Toxicology Reports 8 (2021) 1599–1606

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Angiotensin II type 1 receptor is involved in hypertension and vascular alterations caused by environmental toxicant hexachlorobenzene

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ARTICLE INFO

Handling Editor: Dr. Aristides M. Tsatsakis

Keywords:
Aorta
AT1
Hexachlorobenzene
Hypertension
Losartan

ABSTRACT

Environmental hexachlorobenzene (HCB) increases blood pressure (BP) in female rats, causing alterations in arterial structure and function. Here we study the role of Angiotensin II receptor type 1 (AT1) in HCB-induced hypertension through the use of AT1 antagonist losartan.

HCB-treated male rats showed a 22.7% increase in BP which was prevented by losartan. Losartan blocked HCB-induced changes in arterial morphology (decreased aorta cell number and increased wall thickness). Losartan also prevented HCB-induced alterations in artery relaxation by acetylcholine and nitroprusside but not the reduction in the maximum contraction by phenylephrine. Losartan rescued arterial molecular alterations caused by HCB (i.e. an increase in TGF-β1 and AT1 expression and a decrease in eNOS expression and nitrite levels) and reduced hydrogen sulfide plasma concentration.

In conclusion: in this work we demonstrate that AT1 activity is involved in HCB effects on the vascular system leading to hypertension.

1. Introduction

Hexachlorobenzene (HCB) is a persistent and bioaccumulative dioxin-like environmental pollutant which was once used as a fungicide in crop farming but is currently released into the environment as a by-product of industrial processes. HCB has been classified as a persistent organic pollutant (POPs) [1] and is also known to be hepatotoxic, immunotoxic and an endocrine disruptor [2,3].

Hypertension constitutes a significant risk factor for cardiovascular disease and affects one out of three adults in urban areas [4]. Despite the large body of research exploring the causes that lead to hypertension, only 5% have an identifiable cause [5]. In this sense a direct relationship between POPs and arterial hypertension has been demonstrated [6].

In particular, the effect of HCB on the human cardiovascular system has not been documented yet. Some reports have indeed demonstrated that human exposure to polychlorinated biphenyls (PCBs) and other chlorinated compounds, including HCB, produces an increase in systolic blood pressure (BP) [7,8]. Nevertheless, evidence that this increase in BP is exclusively due to HCB exposure is still inconclusive.

In this context, previous work by our group studied the effect of subchronically administered HCB on the cardiovascular system of rats and demonstrated for the first time an increase in systolic BP, which allowed us to classify this toxicant as a hypertensor [9]. We also showed that HCB caused morphological and functional alterations in arteries.

Abbreviations: Ach, acetylcholine; AhR, aryl hydrocarbon receptor; Ang II, angiotensin II; AT1, angiotensin II receptor type 1; BP, systolic blood pressure; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; HCB, hexachlorobenzene; H&E, hematoxylin and eosin; PCBs, polychlorinated biphenyls; Phe, phenylephrine; POPs, persistent organic pollutant; SNP, nitroprusside; TGF-β1, Transforming Growth Factor-β1.

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https://doi.org/10.1016/j.toxrep.2021.08.009
Received 17 May 2021; Received in revised form 4 July 2021; Accepted 28 August 2021
Available online 31 August 2021

HCB acts, at least in part, through the aryl hydrocarbon receptor (AhR) and increases arterial angiotensin II (Ang II) receptor type 1 (AT1) among other molecules involved in BP regulation [9]. AhR activity has been proven necessary to maintain normal basal BP levels, as AhR-knock-out mice exhibit a reduction in BP [10]. The activity of this receptor has also been shown to control BP through increased AT1 expression in aorta [10].

Our hypothesis is that the AT1 receptor is involved in the mechanism of action of HCB that leads to arterial hypertension. Therefore, the aim of our work is to establish the possible participation of AT1 in the toxic effect of HCB using losartan (2-butil-4-cloro-1-{[2′-(1H-tetrazol-5-yl) bifenil-4-il]metil}-1H-imidazol-5-il)methanol), a selective nonpeptide antagonist of AT1 [11] widely used in clinical practice for several cardiovascular diseases including essential hypertension, heart failure and cardiac infarction.

2. Materials and methods

2.1. Chemicals

HCB (>99% purity), phenylephrine (Phe), acetylcholine (Ach) and nitroprusside (SNP) were purchased from Sigma-Aldrich Co (St Louis, MO, USA). Reverse transcriptase, deoxynucleotide triphosphates (dNTPs), random primers, Taq enzyme polymerase and molecular weight markers were purchased from Biodynamics SRL (Buenos Aires, Argentina). Primers for TGF-β1 and ribosomal protein L19 (L19) were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Anti-proliferating cell nuclear antigen (PCNA) (code: A3271) and anti-β-actin immunoglobulin (code: A1978) were from Sigma-Aldrich Co. Anti-AT1 (code: ab124734) and anti- endothelial nitric oxide synthase (eNOS) (code: ab252439) were purchased from Abcam Inc. (Cambridge, UK). Anti-rabbit IgG (code: 156831), goat anti-mouse IgG (code: 31430), and polyvinylidene difluoride membranes (PVDF) were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). CP-BU plates were purchased from Agfa Gevaert S.A. (Buenos Aires, Argentina). Losartan was generously provided by GADOR S.A. (Buenos Aires, Argentina). All other reagents were of molecular biology grade and at least 99% purity.

