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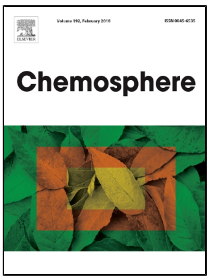

Environmental Pollutant Hexachlorobenzene Induces Hypertension In a Rat Model

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- Hexachlorobenzene (HCB) generates arterial hypertension in rats.
- HCB causes arterial remodeling.
- HCB produces alterations in arterial function.
- TGF-β1, ERα, eNOS, AT1 and DII are involved in the mechanism of HCB action.

ENVIRONMENTAL POLLUTANT HEXACHLOROBENZENE INDUCES HYPERTENSION IN A RAT MODEL

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Abbreviations

AhR: aromatic hydrocarbon receptor

Ach: Acetylcholine

AT_1: angiotensin II type1 receptor

BP: Systolic blood pressure

DII: deiodinase II

eNOS: endothelial nitric oxidide synthase

ERα: estrogen receptor alpha

HCB: Hexachlorobenzene

NA: noradrenalin

NO: nitric oxidide

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

TH: thyroid hormones

VSMC: vascular smooth muscle cells

ABSTRACT

Hexachlorobenzene (HCB) is a dioxin-like environmental pollutant, widely distributed in the environment. New research links exposure to high levels of persistent organic environmental toxicants to cardiovascular disease, however little is known about the effect of HCB on vascular function and on blood pressure. The purpose of the present study was to evaluate biochemical and cardiovascular changes resulting from subchronic HCB exposure. Adult female Sprague-Dawley rats were treated with vehicle or HCB (5 or 500 mg/kg b.w) for 45 days. Systolic blood pressure (BP), recorded by tail cuff plethysmography, was significantly increased at 35, 40 and 45 days of 500 mg/kg HCB-treatment. HCB (500 mg/kg) increased arterial thickness, while both 5 and 500 mg/kg HCB decreased proliferating cell nuclear antigen (PCNA) protein levels and cellular nuclei in abdominal aortas indicating a hypertrophic process. Also, aortas from both groups of HCB-treated rats presented higher sensitivity to noradrenalin (NA) and a significant decrease in maximum contractile response. Arteries from 500 mg/kg HCB-treated rats showed a significant increase in the levels of transforming growth factor-β1 (TGF-β1) mRNA and angiotensin II type1 receptor (AT_1), and a significant decrease in estrogen receptor alpha (ERα), endothelial nitric oxidide synthase (eNOS) protein expression and deiodinase II (DII) mRNA levels. In conclusion, we have demonstrated for the first time that subchronic HCB administration significantly increases BP and alters associated cardiovascular parameters in rats. In addition, HCB alters the expression of key vascular tissue molecules involved in BP regulation, such as TGF-β1, AT1, ERα, eNOS and DII.

Keywords: hexachlorobenzene, hypertension, aorta, TGF-β1, ERα, deiodinase II.

1. INTRODUCTION

Hypertension occurs in one out of three adults in urban populations and represents a significant risk factor for life-threatening cardiovascular disease (Mills *et al*. 2016). Nevertheless, and despite intensive research, only around 5% of cases have an identifiable cause (Carretero and Oparil, 2000). The etiology of hypertension is very heterogeneous and could involve exposure to environmental pollution as a risk factor (EPA, Environmental Protection Agency USA, 2002; Huang *et al.,* 2006).

Dioxins are among the most harmful environmental toxins for humans. The Public Health Statement for Dioxins (1998), reported that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) increases blood pressure (BP) and induces cardiac hypertrophy in laboratory animals (Kopf et al., 2008).

Hexachlorobenzene (HCB) is a widely distributed dioxin-like environmental pollutant. It has been used as a fungicide, and it is still released into the environment as a by-product in several industrial processes. It is very persistent and bioaccumulative, and belongs to the group of persistent organic pollutants (Xu et al., 2013). Chronic exposure of laboratory animals to HCB elicits a number of toxic effects including neurological symptoms, immunological disorders and endocrine disruption (ATSDR, 2002). However, its impact on the cardiovascular system has not yet been evaluated.

It has been reported that HCB alters the expression of molecules involved in BP regulation in several tissues, e.g. it affects the levels of growth factors, interleukins and cytokines such as transforming growth factor-β1 (TGF-β1) (Giribaldi *et al.*, 2011), which, in particular, has been associated to hypertension (Derhaschnig *et al*., 2002). This growth factor also alters estrogen receptor alpha (ERα) levels in several types of cells (Li *et al.*, 2008; Peña *et al.*, 2012). This receptor is in turn involved in BP regulation, increasing the production of

molecules like nitric oxidide (NO) with a relaxing action on vascular smooth muscle cells (VSMC) (Jobe *et al.*, 2013; Pedram *et al.*, 2002).

BP can be altered by an imbalance between relaxation and contraction processes. In this sense, TGF-β1 has been also reported to regulate the expression of angiotensin II type1 receptor (AT_1), the receptor by which angiotensin II acts as vasoconstrictor (Chaudhary and Chaudhary, 2017).

In addition, HCB is known to affect thyroid hormones (TH) homeostasis. In particular, modifications have been reported in the activity and expression of hepatic deiodinase DI and DII enzymes, respectively (Alvarez *et al.*, 2005; Kleiman de Pisarev *et al.*, 1990). TH have multiple effects on the cardiovascular system, including alterations in cardiovascular hemodynamics and the relaxation of coronary artery vascular smooth muscle cells (VSMC) (Gomberg-Maitland and Frishman, 1998).

Despite the evidence discussed above in different tissues, little is known about HCB effect on the expression of TGF-β1, ERα, endothelial NO synthase (eNOS), AT1 and DII in the vascular system. In this context, the aim of this study was to determine whether subchronic exposure of laboratory animals to HCB alters BP, focusing on its possible effects on vascular hemodynamics and the expression of key molecules involved in BP regulation.

2. MATERIALS AND METHODS

2.1. Chemicals

HCB (>99% purity) was purchased from Sigma-Aldrich Co. The reagents used for cDNA synthesis, reverse transcriptase, molecular weight markers, random primers, desoxi-nucleotide triphosphate (dNTPs), and Taq enzyme polymerase, were purchased from Biodynamics SRL (Buenos Aires, Argentina). Specific primers for TGF-β1 and glyceraldehyde 3-phosphate

dehydrogenase (GPDH) were purchased from Invitrogen Life Technology (Carlsbad, CA). Polyvinylidene difluoride membranes (PVDF), goat anti-mouse IgG, and anti-rabbit IgG were purchased from Bio-Rad Laboratories Inc. CP-BU plates were purchased from Agfa, (Gevaert, Argentina S.A). Anti-β-actin immunoglobulin and anti-proliferating cell nuclear antigen (PCNA) from Sigma-Aldrich Co., monoclonal anti-TGF-β1, anti-AT1 and anti-eNOS from Abcam Inc.. All reagents present purification degree of 96% and molecular biology grade.

2.2. Animals

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

Sprague-Dawley rats were maintained on a 12 h/12 h light/dark cycle in a temperature- (21 ± 2 °C) and humidity-controlled (65 ± 5%) environment. Animals had access to food (Purina chow) and tap water *ad libitum*.

2.3. Animals and treatment

Female rats (160 g at the onset of the experiment) were gavage-administered HCB (5 or 500 mg/kg) as a suspension in water, containing Tween 20 (0.5 ml/100 ml), 5 days a week for 45 days. Control animals received equal volumes of vehicle by the same route. Six rats per group were used.

2.4. Blood pressure

To reduce ambient variability in BP measurement, the animals were acclimatized to handling to reduce stress.

