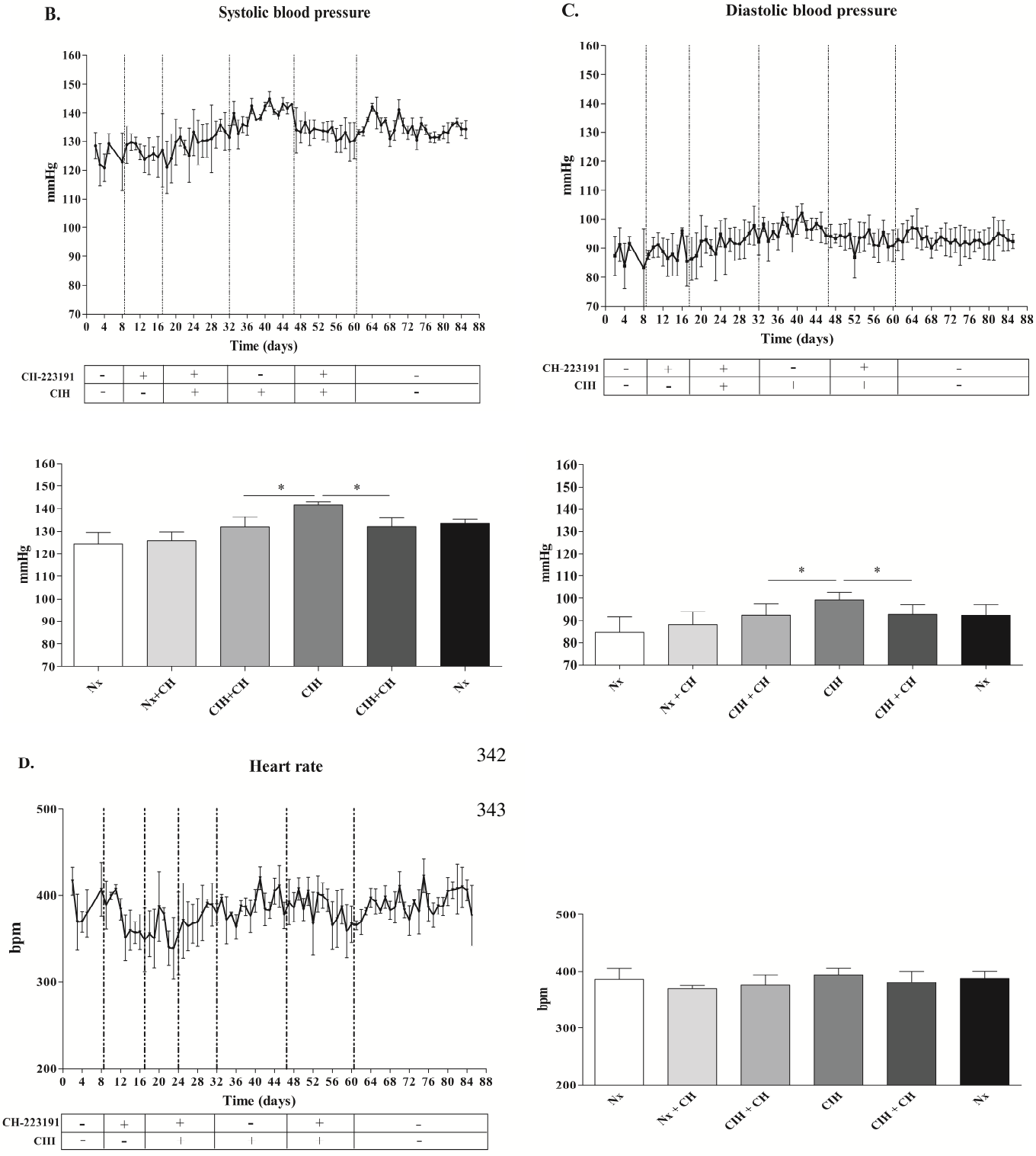
Figure 5A displays the timeline of the experiment, that started by measuring the baseline values for BP and HR in normoxia (Nx; chamber acclimatization). Next, the animals were treated with the CH-223191 for 7 days (5 $mg.Kg^{-1}$, once a day), to assess if the compound could alter these cardiovascular parameters in Nx conditions (Nx+CH) and no changes were observed: 124 ± 5 mmHg vs. 126 ± 4 mmHg for SBP (Figure 5B) and 85 ± 7 mmHg vs. 88 ± 6 mmHg for DBP (Figure 5C). In a separate group of experiments, we confirmed that a longer exposure to CH-