2.2. Animals

All procedures involving animals were conducted according to the principles of the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and protocols approved by the Institutional Committee of Animal Care and Use at the University of Buenos Aires (CICUAL, School of Medicine).

Wistar rats were kept at 21 ± 2 °C and (65 ± 5%) relative humidity with a 12 -h light–dark cycle and had access to tap water and food (Purina chow) *ad libitum*.

2.3. Experimental protocol

Twenty four male Wistar rats (211.1 ± 4.7 g at the onset of the experiment) were divided into the following experimental groups: 1) HCB: gavage-administration of 5 or 500 mg/kg HCB as a suspension in water containing Tween 20 (0.5 mL/100 mL) 3 days a week [12]; 2) losartan: administration in drinking water of 30 mg/kg losartan per day and gavage-administration of Tween 20 (0.5 mL/100 mL) 3 days a week in the same volumes used in group 1; 3) HCB + losartan: gavage-administration of 500 mg/kg HCB as a suspension in water containing Tween 20 (0.5 mL/100 mL) 3 days a week in combination with administration in drinking water of 30 mg/kg losartan per day; 4) control: gavage-administration of Tween 20 (0.5 mL/100 mL) 3 days a week in the same volumes used in group 1. All treatments were administered for 45 days. Six rats per group were used.

The HCB doses were selected on the basis of its endocrine disruptor character and the importance of thyroid hormones (TH) in BP. The lower 5 mg/kg dose is known not to alter TH levels, while the higher 500 mg/kg dose is known to generate hypothyroxinemia [12]. The administration of 500 mg/kg HCB for 45 days rendered a serum concentration similar to the HCB-exposed population [13,14].

The orally administered dose of losartan was chosen on the basis of previous studies using losartan to reduce BP in rats [15–17].

2.4. Blood pressure

Animals were acclimatized to handling in order to reduce stress and prevent variability in BP measurement.

Systolic BP was measured by anesthesia-free tail cuff plethysmography between 11 h a.m. and 12 h a.m. at 7, 16, 22, 30 and 45 days of treatment as indicated in Castilla et al., 2018 [9]. In all cases the the compression interval was less than 20 s. The average of at least 3 readings per session was recorded.

2.5. Sample handling

Rats were euthanized under anesthesia with i.p. 100 mg/kg b.w. Euthanyl® (solution of sodium pentobarbital and sodium diphenylhydantoin). Blood was extracted by ventricular puncture and serum was separated. Aortic tissue was dissected and processed.

2.6. Western blotting

Aortic tissues were homogenized in lysis buffer (10 mM Tris-HCl pH 7.4, 50 mM 2-Mercaptoetanol, 150 mM NaCl, 1 mM PMSF, 50 pg/ml leupeptin, 10 pg/ml pepstatin A and 2 mM EDTA) and then separated by 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto polyvinyldifluoride (PVDF) membranes in a semidry cell transfer at 18 V for 1.5 h. After block overnight at 4 °C with 5% non-fat dry milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 0.5% Tween 20, 150 mM NaCl), the membranes were incubated overnight with the specific primary antibodies: anti-PCNA (1:500), anti-AT1 (1:500), anti-eNOS (1:500) or anti-β-actin (1:500). Then the membranes were washed three times with TBST and incubated with suitable peroxidase-conjugated secondary antibodies: anti-rabbit IgG (1:1000), goat anti-mouse IgG (1:1000) for 2 h. After washing, blots were developed using an ECL detection kit (Amersham Biosciences, Inc., Little Chalfont, UK). The integrated optical densities (IOD) of bands obtained were quantified using Image Quant 5.2. Inc. software.

2.7. Semiquantitative RT-PCR

Total arterial RNA was extracted using Trizol following the manufacturer's instructions (Life Technologies). The reverse transcription was carried out using 2 μg of total RNA and the cDNAs generated were amplified by PCR under optimized conditions using the following primers:

Transforming Growth Factor-β1 (TGF-β1):
Forward: 5′-CTGCTGGCAATAGCTTCCTA-3′
Reverse: 5′-CGAGCCTTAGTTGGACAGGAT-3′
L19: used as housekeeping gene
Forward: 5′-CGTTCGAGAAGCTCTCTGG-3′
Reverse: 5′-ATCACTACACTGGTGCTGTA-3′

The amplification for each mRNA was in the linear range of PCR amplification.