Systolic BP was measured between 11 and 12 h a.m., in rats treated with vehicle or HCB for 30, 35, 40, and 45 days, by anaesthesia-free tail cuff plethysmography. The average of at least 3 readings per session was recorded. A pneumatic pulse transducer positioned on the ventral surface of the tail, distal to the occlusion cuff, detected the return of the pulse following a slow deflation of the cuff using a programmed electro-sphygmomanometer PE-300 (Narco Bio-Systems, Austin, Texas). Pulses were recorded on a Physiograph MK-IIIS (Narco Bio-Systems, Austin, Texas).

2.5. Sample handling

Rats were sacrificed under anesthesia with an i.p. solution of sodium pentobarbital and sodium diphenylhydantoin (Euthanyl®) (100 mg/kg b.w.). Blood samples were obtained by ventricular puncture and plasma was separated. The aortic tissue was removed for further processing.

2.6. Western blotting

Total cellular protein lysates were electrophoresed in 10–12% SDS-polyacrylamide gel (SDS-PAGE), and then transferred to PVDF in a semidry transfer cell at 18 V for 1.5 h. Membranes were blocked for 1 h at 4 °C with 5% non-fat dry milk – 2.5% bovine serum albumin (BSA) in TBST buffer (10 mM Tris–HCl, pH 8.0, 0.5% Tween 20, 150 mM NaCl). Membranes were

incubated overnight at 4 °C with the specific primary antibody (1:500 anti-β-actin immunoglobulin (Sigma-Aldrich Co), 1:500 anti-proliferating cell nuclear antigen (PCNA) (Sigma-Aldrich Co.), 1:500 monoclonal anti-TGF-β1 (Abcam Inc), 1:250 anti-AT1 (Abcam Inc) and 1:500 anti-eNOS (Abcam Inc)) and then washed five times with TBST, and the suitable peroxidase-conjugated anti-specific antibodies were used for protein detection. After washing, blots were developed using and ECL detection kit (Amersham Biosciences, Inc., UK). The quantitative analysis of the integrated optical density (IOD) of bands was done using the Image Quant 5.2. Inc. software.

2.7. Semiquantitative RT-PCR

Total RNA from arteries was extracted using Trizol reagent following the manufacturer's instructions (Life Technologies, Inc.-BRL, Grand Island, NY). The reverse transcription and PCR analyses were made using 2 or 4 μg of total RNA. The cDNAs generated were further amplified by PCR under optimized conditions using the primer pairs listed below.

TGF-β1:

Forward: 5'-CTGCTGGCAATAGCTTCCTA-3'

Reverse: 5'-CGAGCCTTAGTTGGACAGGAT-3'

D II:

Forward: 5′-ACTCGGTCATTCTGCTCAAG-3′

Reverse: 5′-TTCAAAGGCTACCCCATAAG-3′

GPDH (glyceraldehyde 3-phosphate dehydrogenase):

Forward: 5'-ACCCAG AAG ACT GTG GAT GG-3'

Reverse: 5'-CAC ATT GGG GGT AGG AAC AC-3

161 The number of cycles used was optimized for each mRNA to fall within the linear range of
162 PCR amplification.

163 PCR products were resolved on a 1.5% (wt/vol.) agarose gel. The gel images were acquired
164 with the GelPro analyzer (IPS, North Reading, MA). The levels of mRNA were quantified
165 using a computer-assisted image analyzer (ImageQuant 5.2) and the PCR results for each
166 sample were normalized with GPDH mRNA as an internal control.

167

168 **2.8. Thyroid hormone assay**

169 Serum concentration of thyroid hormones were analyzed by RIA as previously described
170 (Obregon *et al.*, 1981).

171

172 **2.9. Vessel preparation and isometric tension recording**

173 The abdominal aortas were carefully dissected, free of connective tissue, immersed in Krebs
174 solution (KS: 130 mM NaCl, 4.7 mM KCl, 1.17 mM Na_2HPO_4, 1.16 mM $MgSO_4$, 24.0 mM
175 HCO_3Na, 2.5 mM $CaCl_2$, and 6 mM glucose), and cut into 3–4 mm wide rings. The ring
176 vessel was gently suspended between two stainless steel wires in a water-jacketed organ bath
177 kept at 37 °C and filled with a KS, bubbled with a mixture of 5% CO_2 and 95% O_2, pH 7.4.
178 The lower wire was fixed to a vertical plastic rod immersed in the organ bath, while the upper
179 one was rigidly attached to a force transducer (Letica TRI-201). After the ring was suspended
180 in the organ bath, a passive force of 2 g was imposed and the preparation was stabilized for 1
181 h, being washed every 20 min. The signals from the force transducers were amplified and
182 driven into an analog–digital board (DT16EZ, Data Translation, Marlboro, MA, USA)

mounted in a computer. On-line recordings and files for later processing were obtained with the software Labtech Notebook Pro (Laboratory Technology, Wilmington, MA, USA).

To assess the contractile response of the abdominal aorta, cumulative doses (10^{-9} to $4x10^{-6}$ M) of noradrenaline (NA) were added to the organ bath sequentially. To avoid variations due to differences in ring size, the measured maximal contractile force for every NA concentration, was normalized to the wet tissue weight and expressed in grams of developed force divided by grams of wet tissue (gF/gW).

KS with a potassium solution was used to elicit maximal contractions by depolarization of the VSMC. The solution was prepared increasing KCl to 80 mM in the KS; NaCl was lowered to keep normal osmolarity.

The Acetylcholine (Ach) relaxation was studied on, aorta precontracted with NA. At the contraction plateau, the effect of cumulative concentrations (10^{-8} -10^{-5}) M of Ach was tested. Maximal relaxant effect was expressed as percent of the maximal contraction obtained with NA.

2.10. Immunohistochemistry

After antigen retrieval with 0.01 M citrate buffer, pH 6, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in 60% methanol. Non-specific binding sites were blocked with 5% BSA. Sections were incubated overnight at 4 °C with 1:200 rabbit polyclonal anti-ERα antibody (Santa Cruz Biotechnology, Inc.) or 1:200 rabbit polyclonal anti-AhR antibody (Santa Cruz Biotechnology, Inc.). After several washes, sections were incubated with secondary antibody biotinylated anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA) for 2 h at room temperature.

To reveal the sites of antigen/antibody binding, an avidin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) and the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA) were used.

For control staining, some sections were incubated with PBS instead of primary antibody and no immunoreactivity was detected. Sections were dehydrated in ethanol, cleared in xylene and mounted in Canada balsam, and then photographed using Nikon Eclipse 80i microscope and Visiopharm Integrator System software.

2.11. Histological evaluations

Abdominal aorta samples (5 mm width) and ventricles from control and HCB-treated rats were fixed with 4% paraformaldehyde. Samples were dehydrated, and embedded in paraffin, serially sectioned in transversal orientation, and stained with Masson Trichrome. Planimetry was performed using a Nikon Eclipse E400 microscope and image analysis software (Image J, National Institute of Health, Bethesda, MD). Sections were magnified and digitalized. Vessel thickness and right and left ventricle cavities were then measured.

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

2.12. Creatinine analysis

Serum creatinine was measured using the enzymatic method Creatinina enzimatica AA (Wiener Lab.) according to the manufacturer instructions.

2.13. Statistical analysis

Data were analyzed by one-way analysis of variance followed by *post hoc* tests (Bonferroni multiple comparison *t*-test) in order to evaluate selected pairs of groups. $p < 0.05$ was considered significant. SPSS™ version 15.0 software was used to analyze data.