223191 (14 days) did not change basal BP in Nx conditions (supplementary figure 2). Then, CH-223191 was administered concomitantly with CIH (14 days), and a moderate non-significant increase in BP (CIH+CH, 131 ± 5mmHg) was observed (Figure 5B and 5C). In order to investigate if the AHR antagonist was preventing a larger increase in BP induced by CIH, we withdrew the compound, while maintaining the animals exposed to CIH for more 14 days (CIH only). Over this period, a bigger and significant increase in the BP was observed (142 ± 1 mmHg), suggesting that the AHR antagonist was able to prevent (CIH+CH vs. CIH) HTN caused by CIH in 56 ± 15% and 44 ± 16% of SBP and DBP, respectively (Figure 5B and C). More importantly, after the rise of the BP that followed the withdrawal of the antagonist (CIH+CH vs. CIH), re-administration of CH-223191 reverted the increase in the BP caused by CIH in 61 ± 18% and 45 ± 13% in SBP (142 ± 1 mmHg vs. 131± 4 mmHg) and DBP (99 ± 4 mmHg vs. 93 ± 4 mmHg), respectively (CIH vs. CIH+CH) (Figure 5B and C). No changes were observed through this longitudinal study in heart rate (Figure 5 D).

A.



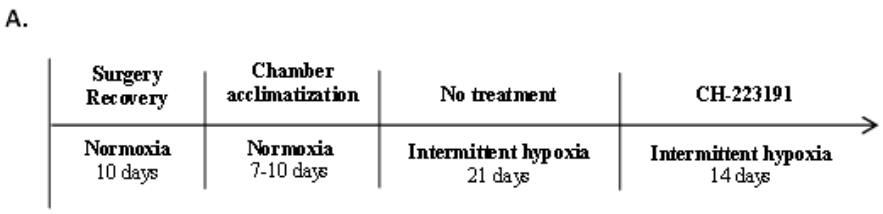

B.
Systolic blood pressure
mmHg
Time (days)
CH-223191
CIH
C.
Diastolic blood pressure
mmHg
Time (days)
CH-223191
CIH
mmHg
Nx
Nx+CH
CIH+CH
CIH
CIH+CH
Nx
mmHg
Nx
Nx + CH
CIH + CH
CIH
CIH + CH
Nx
D.
Heart rate
bpm
Time (days)
CH-223191
CIH
bpm
Nx
Nx + CH
CIH + CH
CIH
CIH + CH
Nx