The PCR products were separated by 1.5% (w/v) agarose gel and stained with 1 μg/ml ethidium bromide. The gel images were acquired with the GelPro analyzer (IPS, North Reading, MA, USA). The levels of mRNA were quantified using ImageQuant 5.2 image analysis software. The results for TGF-β1 mRNA were normalized to L19 mRNA as internal control.

2.8. Vessel preparation and isometric tension recording

The aorta was excised and transferred to a dish filled with Krebs–Henseleit buffer (pH: 7.4; composition in mM: 115.3 NaCl, 4.9 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.46 $CaCl_2.2H_2O$, 0.004 EDTA, 11.1 glucose, 25 $NaHCO_3$ and 0.11 ascorbic acid). After remotion of adhering tissue, the arterial segments were cut into 3 mm-long rings then suspended in organ baths containing Krebs solution constantly bubbled with a carbogenic mix (5% CO_2, 95% O_2) at 37 °C. Aortic intact rings were maintained at 20 mN of basal tension and equilibrated for 1 h. An isometric force transducer (model FT03 B, Grass Instrument Company, West Warwick, RI, USA) coupled to a polygraph (model RPS 7C8, Grass Instrument Company) were used to record aortic ring contraction and relaxation.

The response to cumulative additions of Ach (10^{-9} to 10^{-5} M) or SNP (10^{-9} to 10^{-6} M) on the isometric tone of aortic rings was evaluated in arteries contracted with a submaximal dose of Phe (10^{-7} M). The results were expressed as a percentage of the pre-contracted tone. The response to cumulative concentrations of Phe was also analyzed in intact artery rings. Contractile responses are expressed as a percentage of the maximum response produced by 60 mM KCl. Maximal effect and pD2 (–log EC_{50}) were also calculated using GraphPad Prism version 5 (GraphPad, San Diego, CA, USA).

2.9. Histological evaluations

Aorta samples (5 mm width) were fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. Samples were serially sectioned in transversal orientation and stained with hematoxylin and eosin (H&E) or Masson's trichrome. Planimetry was performed using image analysis software (Image J, National Institute of Health, Bethesda, MD, USA) in digitalized sections.

Arterial wall thickness was evaluated in 10 slices of the same sample, and at least 5 measurements were done of each photograph.

2.10. Plasma hydrogen sulfide determination

To measure plasma hydrogen sulfide (H_2S) concentration, blood was collected in an EDTA anti-coagulated tube and processed according to Suzuki K. et al. [18]. Briefly, plasma samples (100 µl) were added to sealed tubes containing 200 µl zinc acetate (1% w/v). Then, 100 µl N, Ndimethyl-p-phenylenediamine sulfate (20 mM, in 7.2 M HCl) and 133 µl $FeCl_3$ (30 mM, in 1.2 M HCl) were added, and tubes were dark-incubated for 30 min at 37 °C. Afterwards, samples were centrifuged at 5000*g* for 10 min. The absorbance of the resulting solution was measured at 670 nm. H_2S (Sigma-Aldrich) was used to obtain the calibration curve.

2.11. Tissue nitrite determination

Nitric oxide (NO) levels were indirectly determined by their conversion to nitrites, using the standardized Griess method based on the chemical diazotization reaction [19].

Previously, arterial homogenates were deproteinized [20] by dilution with ethanol 1:7 (sample:ethanol v/v). Precipitated proteins were separated by centrifugation at 1000 × *g* for 20 min and the supernatant was used for nitrite determination.

The Griess Reagent System uses sulfanilamide and N-naphthyl ethylenediamine dichloride under acidic conditions (phosphoric acid). Griess reagents were purchased from Promega (Madison, WI, USA) and used according to the manufacturer's recommendations.

2.12. Statistical analysis

Data were analyzed by one-way or two-way analysis of variance (ANOVA) followed by post hoc tests (multiple comparison Tuckey's test and Dunnett's test) as appropriate. $p < 0.05$ was considered statistically significant. SPSS™ version 15.0 software was used for data analysis. Maximal effect and pD_2 (–log EC50) were also calculated using GraphPad Prism version 5 (GraphPad Software Inc., Philadelphia, USA).

3. Results

3.1. Blood pressure

In order to establish HCB effect on BP in male rats, animals were treated with 5 or 500 mg/kg HCB for 45 days and systolic BP was evaluated at different times during treatment.

As shown in Fig. 1, no differences were found in systolic BP across experimental groups at the beginning of the study. BP peaked at 30 days in animals treated with the lowest dose of HCB, but showed a significant increase at 30 days which remained until 45 days in animals treated with the highest dose (22.9% vs control, $p < 0.01$). On the basis of these results, we used 500 mg/kg HCB to evaluate whether losartan might prevent this effect when simultaneously administered. At a dose of 30 mg/kg/day, losartan was effective in preventing the elevated BP induced by HCB treatment (Fig. 1).

Of note, no differences were observed in rat body weight across groups (control: 489 ± 16, HCB 5 mg/kg: 454 ± 13, HCB 500 mg/kg: 463 ± 16, HCB 500 mg/kg + losartan: 442 ± 18, losartan: 465 ± 03). In addition, no deaths occurred during the experiment in any of the groups.