3. RESULTS

3.1. Blood pressure

As dioxin TCDD induces hypertension in rats and HCB is considered a dioxin-like environmental pollutant, we evaluated whether HCB could induce alterations in BP in HCB (5 and 500 mg/kg)-treated rats, for 30, 35, 40 and 45 days (Figure 1).

When animals were treated with the lower dose, 5 mg/kg HCB, BP remain unchanged, but, when animals were treated with the highest dose, 500 mg/kg HCB, BP showed a significant increase at 35 days of HCB treatment which remained until 45 days (Figure 1). Similar BP increase by HCB treatment was observed in a model using male rats (data not shown).

Of note, no differences in neither mortality rate nor rat body weigh was observed between groups (227.6 ± 2.7, 230.3 ± 14.0, 222.0 ± 8.3 g for control, 5 mg/kg HCB and 500 mg/kg HCB respectively).

3.2. Arterial remodeling

It is well known that increased peripheral vascular resistance to blood flow, largely due to vascular remodeling, induces hypertension. In order to investigate whether the effect of HCB on BP can be attributed to this phenomenon, intima-media layer thickness was measured

249 in abdominal aortae. Representative cross-sections of the aortic wall are shown in Figure 2A.
250 Image analyses showed a significant increase (34.6%, $p \leq 0.01$) in the intima-media layer
251 thickness only in 500 mg/kg HCB-treated rats after 45 days of treatment, as compared to
252 control rats (Figure 2B).

253

254 **3.3. PCNA protein level and VSMC number in abdominal aorta**

255 Considering the effect of HCB on abdominal aorta wall thickness, we analyzed PCNA protein
256 expression as an index of cell proliferation and found a decrease in aortae from both 5 and
257 500 mg/kg HCB-treated rats (40.9%, $p<0.01$ and 36.6%, $p<0.01$, respectively) (Figure 2C and
258 D). In addition, the number of cell nuclei per area in the arterial wall was analyzed. A 29.7%
259 ($p<0.05$) decrease was observed at the highest dose of HCB (Figure 2E). Worth pointing out,
260 5 mg/kg HCB-treatment decreased the cell number, even though statistical significance was
261 not reached.

262

263 **3.4. Heart remodeling**

264 Hypertension also causes compensatory structural changes in the heart. For this reason, we
265 analysed the ratio between the left ventricle area, including the interventricular septum, and
266 the left ventricular cavity (Figure 3A). No changes were observed between control and HCB-
267 treated rats, which indicates the absence of adaptive mechanisms (Figure 3B).

268 In addition, the ventricle weights were analyzed and no significant differences between
269 groups were observed (3.35 ± 0.02, 3.49 ± 0.23 and 3.56 ± 0.10 mg of ventricles/g of rat for
270 control, 5 mg/kg HCB and 500 mg/kg HCB, respectively).

271 **3.5. Kidney function**

Hypertension also impacts kidney function, damaging kidney blood vessels and reducing their ability to work properly. Therefore, we evaluated kidney function by determining creatinine concentration in serum of HCB-treated and control rats. Our results show that creatinine levels remain unchanged from control levels, regardless of HCB dose used (Figure 3C).

3.6. Vascular reactivity

With the aim to evaluate arterial function, the contractile response of rings of abdominal aorta to NA was evaluated. In both groups of HCB-treated rats, the aortic contractile response presents higher sensitivity to NA, evidenced by a significant reduction (41.3%, $p<0.01$ and 64.8%, $p<0.001$; respectively) in the half maximal effective concentration (EC_{50}).

In turn, the maximal contractile response to NA was significantly decreased in arteries from both HCB-treated groups compared to control group. Interestingly, 5 mg/kg HCB was more effective than 500 mg/kg HCB, to induce a decrease in the maximal contractile response, (52.4%, $p<0.001$ and 28.6 %, $p<0.05$ respectively) (Figure 4).

We also evaluated the effect of Ach on arterial relaxation after NA contraction. It was observed a significant ($p< 0.05$) diminution in the maximal percentage of relaxation in arteries rings of both 5 and 500 mg/kg HCB-treated rats compared to control (22.93 ± 2.82%, 11.4 ± 2.81% and 12.8 ± 1.6% for control, 5 mg/kg HCB and 500 mg/kg HCB, respectively)

3.7. Key molecules involved in blood pressure regulation

TGF-β1

As abundant evidence shows that TGF-β1 is involved in the pathogenesis of hypertension (Ohta et al., 1994), we analyzed its arterial expression by semiquantitative RT-PCR (Figure 5). Rats treated with 500 mg/kg HCB showed a significant increase in TGF-β1 mRNA levels (35%, $p < 0.05$). Worth pointing out, treatment with 5 mg/kg HCB exhibited an increasing – though statistically non-significant– tendency in growth factor expression.

ERα

An increase in BP could be the consequence of arterial function alterations. The arterial function is finely controlled by contraction and relaxation processes of VSMC. In this sense, a direct relationship has been widely established between vasodilation and ERα expression (Reslan et al., 2013). Therefore, and given that HCB has been shown to alter ERα protein content in mammary gland (Li et al., 2008; Pena et al., 2012), we evaluated ERα expression in aortae from both groups of HCB-treated rats. Immunohistochemistry shows that ERα expression decreased in 500 mg/kg HCB-treated rats (Figure 6A). Similarly, Western blot analysis showed that ERα protein content was significantly decreased (23.6%, $p < 0.05$) by 500 mg/kg HCB, whereas it was unaffected in arteries from HCB (5 mg/kg)-treated rats (Figure 6B).

eNOS

It is well known that estradiol has vessel relaxation effects through the release of several factors. Among them, NO is generated by an increase in eNOS activity, in turn produced by protein induction through ERα. For this reason, we evaluated eNOS expression in arteries from HCB-treated rats. As shown in Figure 6D and E, a significant dose-dependent decrease of 25 ($p < 0.05$) and 40% ($p < 0.01$) eNOS expression was observed in arteries of rats treated with 5 and 500 mg/kg HCB, respectively.

AT1

One of the main mechanisms involved in arterial vasoconstriction is an increment in AT1 expression. Therefore, we evaluated whether HCB also modifies the expression of this receptor. As indicated in Figure 6F and G, a significant increase in AT1 (48.2%, $p < 0.05$) was observed in arteries from rats treated with 500 mg/kg HCB.

TH

TH are involved in the regulation of contraction and relaxation mechanisms. HCB is a hormonal disruptor which impacts TH homeostasis. For this reason, studies were carried out on possible TH alterations in aortic arteries from HCB-treated rats (Table 1). Results rendered no changes in T_3, or TSH levels in serum of rats treated with either dose of HCB. However, T_4 levels were significantly lower in rats with 500 mg/kg HCB (50.9%, ** $p< 0.01$).

Since DII is crucial in the regulation of local T_3 concentrations, HCB effect on abdominal aortae DII mRNA expression (the deiodinase isoform expressed in arteries) was evaluated by semiquantitative RT-PCR. Our results demonstrate that a significant dose-dependent decrease of 31.5 ($p < 0.05$) and 55.4% ($p < 0.01$) in DII mRNA was observed in arteries of rats treated with 5 and 500 mg/kg HCB, respectively (Table 1).

3.8. AhR activation

As a dioxin-like toxicant, HCB exerts both AhR-dependent and independent effects. We studied arterial AhR activation by analysis of its translocation to the nucleus by immunohistochemestry in rats treated with 5 and 500 mg/kg HCB (Figure 6 panel H).

AhR activity in arteries from 5 mg/kg HCB-treated rats showed an increasing tendency although not statically significant, whereas, a statistically greater expression in the nuclear zone in 500 mg/kg HCB treated rats was observed (Figure 6 panel I).