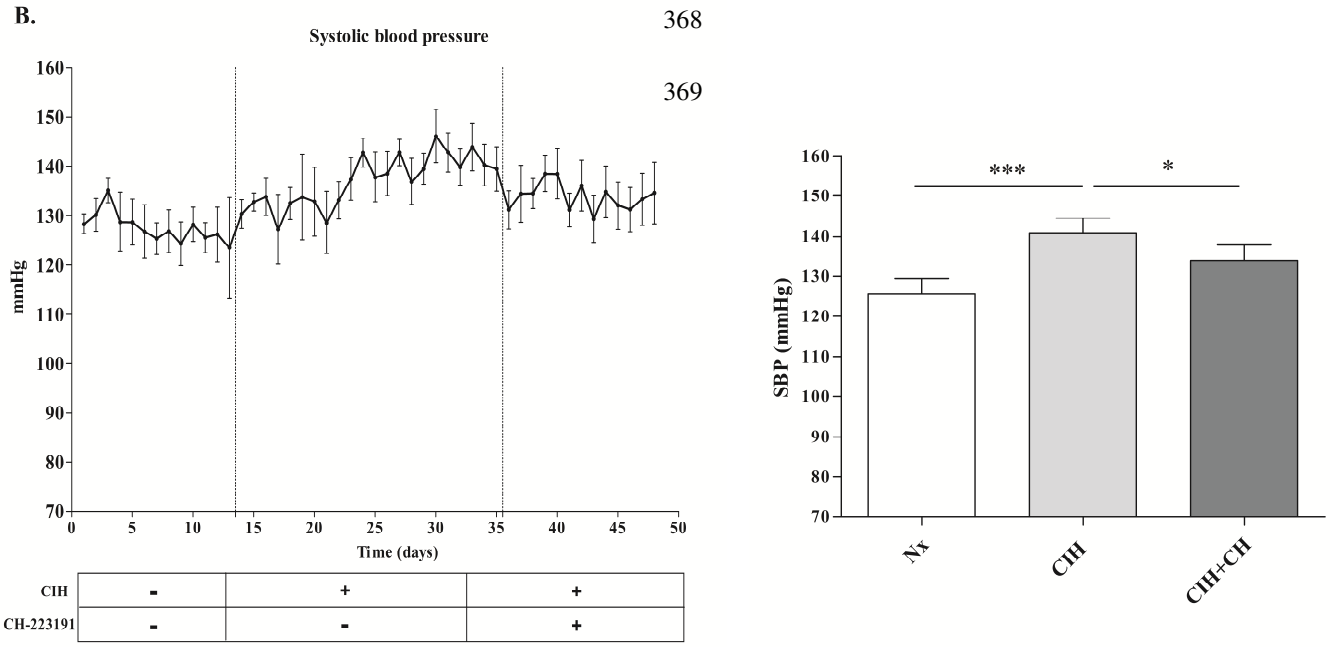
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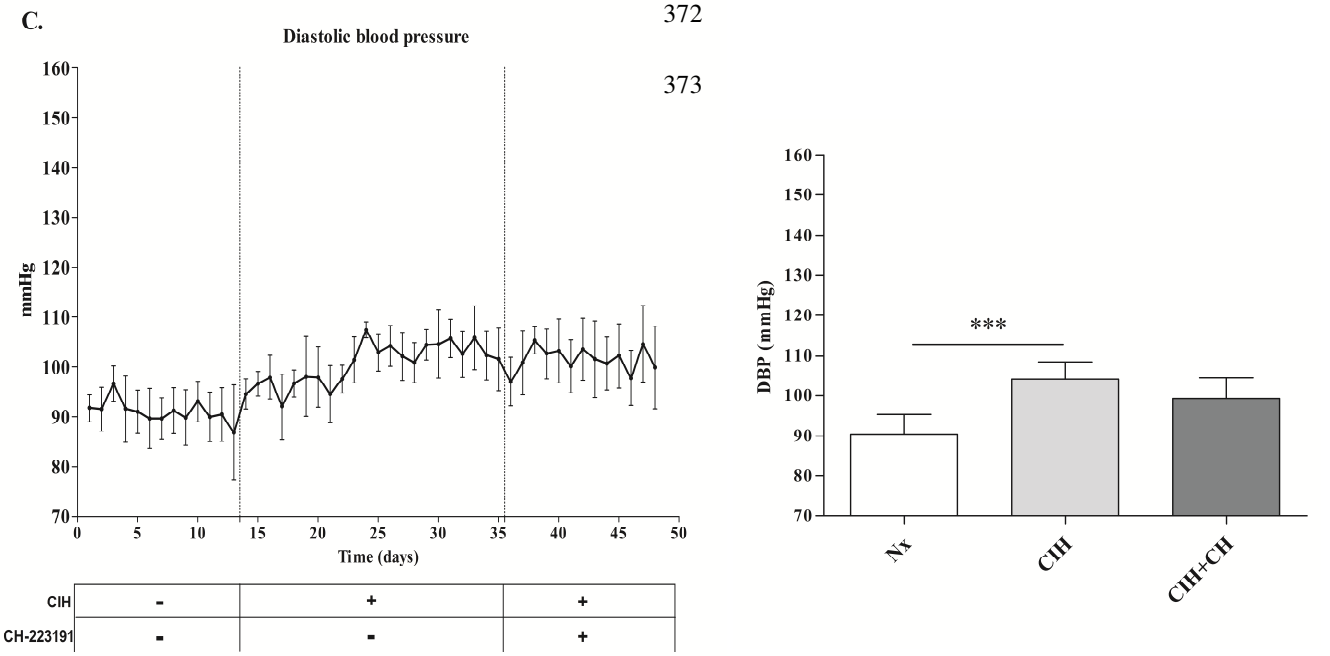
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345 **Figure 5.** A first dose-exploratory experiment (n=3) to clarify the effect of CH-223191 on blood
346 pressure and heart rate. The AHR antagonist, CH-223191 (CH, 5 $mg.Kg^{-1}.day^{-1}$, gavage)
347 prevents and reverts the increase in blood pressure caused by chronic intermittent hypoxia (CIH)
348 exposure. **(A)** Longitudinal study design. The effect of CH-223191 was assessed in **(B)** systolic
349 blood pressure**, (C)** diastolic blood pressure and **(D)** heart rate. Data are presented as mean ±
350 SEM (n=3); * $p<0.05$, paired one-way ANOVA with Bonferroni post-test. Only the last 8 days
351 of each condition were considered for statistical analysis. **Bpm**: beats per minute; **mmHg**:
352 millimeter of mercury; **Nx**: normoxia.
353
354 **3.7 AHR antagonist CH-223191 reverted CIH-induced hypertension**
355 Next, we aimed to confirm that AHR blocking reverses CIH-HTN. For that, a third set of
356 experiments was performed (see also material and methods to compare each set), where the
357 administration of this AHR antagonist was initiated when HTN had been already established (i.e.
358 after 21 days of CIH) (Figure 6A). CH-223191 (5 $mg.Kg^{-1}$ orally, during 14 days) reduces the
359 increase in SBP caused by CIH (21 days) in 52 ± 10% (141 ± 4 mmHg vs. 134 ± 4 mmHg,
360 respectively), while the reduction in DBP (41 ± 13%, 104 ± 4 vs. 99 ± 5 mmHg, respectively)
361 did not achieve statistical significance (Figures 6B and 6C).
362 This effect of CH-223191 in reverting high BP cannot be attributed to its vehicle (vegetable oil)
363 because in control experiments, where the antagonist was replaced by its vehicle, no changes in
364 the effect of CIH in BP and HR were observed (supplementary figure 3).
365
366
367