3.2. Arterial remodeling

It is known that hypertension can alter the vascular wall and produce biochemical and morphological changes. In order to investigate whether AT1 is involved in this effect in HCB-induced hypertension, arterial wall thickness from HCB and HCB + losartan was analyzed in aorta slices stained with Masson's trichrome and H&E.

Representative microphotographs showing aortic wall are shown in Fig. 2A. After 45 days of HCB treatment a significant increase (36.3%, $p < 0.05$) in intima-media layer thickness was observed compared to control rats (Fig. 2B).

In contrast, arterial wall thickness from rats treated simultaneously with HCB and losartan showed no changes in their morphology as compared to control rats (Fig. 2A and B). Worth mentioning, no arterial fibrosis was observed in any of the groups.

In addition, we counted the number of cell nuclei per area in the arterial wall. Results revealed a decrease (38.1%, $p < 0.05$) in animals treated with HCB which was prevented by simultaneous losartan treatment (Fig. 2C).

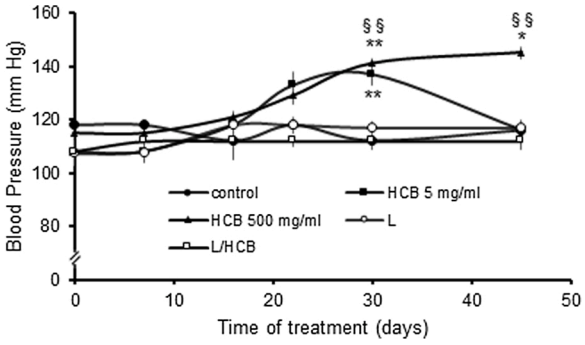

Fig. 1. Systolic BP. Effect of HCB on systolic BP in male rats. Systolic arterial pressure was measured in animals treated with vehicle, 5 mg/kg HCB or 500 mg/kg HCB, 30 mg/kg/day losartan alone or simultaneously with 500 mg/kg HCB at the indicated days of treatment. The kinetics of systolic BP evolution is shown. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$ vs control. §§ $p < 0.01$ vs. HCB/losartan Two-way ANOVA (n = 6 per group).

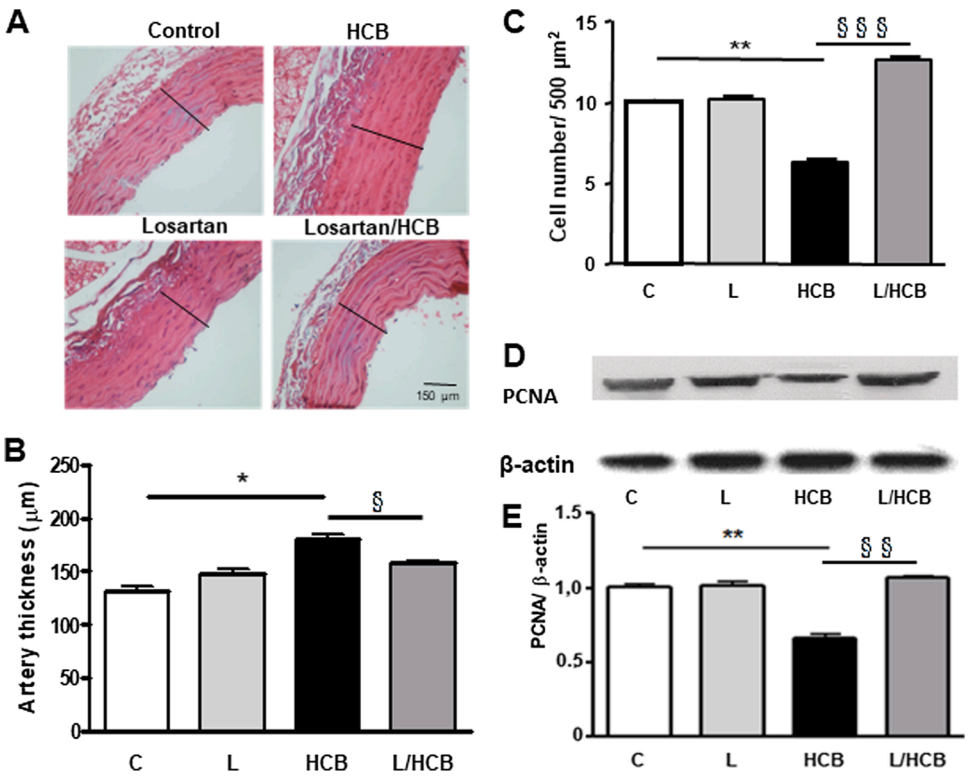

Fig. 2. Arterial remodeling. Arteries from control (C), losartan (L), HCB and losartan-HCB (L/HCB)-treated animals were stained with Masson's Trichome. Panel A: Representative microphotographs are shown. Panel B: quantification of artery wall thickness measured using Image J software (bars indicate how measurements were done). Panel C: quantification of nuclei number of arterial VSMC, Panel D: shows a representative western blot of PCNA, Panel E: quantification of the PCNA protein levels by densitometric scanning of immunoblots. Values are means ± SEM. * $p < 0.05$, **$p < 0.01$ respect to control and, § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ respect to HCB (One-way ANOVA) (n = 6 per group).