4. DISCUSSION

Even when several studies have informed about the effect of HCB on different tissues, data on its influence on the cardiovascular system are scarcely documented (Sjoberg Lind et al., 2013). In this context, the present work constitutes an original initial approach to the analysis of the effect of HCB on the cardiovascular system and unveils some of the molecular mechanisms involved.

The effect of HCB on BP regulation was evaluated using two different gavage-administered doses of HCB during 45 days. The criteria used for dose selection were based on TH levels. The lower dose (5 mg/kg) does not alter TH levels but the highest dose (500 mg/kg) generates hypothyroxinemia (Kleiman de Pisarev et al., 1990). According to previous reports, the administration of 500 mg/kg HCB for 30 days results in an HCB serum concentration of 6 ng/ml, which is equivalent to HCB serum of a widely exposed population (4.1 – 10.6 ng/ml) (Ohta et al., 1994). For this reason, our findings show a new pathological aspect to take into account in the evaluation of human populations with high HCB exposure.

Worth pointing out, the high discrepancy observed between the HCB administered and the serum concentrations responds to the fact that only 2-5% HCB is absorbed when administered as an aqueous suspension (Koss and Koransky, 1975).

We observed an increase in systolic BP in rats exposed to the high dose of HCB, which allowed us to classify this toxic as a hypertensor. Worth pointing out, this increase in systolic BP was evident as late as 35 days of HCB treatment, suggesting an indirect effect of the toxic on vessels. Of note, our results are in line with those reported by Kopf et al., who demonstrated that dioxin TCDD induces hypertension *in vivo* and *in vitro* models (Kopf et al., 2008) and also in agreement with previous work by Dalton et al. which did not show significant increases in BP until day 23–29 of TCDD treatment (Dalton et al., 2001).

364 The persistent increment in vascular resistance is the main hemodynamic characteristic of
365 hypertension (Intengan and Schiffrin, 2000). This can be produced by functional
366 vasoconstriction or structural vessel wall thickening as a consequence of hypertrophy,
367 hyperplasia or both phenomena together. In this work, 500 mg/kg HCB-administered rats
368 exhibited wall thickening and a decrease in PCNA expression in abdominal aorta. In
369 agreement, the number of aorta nuclei was decreased in HCB-treated rats. Although we have
370 not studied the arterial extracellular matrix content, these results suggest that wall thickening
371 may be due to the hypertrophic effect of HBC on VSMC and not to hyperplasic growth.

372 Hypertension is also known to participate in heart remodeling. Sjoberg *et al.,* (2013) showed
373 that long exposure to HCB is related to increased wall thickness of the left ventricle and
374 concentric left ventricular remodeling (Sjoberg Lind et al., 2013) . However, in this work, no
375 compensatory structural changes were observed in the hearts of HCB-treated rats. It is
376 possible that this absence of heart remodeling responds to the relatively short duration of the
377 hypertensive state.

378 In turn, kidney plays a key role in and is affected by BP regulation, as high BP can damage
379 kidney blood vessels, reducing their ability to work properly. In this work a conserved kidney
380 function was observed in HCB-treated rats. These results are in line with previous studies
381 covering less than 7 weeks of intoxication (Andrews *et al*., 1988; Richter *et al.,* 1981)
382 although an increase in kidney weight was reported in longer HCB exposure (Andrews *et al.,*
383 1989; Den Besten *et al.,* 1993; Smith *et al.*, 1985). Furthermore, HCB nephrotoxicity is a sex-
384 dependent phenomenon, as it is observed in male but not female rats (Smith *et al*., 1985;
385 Arnold *et al*., 1985).

386 Alterations in arterial function, finely controlled by contraction and relaxation processes of
387 VSMC, can trigger an increase in BP. In this work, these alterations were observed in
388 abdominal aorta from HCB-treated animals, reflected by an increase in NA sensitivity and a

decrease in maximal response to NA. An increase in NA sensitivity could contribute to vascular resistance when the sympathetic neurotransmitter is released and, in this way, increase BP in HCB treated animals. The decrease in maximal response to NA could indicate a imbalance in molecules with relaxing or contractile actions or a toxic effect of HCB on the contractile machinery of VSMC. It is worth highlighting that this decrease was sharper in arteries from 5 mg/kg than 500 mg/kg HCB-administered rats, suggesting that HCB could act by different mechanisms depending on the dose used. In addition, the fact that BP is not modified at 5 mg/kg HCB despite the lower capacity of contraction by NA could be explained by compensatory responses that lead to BP normalization. In contrast, the increase in BP in the 500 mg/kg HCB group may be explained by structural changes in the arterial wall interfering with such compensatory mechanisms.

Among the components of the vascular wall, the endothelium plays an important role in vascular remodeling as a reaction to disruptive stimuli. Shear stress induced by blood flow on endothelial cells can result in structural and functional modifications affecting the production or release of several vasoactive factors, as well as a response to these factors (Urschel et al., 2012). In this sense, endothelial cells produce TGF-β1, among others, which exerts its effect on VSMC (Simionescu et al., 2005).

In previous work we demonstrated an increase in TGF-β1 expression in liver from HCB-treated rats which correlated with a imbalance between cell proliferation and apoptosis (Giribaldi et al., 2011). In this work, an increment in TGF-β1 was observed in abdominal aortae from HCB-treated rats, which could constitute an adaptive endothelial homeostatic response to structural alterations in order to regulate cell proliferation. The increase in TGF-β1 could also explain, in part, the reduction observed in aortic cell proliferation in the 500 mg/kg HCB group. This is in line with results reported by Sato *et al.,* (1995) showing a role

413 for TGF-β1 in the control of VSMC growth. TGF-β1 inhibits serum-induced proliferation of
414 rat aortic VSMC and concurrently induces cellular hypertrophy (Sato *et al.*, 1995).

415 In this study, a significant reduction was observed in ERα expression in abdominal arteries of
416 high-dose HCB-treated rats, in line with previous results indicating that HCB alters ERα
417 protein content in mammary gland (Li et al., 2008; Pena et al., 2012). Of note, it has also been
418 demonstrated that TGF-β1 diminishes the expression of ERα in several tissues (Petrel and
419 Brueggemeier, 2003). Moreover, we have recently reported that an increase in TGF-β1
420 expression in the intimal layer of coronary arteries correlates with a decrease in ERα
421 expression (Castilla et al., 2014). Also, TGF-β1 has been shown to produce increased
422 proteasome-dependent degradation of ERα in cancer cell lines (Petrel and Brueggemeier,
423 2003). ERα is a relevant molecule in the mechanism of arterial relaxation and it is involved in
424 the protective effect of estrogens on the endothelium (Arnal *et al.*, 2007; Jobe *et al.*, 2013).
425 Estradiol increases eNOS activity through ERα by both Akt-mediated phosphorylation and
426 protein induction. This results in the generation and release of NO (Chambliss *et al*., 2000;
427 Rosenfeld *et al*, 2003, Jobe, S.O. 2013), which in turn relaxes VSMC, thus producing vessel
428 relaxation. Accordingly, in this work, a decrease was also observed in eNOS expression in
429 HCB treated rats. The decrease in NO levels in HCB-treated rats was also indirectly
430 corroborated by the Ach relaxation tests, since it acts through NO production.

431 In addition, an increase in arterial AT1 expression, the receptor by which angiotensin II acts
432 as a vasoconstrictor, was observed in HCB-treated rats, in agreement with results reported in
433 previous work in hypertensive rats (Romero-Nava *et al*., 2016). Worth highlighting, TGF-β1
434 was also shown to enhance AT1 gene expression in several tissues such as lung fibroblasts
435 (Renzoni *et al.*, 2004), adrenal cells (Lebrathon *et al*., 1994.), and trophoblasts (Tower *et al*.,
436 2005.) but to decrease AT1 levels in VSMCs (Zhang *et al*., 2012), which indicates the
437 importance of the physiological cell microenvironment in the final expression pattern.