B.
Systolic blood pressure
mmHg
Time (days)
CIH
CH-223191
SBP (mmHg)
***
*
Nx
CIH
CIH+CH


C.
Diastolic blood pressure
mmHg
Time (days)
CIH
CH-223191
DBP (mmHg)
***
Nx
CIH
CIH+CH

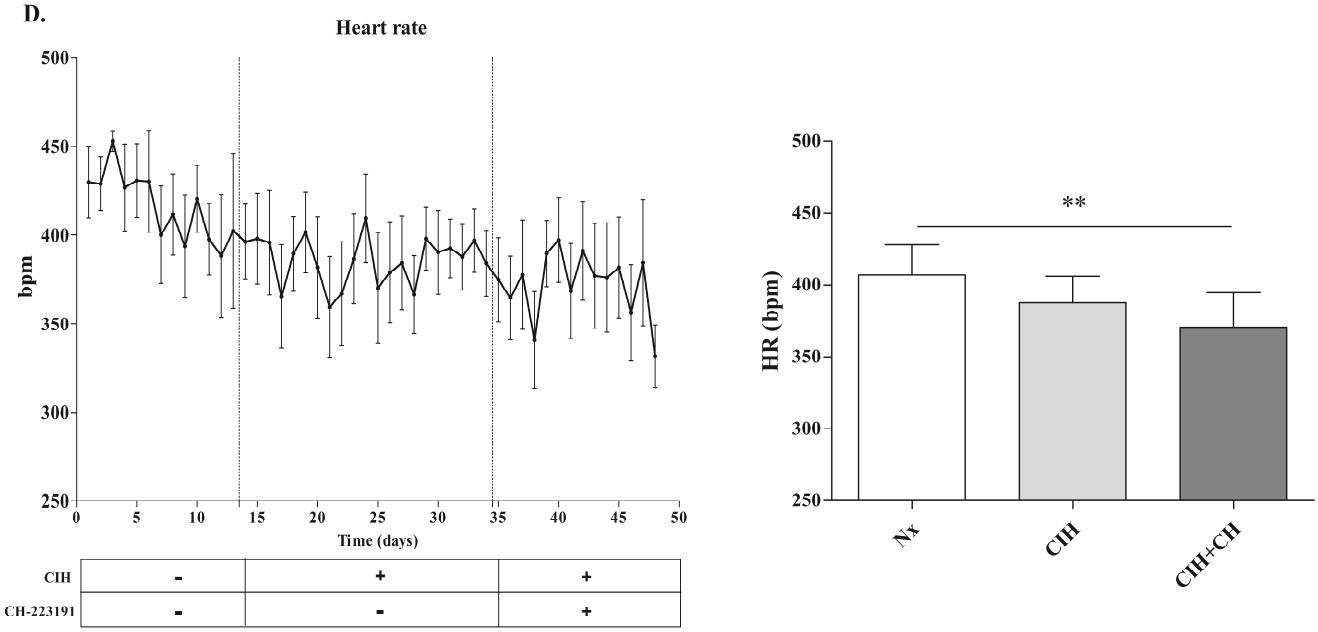


Figure 6. The AHR antagonist, CH-223191 (CH, 5 mg.Kg^{-1}.day^{-1}, gavage) reverts the blood pressure increase caused by chronic intermittent hypoxia (CIH). **(A)** Longitudinal study design (n=5). The effect of CH-223191 was assessed in **(B)** systolic blood pressure, **(C)** diastolic blood pressure and in **(D)** heart rate. Data are expressed as mean ± SEM (n=5); * p<0.05; **p<0.01; *** p<0.001; paired one-way ANOVA with Bonferroni post-test. Only the data of the last 8 days of each sub-group (Nx, CIH or CIH+CH), were used for statistical analysis. **Bpm**: beats per minute; **mmHg**: millimeter of mercury; **Nx**: normoxia.