Furthermore, we studied PCNA expression as an index of cell proliferation [21] (Fig. 2D). Aortae from HCB-treated rats exhibited a decrease (35.71%, $p < 0.05$) in PCNA expression (Fig. 2E), while aortae treated simultaneously with HCB and losartan showed no differences from control.

3.3. Aorta function

The contractile response of aorta rings to Phe and the relaxation response to Ach and SNP were evaluated as a measure of arterial function (Fig. 3). Arteries from HCB-treated rats showed lower maximum contraction by Phe ($p < 0.05$ vs control), which remained altered in losartan- ($p < 0.05$ vs control) and HCB + losartan-treated animals (control: 151.1 ± 6.4%, HCB: 128.1 ± 6.6%, HCB + losartan: 126.4 ± 6.44%, losartan: 120.1 ± 9.4, $p < 0.05$) (Fig. 3A). However, no differences in pD_2 were observed across groups (control: 6.63 ± 0.19, HCB: 6.26 ± 0.09, HCB + losartan: 6.41 ± 0.09, losartan: 6.70 ± 0.13) (Fig. 3A).

In addition, aortae from rats intoxicated with HCB showed a decrease in maximal relaxation by Ach stimulus ($p < 0.01$ vs control) which was not observed in arteries from control, losartan, or HCB + losartan rats (control: 90.5 ± 6.1%, HCB: 61.5 ± 4.3%; HCB + losartan: 93.6 ± 4.7%; losartan 91.2 ± 5.7) (Fig. 3B). Also, no differences were detected in pD_2 across groups (control: 6.4 ± 0.23; HCB: 6.26 ± 0.23; HCB + losartan: 7.07 ± 0.18; losartan: 6.65 ± 0.13) (Fig. 3B).

The analysis of endothelium-independent relaxation by response to SNP showed a right shift in the response curve in aorta rings treated with HCB ($p < 0.05$ vs control) (Fig. 3C). In contrast, this effect was not detected in arteries from control, losartan, or HCB + losartan animals (pD_2: control: 8.21 ± 0.22; HCB: 7.56 ± 0.19; HCB + losartan: 8.28 ± 0.10; losartan 8.65 ± 0.27). In addition, no differences were observed in maximal relaxation (control: 101.7 ± 1.3%, HCB: 100.9 ± 1.5%; HCB + losartan: 98.1 ± 3.4%; losartan 101.0 ± 1.8%) (Fig. 3C). For both Emax and pD_2 analysis one-way ANOVA test was used.

3.4. Key molecules involved in BP regulation

As TGF-β1 cytokine and AT1 are regulators of BP, we analyzed their arterial expression by semiquantitative RT-PCR and western blot, respectively (Fig. 4). Rats treated with HCB showed a significant increase in TGF-β1 mRNA levels (40%, $p < 0.01$) which was not observed upon combined treatment with losartan (Fig. 4A). In addition, analyses of AT1 expression levels showed, as expected, a 66.6% increase ($p < 0.05$) in arteries from HCB-treated rats but not in rats undergoing combined treatment (Fig. 4B).

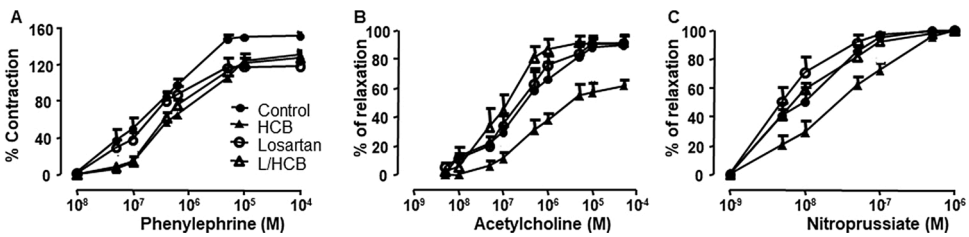

Fig. 3. Arterial function. The arterial function was studied in aorta rings from control (●), HCB (▲), losartan (○) and losartan/HCB (△)-treated rats Panel A: contraction by Phe. Panel B: relaxation by Ach in previously precontracted aorta by Phe. Panel C: relaxation by SNP in previously precontracted aorta rings by Phe. Data represent mean ± SEM (n = 6 per group).

