RESEARCH

Wnt/β-catenin signaling in the adrenal glands of rats in various types of experimental hypertension

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Abstract

Wnt/β-catenin signaling plays a key role in maintaining homeostasis, which is disturbed in hypertension. Taking into account the lack of literature describing changes in the Wnt/β-catenin pathway in the adrenal glands under conditions of elevated arterial pressure, here we compare the expression of WNT4, WNT10A, β-catenin, and GSK-3β in the adrenal glands of hypertensive rats of various etiologies. The studies were carried out on the adrenal glands of rats with spontaneous hypertension (SHR), renalvascular (2K1C), and deoxycorticosterone acetate (DOCA)-salt. Immunohistochemical and PCR methods were used to identify the molecular components of the canonical signaling pathway and to evaluate gene expression. Immunoreactivity and expression of WNT4, WNT10A, β-catenin, and GSK-3β in adrenals of SHR was decreased, compared to control rats. In adrenals of 2K1C rats, intensity of immunohistochemical reaction and expression of WNT4 and β-catenin was lower, while immunoreactivity and expression of WNT10A and GSK-3β were higher, compared to normotensive animals. Significantly stronger immunoreaction and expression of WNT4, β-catenin and GSK-3β but weaker immunoreactivity and expression of WNT10A were noted in adrenals in DOCA-salt rats, compared to control rats. In conclusion, our data provide new molecular information indicating that the canonical WNT pathway is disrupted in the adrenal glands of hypertensive rats. They show that the dysregulation of the WNT pathway depends on the etiology of hypertension.

Key Words

- Wnt
- β-catenin
- hypertension
- adrenal glands
- experimental model

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Introduction

In recent years, much attention has been paid to the role of Wnt/β-catenin pathway in various biological processes of the body (1). This signaling includes extracellular WNT ligands, specific Frizzled membrane receptors (Fzd), and β-catenin – the main pathway effector. In the absence of binding of WNT ligands to Fzd receptors, intracellular β-catenin is kept very low due to constant degradation. The level of cytoplasmic β-catenin is regulated by a multi-protein complex called the β-catenin destruction complex, consisting of axin, adenomatous polyposis coli, casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK-3β). In this complex, CKI and GSK-3β sequentially phosphorylate β-catenin and then phosphorylated β-catenin is ubiquitinated and is targeted for proteasomal degradation. After attachment of the WNT ligand and activation of Fzd receptors, destruction of β-catenin is inhibited and the peptide accumulates in the cell. Then β-catenin is transferred to the nucleus, where it regulates the expression of WNT target genes (1).

One aspect that is currently the focus of attention is the involvement of the Wnt/β-catenin pathway in the regulation of adrenal development, physiology, and carcinogenesis (2, 3, 4, 5, 6). Wnt/β-catenin signaling determines key physiological processes of the adrenal

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glands such as gland development, organ architecture, differentiation of glandular cells, regeneration processes, and hormone production (2, 3, 4, 5, 6). Inactivation or silencing of the genes encoding the Wnt/β-catenin components in mice leads to a gradual thinning of the adrenal cortex, reduction in the number of adrenocortical cells, and a decrease in mineralocorticoid production (7, 8). In mice with a targeted disruption of the β-catenin gene, Kim *et al.* (7) noted adrenal aplasia, reduced thickness, and disorganization of the adrenal cortex compared to WT animals. Experimental data showed that the Wnt-4-deficient mice have lower aldosterone production compared to non-mutant mice (8). While in mice with constitutive activation of β-catenin, Berthon *et al.* (9) found adrenal hyperplasia, hyperaldosteronism, increased proliferation of glandular cells, disturbed differentiation of endocrine cells, accumulation of undifferentiated cells under the glandular capsule, and adrenal tumor formation within 17 months. *In vitro* studies demonstrated that WNT4 delivered by adenovirus significantly enhanced steroidogenesis in primary adrenal cortical cell cultures (10). Clinical trials have shown frequent activation of Wnt/β-catenin signaling and constitutive accumulation of β-catenin in the adrenal glands of patients with primary aldosteronism or adrenocortical tumors (11, 12, 13).

Hypertension proceeds with significant dysregulation of internal homeostasis not only in terms of disturbed mechanisms regulating blood pressure but also in altered secretion of hormones by endocrine organs (14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27). Adrenal hormones, which cause the heartbeat to accelerate, have vasoconstrictive properties, as well as regulate water–electrolyte balance and body fluid volume, play a key role in the pathogenesis of hypertension. A substantial amount of evidence indicates increased catecholamine secretion as well as the abnormal release of mineralocorticoids and glucocorticoids in hypertension (14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27).