3.8 AHR antagonist CH-223191 interference in other CIH-induced cardiometabolic changes

Although no impact of CIH or CH-223191 on HR was observed in second set of experiments (figure 5 D), in the reversion protocol (third set) (figure 6), the exposure of CIH together with CH-223191 decreased the HR in 9±2% (from 407 ± 21 beats per minute (bpm) to 370 ± 24 bpm), when compared to Nx (figure 6D). An effect confirmed also in the vehicle-treated animals, with

HR decreasing with CIH (415 ± 12 bpm vs 375 ± 11 bpm, Nx and CIH, respectively) and maintained with the vehicle administration (364 ± 15 mmHg) (supplementary figure 3C).

Additionally, Figure 7 shows that CIH reduces daily food consumption, an effected not reverted by CH-223191 (5 mg.Kg^{-1}/day, 14 days) and not observed in Nx upon 7 days or 14 days of CH-223191 exposure (n=3). Also, no changes in water consumption were found in any condition. Animals exposed to 35 days of CIH showed a lower increase in body weight, when compared to control animals exposed to Nx, respectively 12 ± 2% versus 31 ± 2%. This slower growth weight was not modified by CH-223191 (15 ± 2%), nor by its vehicle (18 ± 3%).

We also found that CIH increases *Pepck1* mRNA expression (AHR target gene, pivotal in gluconeogenesis) in liver (2.6-fold change) and kidney (2-fold change), but not in VAT.

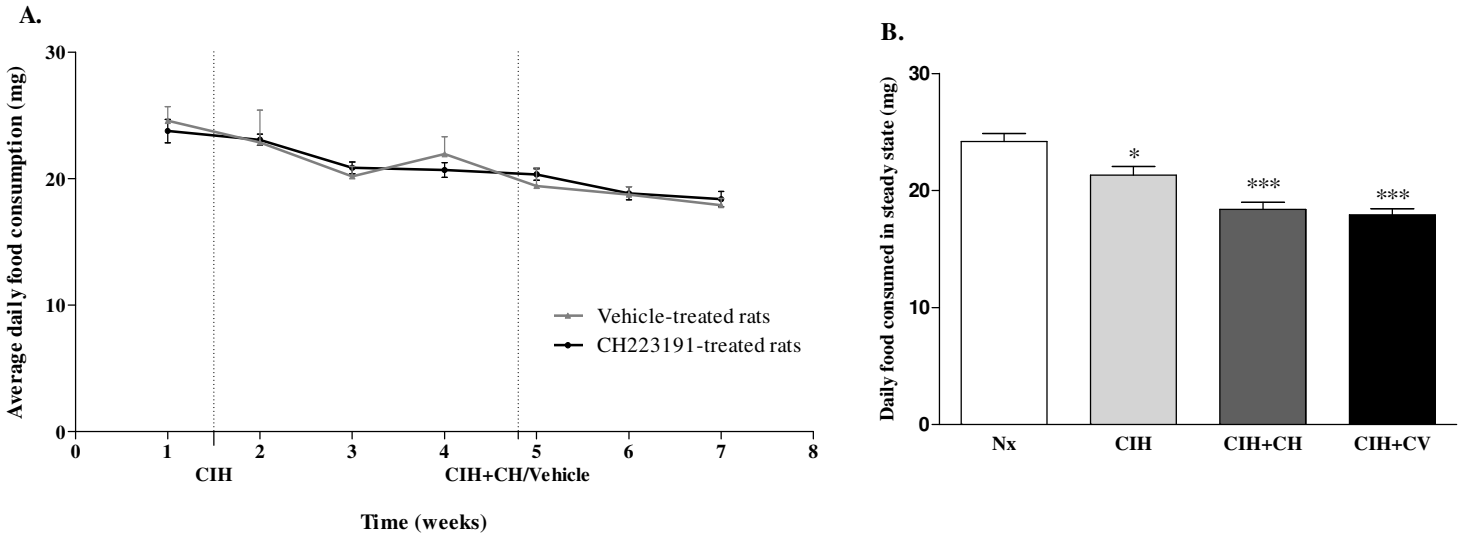


Figure 7. The decrease in daily average food consumption caused by chronic intermittent hypoxia (CIH) was not modified by blocking AHR with CH-223191 (CH). **(A)** Temporal variation of average daily consumption in CH-223191 (n=5) and vehicle-treated animals (CV, n=5). **(B)** Comparison between subgroups corresponding to the experiments described in figure

6 and supplementary figure 3. Data are expressed as mean ± SEM; * $p<0.05$; *** $p<0.001$ using one-way ANOVA with Bonferroni post-test, in comparison with Nx group. For statistical analysis, only the last week of each sub-group (Nx, CIH or CIH+CH/CV) was used. **mg**: milligram; **Nx**: normoxia.