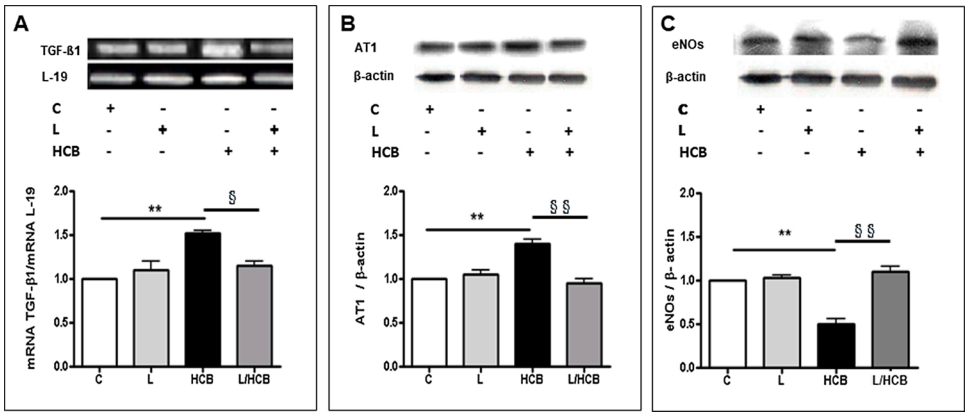

Fig. 4. TGF-β1, AT1 and eNOS expression These molecular markers were analyzed in arteries from control (C), losartan (L), HCB and losartan−HCB (L/HCB)- treated rats. Panel A: representative pattern of semiquantitative RT-PCR amplification of TGF-β1 and L19 cDNA and the quantification of cDNA normalized to L19 cDNA. Panel B: representative Western blot of AT1 and the quantification of Western blot AT1 IOD normalized to the corresponding β-actin signal. Panel C: representative Western blot of eNOS and the quantification of eNOS IOD normalized to the corresponding β-actin signal. Data are normalized to the average value of control and represent the mean ±SEM. **p < 0.01 respect to control and, § p < 0.05, §§ p < 0.01 respect to HCB (One-way ANOVA) (n = 6 per group).

On the other hand, we evaluated the effect of losartan on eNOS protein level in rats treated with HCB. HCB produced a 48% ($p < 0.01$) decrease in eNOS expression, while losartan prevented this reduction, rendering eNOS expression levels similar to control (Fig. 4C). In addition, arterial NO concentration was indirectly evaluated by means of nitrite determination (Fig. 5A). HCB decreased nitrite levels by 70% ($p < 0.001$) compared to control. However, concomitant treatment with losartan prevented this reduction, maintaining levels similar to control (Fig. 5A).

As Ang II-induced hypertension has been shown to decrease H_2S [22], we studied plasma H_2S concentration in HCB-treated rats. As shown in Fig. 5B, plasma H_2S levels were lower in HCB-treated rats than in control but remained at unaltered in HCB + losartan animals.

4. Discussion

This work focuses on the involvement of AT1 in the HCB mechanism of action in the cardiovascular system.

The effect of HCB on BP regulation was assessed using two different gavage doses for 45 days. BP was measured indirectly by the tail cuff method, which is considered less exact than a direct method involving a pressure transducer into the left carotid artery. However, Fritz et al., using BP measurement under conditions similar to those used in this work, have shown that only slightly lower values were obtained compared to directly measured BP [23].

Male rats exposed to the higher dose of HCB showed an sustained increase in systolic BP until 45 days. However, when rats were treated with the lower dose of 5 mg/kg HCB, BP transiently increased, which suggests the activation of compensatory responses leading to BP normalization. It is worthy of note that the evidence of the increase in systolic BP was evident after 30 days of HCB treatment suggests a long-term effect of this toxicant. These results are in line with those previously obtained in female rats by our group [9]. Moreover, the moderate HCB-induced increase in BP may find support in the results of an important study involving 7557 men exposed to pesticides, including HCB [7], although these findings fail to single-out HCB effects on BP.

Simultaneous treatment with 30 mg/kg losartan prevented the increase in BP, which indicates that AT1 is involved in the hypertensive effect of 500 mg/kg HCB.

Although it is well established that microvessels resistance is a key factor in BP regulation, we conducted the present study in aorta, given that the role of the renin-angiotensin system (RAS) in high capacitance vessels has been extensively studied and validated [24]. However, as arterioles express AhR [10], which mediates HCB action [9], HCB may be inferred to also affect these small vessels.

In this work, and as previously observed in arteries of 500 mg/kg HCB-administered female rats [9], male rat arteries showed alterations in arterial histology such as wall thickening a decrease in PCNA expression and the number of aorta nuclei. Altogether, these results indicate that wall thickening may be due to the hypertrophic effect of HCB on vascular smooth muscle cells (VSMC) and not to hyperplasic growth. The hypertrophic remodelling has been reported to predominate in secondary hypertension [25].

All effects stated above were prevented when losartan was added simultaneously with HCB treatment, which is in agreement with previous studies showing an important role for local RAS and its receptors in the control of structural and functional remodeling in the vasculature [26]. It is very well known that Ang II stimulates hypertrophy of VSMC through AT1 both *in vitro* and *in vivo* [27]. Furthermore, elevated Ang II is known to cause VSMC apoptosis [28].