There are several reports in the literature describing the location of components of the Wnt/β-catenin pathway (e.g. some WNT ligands, Fzd receptors, and β-catenin) in rodent adrenals under physiological conditions (2, 5, 7, 9). However, no studies have yet been carried out to determine whether the state of elevated blood pressure leads to changes in Wnt/β-catenin signaling activity in the adrenal glands. This prompted us to undertake research aimed at identifying and evaluating the WNT/β-catenin pathway in the adrenal glands of hypertensive rats of various etiologies.

The aim of the present study was to compare the expression of WNT4, WNT10A, β-catenin, and GSK-3β in the adrenal glands of rats with spontaneous hypertension (SHR), renovascular (2K1C), and deoxycorticosterone acetate (DOCA)-salt hypertension.

Materials and methods

Experimental animals

The assumptions, the aim, and the plan of the study, as well as the approach to animals were approved by the local Ethics Committee for Studies on Animal Subjects in Olsztyn.

The study was performed on 5 normotensive male Wistar Kyoto rats (WKY), 7 male SHR rats and 24 ($n = 24$) young male Wistar rats. The rats were 6 weeks of age, and their body weight at the beginning of the experiment was within 170–200 g. The animals were housed at constant humidity (60 ± 5%) and temperature (22 ± 1°C) and were kept under a 12 h light:12 h darkness cycle. The rats had free access to standard granulated chow and a normal drinking water (28).

The experimental animals were divided into six groups (28):

(1) SHR: seven rats with genetically determined systemic hypertension, inbred strain established from Wistar rats selected for high blood pressure.
(2) WKY: five normotensive Wistar Kyoto rats, being the reference for SHR rats.
(3) 2K1C: Seven Wistar rats with renovascular hypertension induced by ligation of the artery supplying the blood to the left kidney (two kidney, one clip model of hypertension).
(4) Sham-operated: five Wistar rats underwent sham operation (submitted to the same surgical procedure as the hypertensive rats, however, without arterial ligation), being the reference for 2K1C.
(5) DOCA-salt: seven Wistar rats which were uninephrectomised, then rendered hypertensive by high-salt diet and deoxycorticosterone acetate (DOCA) injections.
(6) UNX: five Wistar rats uninephrectomised only, being the reference for DOCA-salt hypertensive rats.

2K1C renovascular hypertension

Induction of experimental hypertension was performed according to the procedure by Goldblatt *et al.* (29). After the rats were anesthetized by exposure to pentobarbital (40 mg/kg, i.p.), a 3-cm retroperitoneal flank incision was performed under sterile conditions. The left kidney was

exposed and the renal artery was carefully dissected free of the renal vein. The renal artery was then partially occluded by placing a silver clip with an internal diameter of 0.20 mm on the vessel. The wound was closed with a running 3-0 silk suture ($n = 10$). Sham-operated rats ($n = 5$) underwent identical surgical procedures, except that a clip was not applied to the renal artery. After the surgery, the rats were kept in single cages till wound healing. Normotensive control rats were submitted to the same surgical procedure as the hypertensive rats, however, without arterial ligation (underwent sham operation) (28).

DOCA–salt hypertension

Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (300 μmol or ~70 mg/kg of body weight). The right kidney was removed in all rats via a right lateral abdominal incision. After a 1 week recovery period, hypertension was induced over a time period of 4 weeks by subcutaneous injections of DOCA (67 μmol or ~25 mg/kg in 0.4 mL/kg of dimethylformamide; DMF) twice weekly and replacement of drinking water with 1% NaCl solution. Normotensive control rats were also uninephrectomized but received the vehicle for DOCA (DMF, 0.4 mL/kg, s.c.) twice weekly and drank tap water.

Blood pressure measurement by an indirect method in wakeful rats

After 6 week period of the experiment, the systolic blood pressure (BP) was measured in all animals by using a non-invasive tail-cuff method (using a Rat Tail Blood Pressure Monitor, Hugo Sachs Elektronik-Harvard Apparatus, March–Hugstetten, Germany). BP measurements were considered valid only when three consecutive readings did not differ by more than 5 mmHg. The average of the three measured values was then recorded. The measurements of BP proved systolic hypertension in SHR, 2K1C, and DOCA-salt rats (those animals had values of SBP equal to or higher than 150 mmHg).