4. Discussion

Experiments to address how CIH modifies AHR-CYP1A1 pathway were performed using a rat model exposed to a mild paradigm of CIH for 21 days. This represents the time needed for HTN development in this model. Animals kept 21 days in normoxia (Nx) were used as control group. *Cyp1a1* mRNA expression was used as a sensitive indicator of AHR activation and assessed in several tissues (kidney cortex, kidney medulla, liver and visceral adipose tissue). Then we validated the protocol investigating the mRNA expression of classical biomarkers of CIH including HIF, NF-kB and the renin-angiotensin-aldosterone (RAA) pathway that have also been shown to be related to AHR. As AHR not only regulates xenobiotic metabolism, we have also analyzed several panels of markers of AHR-related processes such as fibrosis [39], EMT [35], inflammation and gluconeogenesis [40].

The identification of new pathways related to OSA-associated HTN is needed to deal with the high prevalence of resistant hypertension in this population. Our hypothesis was based on the assumption that the AHR pathway is activated by CIH and that this can contribute to HTN associated to this condition. Overall, the results confirmed our initial hypothesis and provided new insights about AHR signaling linking different co-morbidities associated to OSA. In fact we demonstrated that CIH overexpresses the classical AHR target gene, the xenobiotic metabolizing enzymes (*Cyp1a1*), particularly in renal tissues. Moreover, blockade of the AHR with CH-

223191 prevented and reverted the HTN induced by CIH. The overexpression of EMT genes found in the kidney of animals exposed to CIH is also in line with an endogenous activation of the AHR pathway by CIH. The increase in *Pepck1* mRNA-expression (the rate-controlling step of gluconeogenesis) also suggests that AHR pathway might also be linked with other comorbidities associated to CIH such as glucose intolerance. In contrast, the inflammation of the visceral adipose tissue induced by CIH might represent an AHR-independent event, because it showed a completely different pattern of genes induced by CIH: no change in the typical AHR pathway induced genes; a selective overexpression of *Il6* without overexpression of the canonical RelA/NF-kB activation and an overexpression of the *p49/p10*0 NF-kB. We also found a remarkable increase in the mRNA expression of *Hif3a* in the kidney. This finding suggests that CIH might stimulate the inhibitory control of HIF3α over HIF-1/2α or that HIF3α has transactivation activity partially overlapping that of HIF-1α, as already proposed by others [41-43].

Inference of AHR activation was mainly based on the assumption that CYP1A1 is a hallmark of AHR activation [19] and that CYP1A2 and CYP1B1 are also regulated in an AHR- independent manner [44]. To the best of our knowledge, the effects of CIH on CYP1A1 have never been addressed. A decrease (0.5-fold) in hepatic CYP1A2 mRNA and protein levels caused by CIH was described in mice by Zhang and co-authors [45], while in rats CYP1A2 mRNA did not change. AHR mRNA expression was slightly increased in kidney and liver. Its binding partner, shared with HIF family pathways, ARNT, was not upregulated by CIH in the kidney, but exhibit increased expression in liver (2.1-fold) and visceral adipose tissue (2.4-fold). The meaning of these results is unknown once the regulation of ARNT expression is far from being elucidated.

As CIH seems to activate the AHR pathway, we logically performed *in vivo* experiments with an antagonist of the receptor to assess the effects of the AHR on the development of HTN. CH-223191 has been developed in 2006 and shown to prevent activation of the AHR by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or other related halogenated aromatic hydrocarbons (HAHs) [30, 46]. More recently the first *in vivo* study in rats showed that an oral administration of CH-223191 (8 $mg.Kg^{-1}$) reversed the development of pulmonary HTN caused by the AHR agonist Sugen, followed by exposure to sustained hypoxia, normalizing as well pulmonary CYP1A1 expression [31]. The phenotype of sustained hypoxia differs from CIH because it has been associated to pulmonary HTN mediated by HIF-1α stabilization, not causing systemic HTN [47]. In contrast, the high prevalence of cardiovascular disease in OSA patients has been attributed to a predominant activation of inflammatory (e.g. NF-kB) over HIF-1 dependent-transcription in experimental models of CIH and in OSA patients [12]. In fact, the increase of inflammatory cytokines, such as interleukin 6 (Il6) and tumor necrosis factor α (TNF-α), was already reported in plasma and kidney in several CIH models (40 cycles/hour, 5-21% of O_2, 28 days and 60 cycles/hour, 6-21% of O_2, 60 days) [48, 49]. These *in vivo* results are also in accordance to what was observed in OSA patients [50, 51].