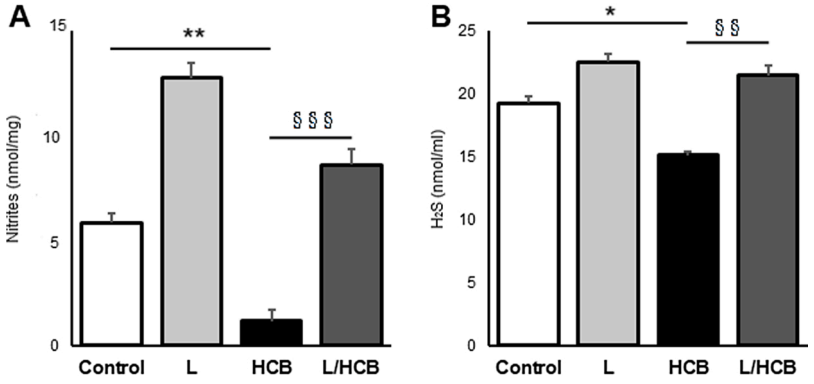

Fig. 5. Gasotransmitters. Arterial NO concentration was indirectly estimated by arterial nitrite levels. Panel A: nitrite quantification. Panel B: plasma H_2S concentration. Samples analyzed were from control (C), losartan (L), HCB and losartan + HCB (L/HCB)-treated rats. Data represent mean ± SEM. * $p < 0.05$, ** $p < 0.01$ respect to control, § $p < 0.05$, §§§ $p < 0.001$ respect to HCB (One-way ANOVA) (n = 6 per group).

As stated above, we previously demonstrated that HCB acts through AhR [9]. AhR activity has been proven necessary to maintain normal basal BP levels, as AhR-knock-out mice exhibit a reduction in BP [10]. The activity of this receptor has also been shown to control BP through an increase in AT1 expression in aorta [10]. In the current work, HCB increased AT1 enhancing Ang II sensitivity which produced both an increase in BP and vascular remodeling. The latter could may be regarded as independent of hypertension produced through mechanisms other than AT1, although this point deserves further analysis.

An increase in BP can be triggered by alterations in arterial function, finely controlled by VSMC contraction and relaxation processes. In the current study a decrease in maximal response to Phe was observed in aortae from HCB-treated animals, in agreement with previous results obtained in both arteries from female HCB-treated rats [9] and from hypertensive patients [29]. This decrease in maximal response to Phe may hint at an imbalance between relaxing and contractile molecules or a toxic effect of HCB on the contractile machinery of VSMC. It is worth highlighting that losartan did not prevent the decrease induced by HCB and, moreover, induced per se a reduction in maximal response to Phe, as previously reported [30]. This evidence leads us to infer that the reduced response to Phe is independent of high BP.

In addition, our results have shown that the endothelium-dependent relaxation induced by Ach is impaired in aortic rings from HCB-treated rats, which was effectively antagonized by concomitant losartan therapy. Hypertensive animals exhibit endothelial dysfunction due to shear stress and activation of RAS. In this situation, endothelial cells ability to release NO could be compromised, which triggers a failure in vascular tone modulation. In this sense, the reduction in eNOS expression observed in the HCB group could explain, at least in part, the failure in endothelium-dependent relaxation. Aditionally, the fact that losartan avoided HCB-relaxation alteration indicates a negative correlation between BP and relaxation. A similar observation has been previously reported in spontaneously hypertensive rats as a result of long-term BP elevation [31]. Moreover, AT1 blockage by losartan is known to favor Ang II binding to Ang II receptor type 2 (AT2), located on endothelial cells, which in turn increases NO-cGMP activity inducing vascular dilation [32]. Furthermore, vasodilation may also result from increased angiotensin (1–7) via the Mas receptor pathway [33], which contributes to the relaxation effects of losartan. In other words, losartan effectively decreases VSMC constriction and simultaneously increases endothelium-dependent dilation.

On the other hand, maximal relaxation induced by SNP, remained unaffected by treatments, although a significant change in sensitivity (pD2) was observed in arteries from HCB-treated rats. It is worth stressing that, a similar response pattern has been previously observed in aortic rings from Ang II-induced hypertensive rats [22]. In the current work, the decrease in relaxation was prevented by losartan treatment, which indicates the role of AT1 in HCB-induced modification of SNP sensitivity (pD2).

Furthermore, the analysis of arterial AT1 expression showed an increase in HCB-treated rats (Fig. 6), in agreement with results reported in our previous work [9] and in Ang II-induced hypertensive mice [22] and hypertensive rats [34]. In addition, an arterial increase in TGF-β1 was observed in HCB-treated rats (Fig. 6).