Altogether, these findings could partly explain the decrease in NA-induced vessel contraction observed in arteries from 500 mg/kg HCB-treated rats. Further studies should be conducted to clarify the mechanisms underlying the lower contraction observed at low doses of toxic in aorta rings. Likewise, such alterations observed *in vitro* assays at 5 mg/kg HCB did not correlate with a BP increase in the *in vivo* model.

Furthermore, and given that HCB is a hormonal disruptor, analyses were conducted on TH concentration, of strong impact on vascular resistance and contraction. Yoneda et al. (1998) have demonstrated that a single T_3 or T_4 injection promotes fast vasodilation in rat coronary arteries. In contrast, the loss of vasodilation has been associated with an increase in systemic vascular resistance, as observed in hypothyroidism (Duntas and Biondi, 2011; Klein and Ojamaa, 2001). In this work, we also observe that HCB generates hypothyroxinemia, which agrees with our previous report (Kleiman de Pisarev *et al.*, 1990). Hypothyroxinemia, in turn, can affect tissue concentration of TH and their possible effects on downstream intracellular signal transduction.

This work also evaluated the levels of arterial DII expression, as it is responsible for T4 metabolism to generate T3. A dose-dependent decrease in DII expression was observed in abdominal arteries from HCB-treated rats, which, together with the lower levels of T4 in serum, could be responsible for the decrease in arterial T3 levels. Moreover, it has been widely established that a decrease in T3 causes a reduction in ERα expression (Davis *et al.*, 2002; Faustino *et al.*, 2015) which could be in line with the decrease in ERα expression observed in this work.

As a dioxin-like toxicant, HCB exerts both AhR-dependent and independent effects. AhR is a transcription factor that modulates processes such as apoptosis, cell proliferation and migration. Even though AhR is located in the cytosol, the binding of HCB to AhR can trigger the toxic- AhR complex translocation to the nucleus in order to modulate the expression of

463 genes with dioxin response elements (DREs) in their promoters. In this work we have shown
464 artery AhR activation by 500mg/kg HCB but not by 5 mg/kg HCB, which indicates that the
465 mechanism by which these doses act is different. In addition, we conclude that at least part of
466 500 mg/kg HCB effects on the vascular system are exerted through AhR.

467 CONCLUSIONS

468 This work shows for the first time that subchronic administration of HCB generates arterial
469 hypertension in rats, associated with impaired vascular reactivity. In this scenario, TGF-β1,
470 ERα, eNOS, AT1 and DII could constitute key molecules involved in the mechanism of HCB
471 action and the development of hypertensive pathology. These findings may facilitate an
472 experimental model for the development of more precise therapeutic targets in the treatment
473 of HCB-induced hypertension.

474

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479

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629

FIGURE LEGENDS

Figure 1: **Systolic BP**. Systolic arterial pressure was measured at the indicated days of HCB treatment. White bands indicate control animals; grey and black bands indicate rats treated with 5 and 500 mg/kg HCB, respectively. Data represent the mean ± SEM of three independent experiments * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$ vs. control at each time; + $p< 0.05$; ++ $p< 0.01$,+++ $p< 0.001$ vs. 5 mg/kg HCB group (n = 6 per group).

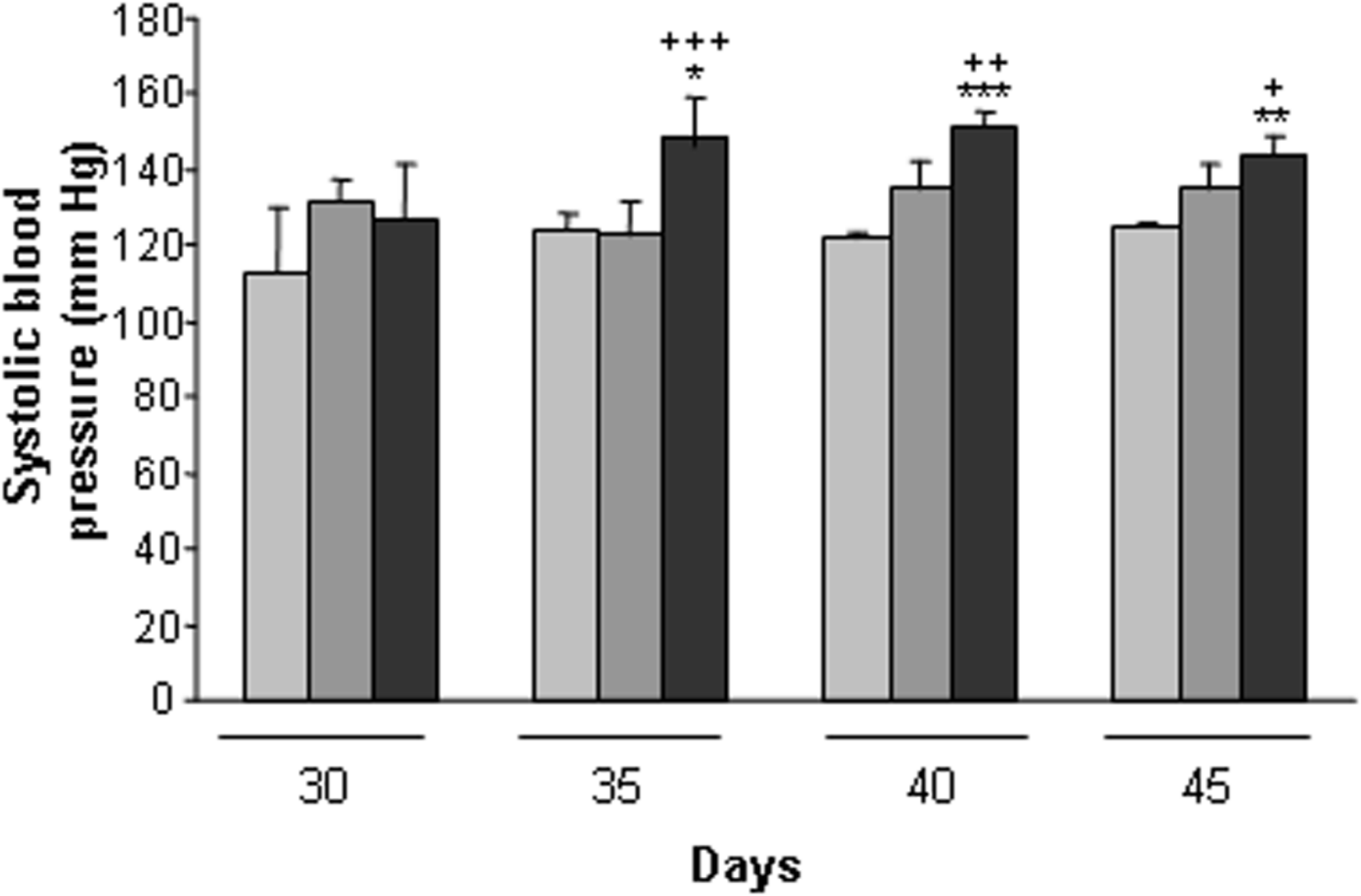
Figure 2*:* **Abdominal aorta structure and PCNA protein level:** Arteries from control and HCB-treated rats were stained with Masson Trichrome. Panel A: Representative microphotographs. Artery wall thickness was measured in the arterial photographs using Image J software (bars indicate how measurements were done). Panel B: Quantification of arterial wall thickness. Panel C: Representative Western blot of PCNA from control and HCB-treated rats. Panel D: Quantification of PCNA protein levels by densitometric scanning of immunoblots. Panel E: Quantification of VSMC number in the arterial wall. Data represent the mean ± SEM of three independent experiments * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$ vs. control (n = 6 per group).