However, the finding that the AHR antagonist reverses both pulmonary [31] and systemic HTN (present work) point to the need of an in-depth understanding of HIF-AHR interactions in conditions associated to hypoxia.

The effects of CIH on HR demonstrate some inconsistencies. In fact, both the seminal works of Fletcher's group [34, 52] and a more recent one [33] found no changes in HR in animals submitted to 35 days of CIH, but other works found elevated values of HR in both male [53] and female [54] rats exposed to CIH during 35 days. In all the studies, HR recordings were obtained

475 in conscious rats, however, while the two former studies used implanted telemeters/catheters and
476 daily monitoring, the later analyzed HR through intra-femoral catheters introduced at the end of
477 the CIH exposure, in anaesthetized animals.
478 We have paid particular attention to the kidney due to its pivotal importance in BP regulation,
479 because renal sympathetic nerve ablation has been a common strategy to control resistant HTN
480 [55, 56] and because the effects of CIH have been poorly investigated in the kidney. A few
481 studies have addressed the renal effects of CIH in animal models [10, 36, 49, 57]. Long-term IH
482 exposure (2-4 months) induces kidney damage, with the presence of renal inflammation,
483 oxidative damage, endoplasmic reticulum (ER) stress, glomerular hypertrophy, mesangial matrix
484 expansion, increased expression of glomerular growth factors (TGF-β1, CTGF and VEGF) cell
485 death and fibrosis [57]. These observations were also reported by others [48, 58].
486 The added value of the present work is to note that the link between CIH and ER stress is already
487 apparent in the kidney and liver after 21 days of CIH exposure and to highlight the putative
488 association of EMT/fibrosis caused by CIH with the endogenous activation of AHR and *Cyp1a1*
489 overexpression.
490 The effect of CIH on body weight herein observed was expected and consistent with CIH
491 causing a reduction in rats' growth curve, an effect well established in this animal model [33,
492 59]. However, our results suggest that the decrease in body weight can be attributed to a decrease
493 in appetite/food intake and not only to a higher metabolism and energy expenditure as proposed
494 before by others [60]. Apparently, the growth delay and reduction in food consumption caused
495 by CIH are not mediated by activation of AHR, since CH-223191 (5 $mg.Kg^{-1}.day^{-1}$, 2 weeks) did
496 not modify it. In contrast, CH-223191 (10 $mg.Kg^{-1}.day^{-1}$, 5 weeks) suppressed severe wasting
497 with significant weight loss characteristic of AHR agonists, such as TCDD [30]. This

498 inconsistency might reflect the need of higher doses of CH-223191. As a consequence, different
499 doses and intensities of CIH exposure should be further investigated before discarding the
500 participation of AHR activation on the metabolic effects triggered by CIH**.** In the present work
501 we found an increase in liver and kidney mRNA expression of *Pepck1,* a major regulator of
502 hepatic gluconeogenesis controlled by AHR [61, 62] among others. Although we ignore at which
503 extent this increase in PEPCK involves changes in AHR activation, it seems that CIH shares with
504 AHR agonists a decrease in appetite but the opposite effects in PEPCK activity [55, 57]. The
505 increase in *Pepck1* expression caused by CIH (21 days) might explain why animals submitted to
506 CIH show increased levels of insulin [9, 63] and is consistent with the pattern of intolerance to
507 glucose observed in sleep apnea patients [64]. These results support not only the activation of
508 AHR pathway by CIH but also its participation in CIH-cardio-metabolic comorbidities other than
509 HTN. However, since renal PEPCK is also regulated by other transcriptional factors such as NF-
510 kB [65], we cannot exclude the involvement of other pathways.
511 This study has several limitations related to the experimental model used (whole animal *in vivo*)
512 that is more oriented to functional drug effects than to characterization of molecular mechanisms.
513 Although we have provided evidence that CIH stimulates AHR signaling, particularly in the
514 kidney, we cannot state that the anti-hypertensive effect of the AHR antagonist, CH-223191 was
515 mediated by a direct effect on the kidney. CH-223191 is a highly lipophilic drug, and we did not
516 investigate the effect of CIH on AHR pathway in the central nervous system and carotid body,
517 key players in BP control. Other limitations of the study include the use of only one antagonist in
518 a single dose and a relative short time exposure to CH-223191 and the lack of data on protein
519 quantification of AHR hallmarks. Anyway, the functional effect of CH-223191 in HTN is
520 relevant enough to support further exploitation of the mechanisms behind.