As stated above, treatment with HCB generates functional and structural alterations in the vasculature. Likewise, these alterations can affect the production, release, or function of vasoactive factors such as TGF-β1. Vasoactive factor alterations or production may respond to shear stress suffered by endothelial cells through blood flow when BP increases [35]. Another explanation could be that TGF-β1 is upregulated by Ang II through AT1 [36]. Even if we cannot rule out that TGF-β1 levels increase as a consequence of pressure, however unpublished data of our laboratory indicate that HCB can induce TGF-β1 in a pressure-independent way in endothelial cell cultures (Ea-hy 926) (data not shown).

In this study we demonstrated that the increase in both AT1 and TGF-β1 was prevented by losartan, which could be attributed to a decrease in BP and a consequent reduction in the physical stress targeting the vascular wall. However, a reduction in both AT1 and TGF-β1 expression by losartan has been reported independent of its hypotensive effect [36, 37].

In this work, a downregulation of eNOS expression was also observed in HCB-treated rats, which would indicate a reduction in NO levels. This was indirectly corroborated by means of Ach relaxation tests, as Ach acts through NO production, and also by the nitrite production (Fig. 6). In addition, a decrease was detected in plasma H_2S levels in HCB-treated rats which remained unchanged in the presence of losartan. H_2S is a gasotransmitter, vasodilator and regulator of BP which reduces oxidative stress [38,39]. In this sense, a reduction in H_2S production has been reported in the aorta of mice chronically infused with Ang II [22].

The decrease in eNOS expression in HCB-treated rats could be explained by the increase in TGF-β1 expression levels (Fig. 6). This cytokine negatively regulates ERα expression, which, in turn, is in part responsible of eNOS induction [40,41]. Furthermore, eNOS downregulation may be related to the decrease in H_2S, as H_2S donors increase eNOS expression [42] (Fig. 6). Moreover, the fact that losartan prevented the decrease in eNOS could be explained by its role in preventing the increment in TGF-β1 expression. In addition, it is well known that Ang II acting through AT2 −through which it acts in the presence of losartan− produces an increase in eNOS expression [43] which, in turn, explains the vasodilator effect of losartan.

Although further studies should be conducted to elucidate the mechanisms by which HCB induces AT1, both direct and indirect mechanisms may be thought to take part. A direct mechanism may

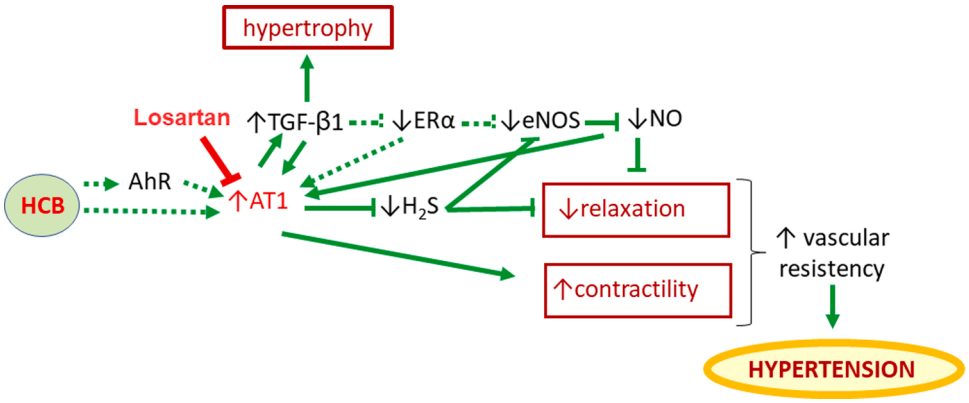

Fig. 6. Proposed mechanisms of action of HCB on the vascular system leading to hypertension. Solid lines represent the mechanisms studied in this work. Dotted lines represent mechanisms studied in previous reports. In this scheme, a vascular disbalance between relaxation and contraction is proposed as the main cause of hypertension developed by HCB intoxication.

involve AhR activation, as it has been demonstrated that this receptor activity produces an increase in AT1 expression in aorta [10,44] (Fig. 6). As an alternative mechanism, low density lipoprotein (LDL) may increase AT1 expression in VSMC [45], as HCB has been reported to produce an increase in serum cholesterol levels [46]. AT1 expression may then expand through positive feedback by TGF-β1 [44,47]. In addition, the decrease in NO and ERα induced by HCB [9] could also explain the increase in AT1 expression, as both NO and ERα are known to play inhibitory roles [48,49] (Fig. 6).

Conclusion: In this work is demonstrated for the first time that AT1 activity is involved in HCB effects on the vascular system leading to hypertension.

Sources of funding

This work was supported by grants from the Universidad de Buenos Aires, Argentina (20020190200194BA and 20020170200178BA).

Author contributions

RC and LA designed the study and acquired funds. GRC, ZD, SG, MIR and LA contributed to the experimental set-up and conducting the study. GRC, SG, PB produced and analyzed the data. GRC analyzed histopathology. RC and LA wrote the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Dr. Hernán Gómez Llambi for his helpful suggestions in this work.

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