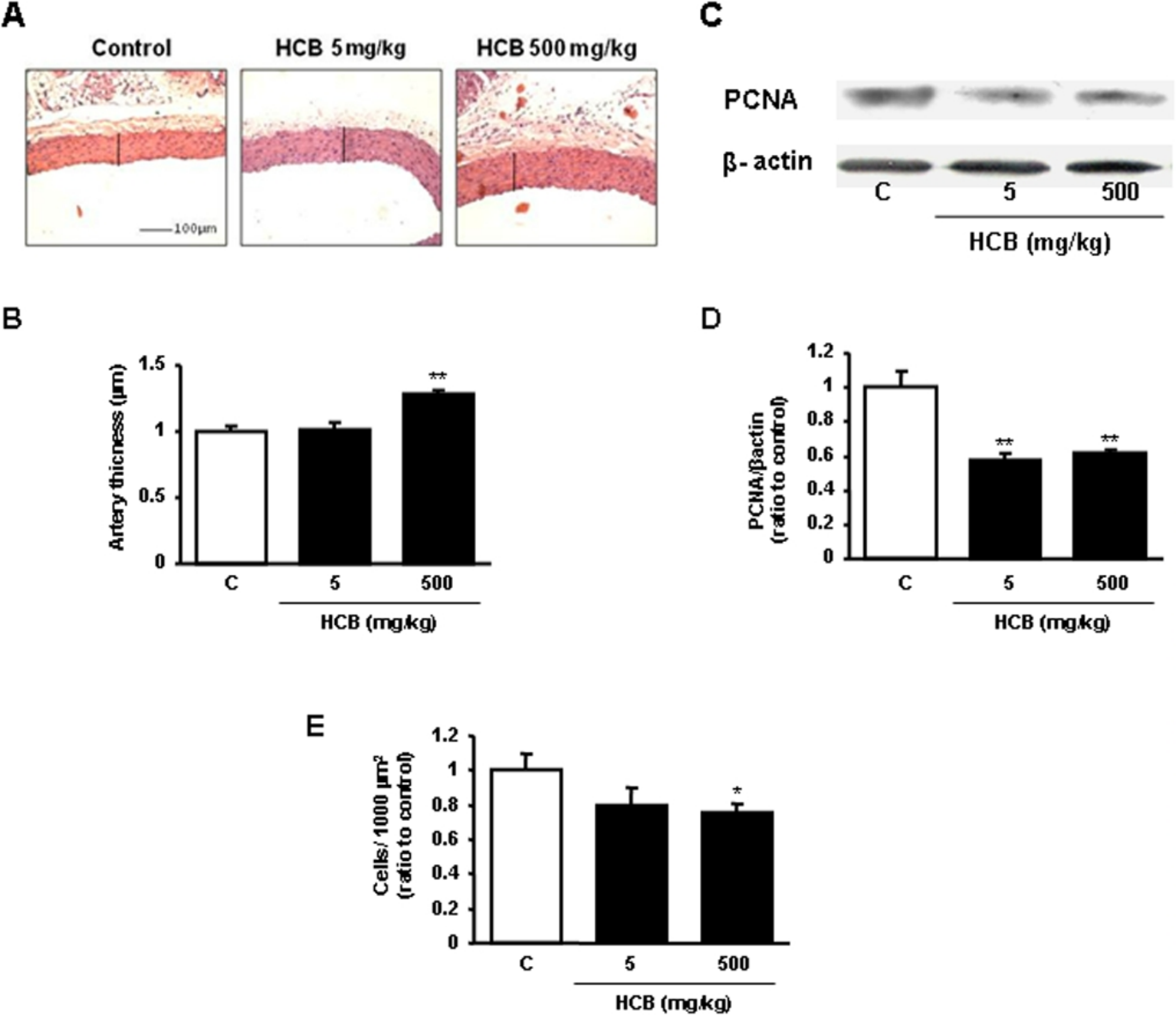
Figure 3: Heart remodeling and serum creatinine levels. Heart sections from control and HCB-treated rats were stained with Masson Trichrome. Panel A: Representative photographs. Panel B: Morphometric quantification. Panel C: serum creatinine concentration from control and HCB-treated rats. Data represent the mean ± SEM of three independent experiments (n = 6 per group).

Figure 4: **Abdominal aorta ring response to NA.** The arterial contractile response to NA was analyzed *in vitro* Data represent the mean ± SEM of three independent experiments * $p < 0.05$, ** $p < 0.01$ vs. control; + $p < 0.05$ vs. 5 mg/kg HCB group (n = 6 per group).

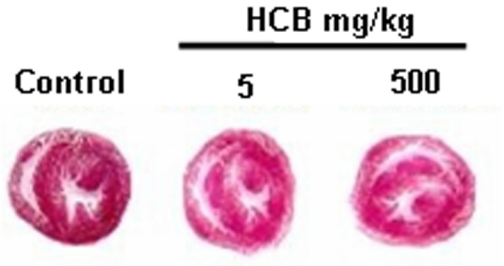
Figure 5: TGF-β1 expression. Rats were treated with HCB (5 and 500 mg/kg) for 45 days. Panel A: Representative pattern of semiquantitative RT-PCR amplification of TGF-β1 and GPDH cDNA. Panel B: Quantification of cDNAs normalized to GPDH cDNA. Panel C. Representative Western blot of TGF-β1□. Panel C: Quantification of western blot TGF-β1 IOD normalized to the corresponding β-actin signal. Data represent the mean ± SEM of three independent experiments. * $p < 0.05$ *vs.* control; + $p < 0.05$ vs. 5 mg/kg HCB group (n = 6 per group).

Figure 6: ERα, eNOS and AT1 expression, and AhR activation. Rats were treated with HCB (5 and 500 mg/kg) for 45 days. Panel A: Representative image of ERα arterial immunohistochemistry. Panel B: Representative Western blot of ERα□. Panel C: Quantification of western blot ERα IOD normalized to the corresponding β-actin signal. Panel D: Representative Western blot of eNOS. Panel E: Quantification of eNOS IOD normalized to the corresponding β-actin signal. Panel F: Representative Western blot of AT1. Panel G: Quantification of AT1 IOD normalized to the corresponding β-actin signal. Panel H: Representative image of AhR arterial immunohistochemistry. Panel I: Quantification of cell number with nuclear AhR signal. Arrows indicate positive signal. Data represent the mean ± SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control; + $p < 0.05$ vs. 5 mg/kg HCB group (n = 6 per group).