In conclusion, the pharmacological blockade of AHR had a significant impact reverting already established HTN caused by CIH without hypotensive effects in normoxic conditions. We also noted that AHR pathway might also be a common link with other comorbidities associated to CIH such as glucose intolerance.

Systemic HTN is a major cause of resistant HTN in OSA patients. Knowing the increasing prevalence associated with this condition, the research on novel therapeutic approaches to treat this condition is of utmost importance. Thus, our work provides an innovative view over the molecular mechanism of HTN-CIH, presenting AHR signaling as a new player in the pathophysiology of HTN-CIH, calling upon more research efforts to unveil the exact mechanisms linking AHR with HTN-CIH.

Authors contribution

Designed the research: CT-SP-EM-RB-XC; Performed research: NC-CT-CD-MJC; Analyzed data: NC-CT-SP-RB-EM-XC; Wrote the paper: NC-SP-EM-XC.

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Declarations of interest: none

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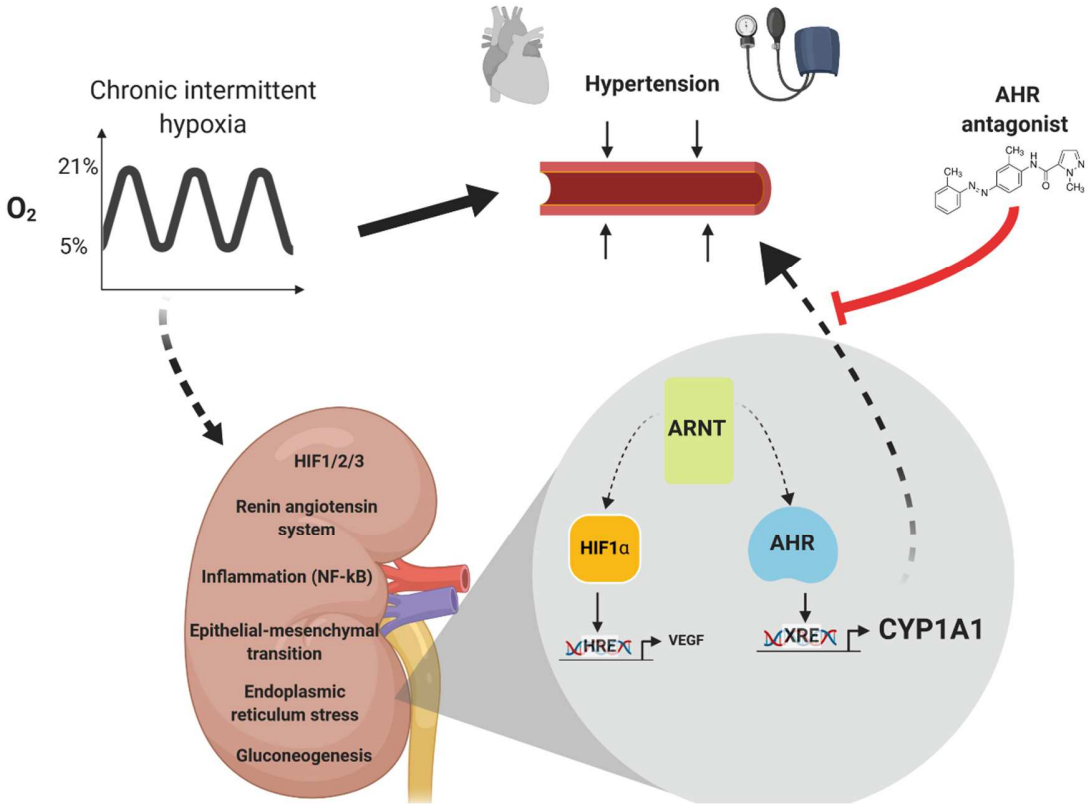
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Chronic intermittent hypoxia
21%
5%
O2
Hypertension
AHR antagonist
HIF1/2/3
Renin angiotensin system
Inflammation (NF-kB)
Epithelial-mesenchymal transition
Endoplasmic reticulum stress
Gluconeogenesis
ARNT
HIF1α
AHR
HRE
VEGF
XRE
CYP1A1