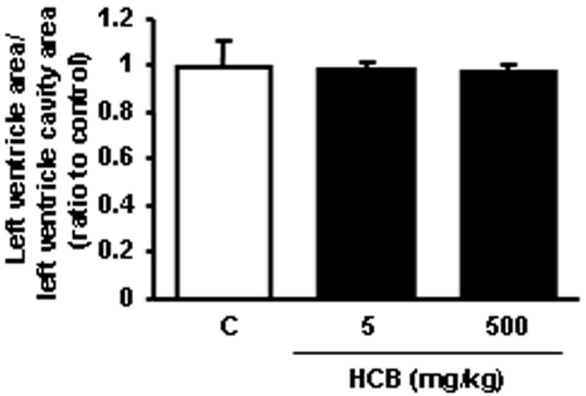

Systolic blood pressure (mm Hg)
180
160
140
120
100
80
60
40
20
0
+++
*
++
***
+
**
30
35
40
45
Days


A
Control
HCB 5 mg/kg
HCB 500 mg/kg
100μm
C
PCNA
β- actin
C
5
500
HCB (mg/kg)
B
Artery thicness (μm)
1.5
1
0.5
0
**
C
5
500
HCB (mg/kg)
D
PCNA/βactin (ratio to control)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
C
5
500
HCB (mg/kg)
E
Cells/ 1000 μm² (ratio to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
C
5
500
HCB (mg/kg)

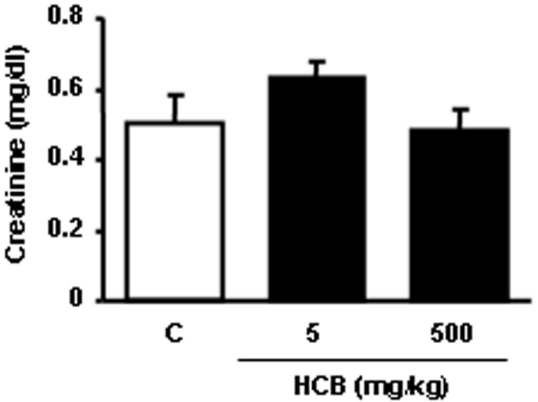
A

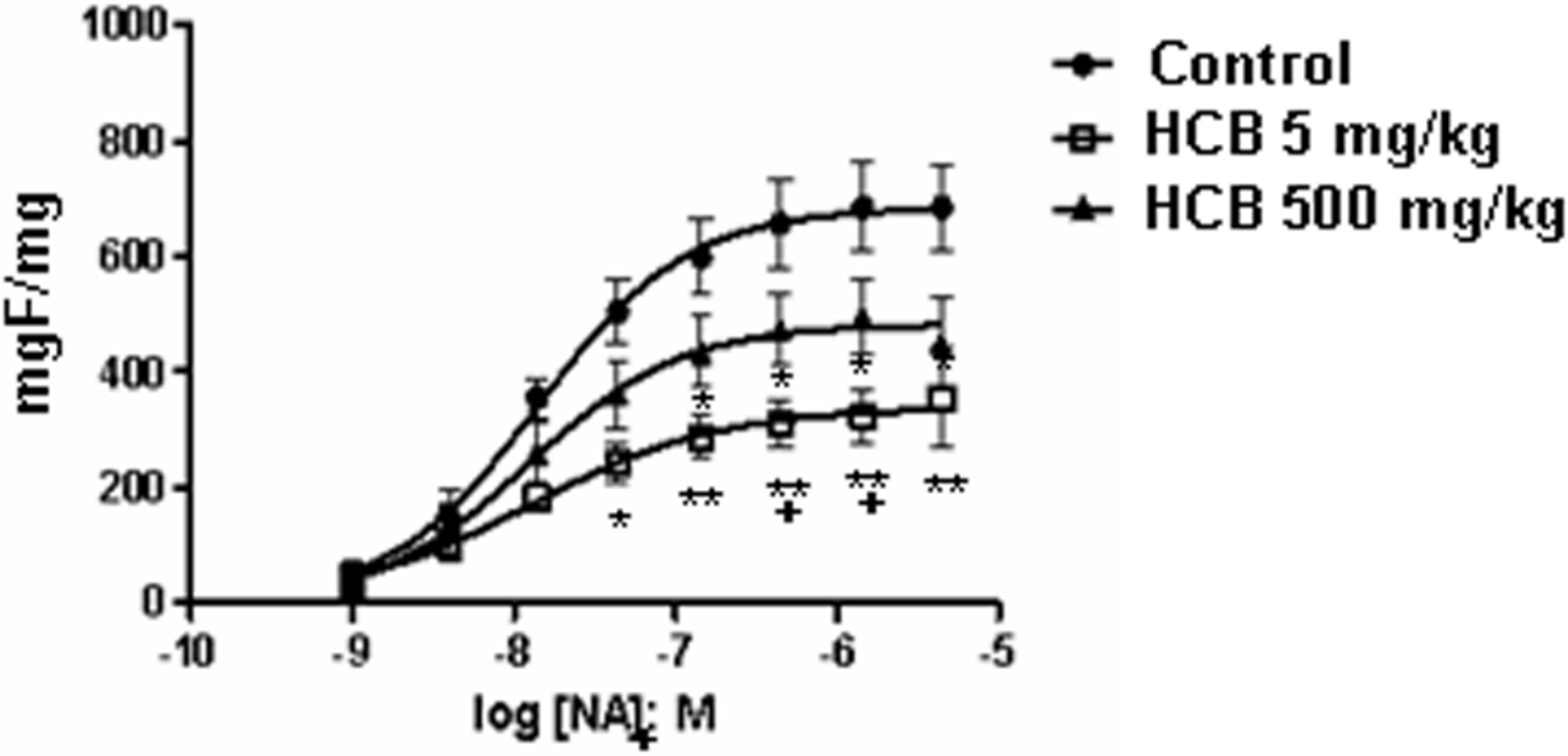

HCB mg/kg
Control
5
500

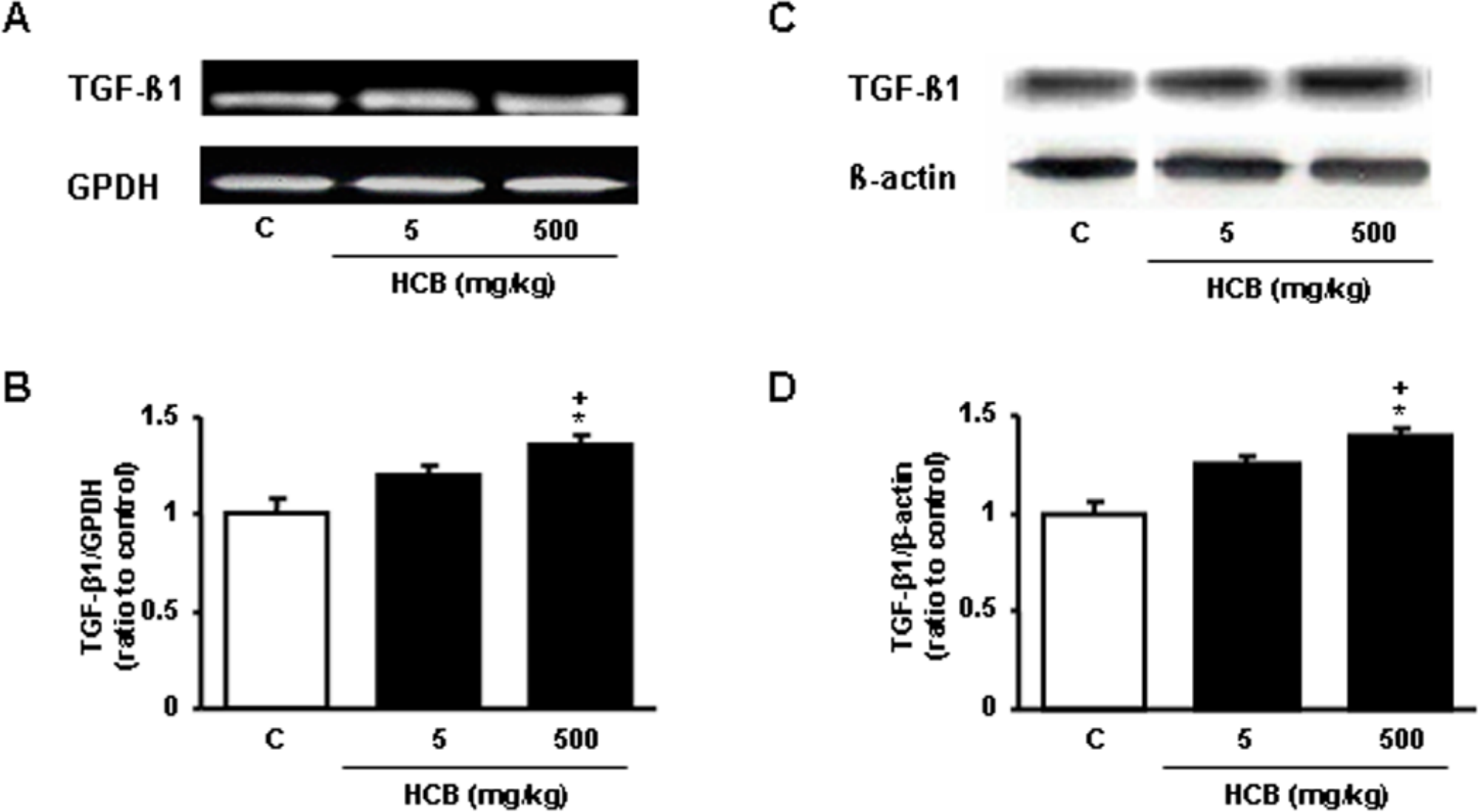
B


Left ventricle area/
left ventricle cavity area
(ratio to control)
1.2
1
0.8
0.6
0.4
0.2
0
C
5
500
HCB (mg/kg)

C


Creatinine (mg/dl)
0.8
0.6
0.4
0.2
0
C
5
500
HCB (mg/kg)


1000
800
600
400
200
0
mgF/mg
-10
-9
-8
-7
-6
-5
log [NA]; M
Control
HCB 5 mg/kg
HCB 500 mg/kg


A
TGF-ß1
GPDH
C
5
500
HCB (mg/kg)
C
TGF-ß1
ß-actin
C
5
500
HCB (mg/kg)
B
TGF-ß1/GPDH (ratio to control)
1.5
1
0.5
0
++
C
5
500
HCB (mg/kg)
D
TGF-ß1/ß-actin (ratio to control)
1.5
1
0.5
0
++
C
5
500
HCB (mg/kg)

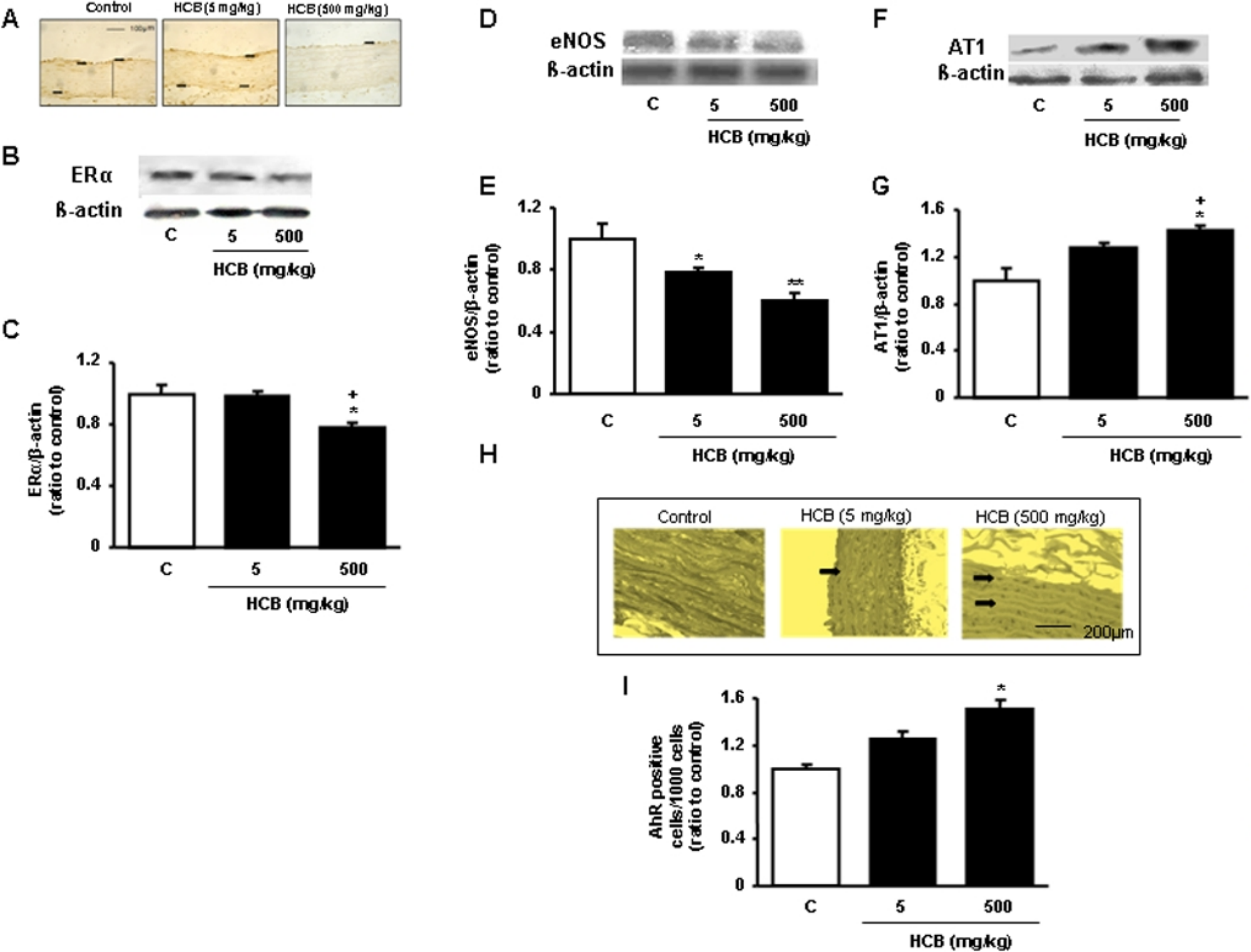

A
Control
HCB (5 mg/kg)
HCB (500 mg/kg)
B
ERα
ß-actin
C
5
500
HCB (mg/kg)
C
ERα/β-actin (ratio to control)
1.2
0.8
0.4
0
+
*
C
5
500
HCB (mg/kg)
D
eNOS
ß-actin
C
5
500
HCB (mg/kg)
E
eNOS/β-actin (ratio to control)
1.2
0.8
0.4
0
*
**
C
5
500
HCB (mg/kg)
F
AT1
ß-actin
C
5
500
HCB (mg/kg)
G
AT1/β-actin (ratio to control)
1.6
1.2
0.8
0.4
0
+
*
C
5
500
HCB (mg/kg)
H
Control
HCB (5 mg/kg)
HCB (500 mg/kg)
200μm
I
AhR positive cells/1000 cells (ratio to control)
1.6
1.2
0.8
0.4
0
*
C
5
500
HCB (mg/kg)

TABLE 1: TH serum levels and aortic DII expression

	TSH (ng/ml)	**T_4 (µg/dl)**	**T_3 (µg/dl)**	**mRNA DII/GPDH**
Control	1.90 ± 0.30	4.16 ± 0.43	74.00 ± 4.60	9.2 ± 0.2
HCB 5 (mg/kg)	1.99 ± 0.38	3.95 ± 0.27	76.00 ± 2.90	6.3 ± 0.1*
HCB 500 (mg/kg)	2.07 ± 0.27	2.01 ± 0.20**+	72.50 ± 5.50	4.1 ± 0.1**+

Table 1: Data represent the mean ± SEM of three independent experiments, * $p < 0.05$ and ** $p < 0.01$ vs. control; + $p < 0.05$ vs. 5 mg/kg HCB group (n = 6 per group).