Method of experimental material collection and fixation

At 6 weeks of the experiment, the fragments of adrenals were collected under deep pentobarbital anesthesia (50 mg/kg of body weight) from all rats. Obtained adrenal tissues were immediately fixed in 10% buffered formalin solution and routinely embedded in paraffin or placed in RNA-later solution (AM7024 Thermo Fischer) and stored at –80°C.

Adrenal paraffin blocks were cut into sections of 4 μm thickness and then stained with hematoxylin–eosin for general histological examination and processed by immunohistochemistry to detect WNT4, WNT10A, β-catenin, and GSK-3β. Material stored in RNA-later solution was processed by real-time PCR to evaluate the expression of genes coding WNT4, WNT10A, β-catenin, and GSK-3β.

Identification of WNT4, WNT10A, β-catenin, and GSK-3β by immunohistochemistry

In the immunohistochemical study, the EnVision method was used, as previously described by Kasacka *et al.* (28). Immunohistochemistry was performed, using a REAL™ EnVision™ Detection System, Peroxidase/DAB, and Rabbit/Mouse detection kit (K5007; Dako Cytomation). Immunostaining was performed by the following protocol. Paraffin-embedded sections were deparaffined and hydrated in pure alcohols. For antigen retrieval, the sections were subjected to pre-treatment in a pressure chamber heated for 1 min at 125°C. During antigen retrieval sections for detection of WNT4, WNT10A and β-catenin were incubated with Target Retrieval Solution Citrate pH of 6.0 (S 2369 Dako Cytomation) and sections for detection of GSK-3β were incubated with Target Retrieval Solution with pH of 9.0 (S 2367 Dako Cytomation). After cooling down to room temperature, the sections were incubated with Peroxidase Blocking Reagent (S 2001 Dako Cytomation) for 10 min to block endogenous peroxidase activity. Subsequently, the sections were incubated with the primary antibody for WNT4 (rabbit polyclonal antibody to WNT4, PA5-27321 Invitrogen), WNT10A (rabbit polyclonal antibody to WNT10A, ab106522, Abcam), β-catenin (rabbit MAB to β-catenin, ab32572, Abcam, antibody that recognizes total β-catenin) and GSK-3β (rabbit MAB to GSK-3β, ab68476, Abcam). The antisera were previously diluted in Antibody Diluent (S 0809 Dako Cytomation,) in relation 1:250 for WNT4, 1:200 for WNT10A, 1:2000 for β-catenin, and 1:100 for GSK-3β. Incubation with primary antibodies lasted overnight and was carried out at 4°C in a humidified chamber. The procedure was followed by incubation with a secondary antibody (conjugated to horseradish peroxidase-labeled polymer). The bound antibodies were visualized by 1 min incubation with liquid 3,3'-diaminobenzidine substrate chromogen. The sections were finally counterstained in hematoxylin QS (H – 3404, Vector Laboratories; Burlingame, CA, USA), mounted, and evaluated under a light microscope. Appropriate washing

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with Wash Buffer (S 3006 Dako Cytomation) was performed between each step.

Specificity tests performed for the WNT4, WNT10A, β-catenin, and GSK-3β antibody included negative control, where the primary antibodies were omitted, only antibody diluent was used, and a positive control was prepared with specific tissue as it was recommended by the manufacturer. Histological preparations were evaluated using an Olympus BX43 light microscope (Olympus 114 Corp.) with an Olympus DP12 digital camera (Olympus 114 Corp.) and documented.

Real-time PCR

Samples of adrenal (1 cm^3) were taken from each rat and placed in an RNA-later solution. Total RNA was isolated using NucleoSpin® RNA Isolation Kit (Machery-Nagel). Quantification and quality control of total RNA was determined using the spectrophotometer – NanoDrop 2000 (Thermo Scientific). Only RNA samples for which the absorbance ratio at wavelength 260 nm/280 nm was 1.8–2.1 were adopted for the next analysis steps. The mentioned absorbance ratio proves that isolated RNA is of high quality. An aliquot of 1 µg of total RNA was reverse transcribed into cDNA using iScript™ Advanced cDNA Synthesis Kit for real-time PCR (RT-qPCR) (Bio-Rad). Synthesis of cDNA was performed in a final volume of 20 µL using an Thermal Cycler (Model SureCycler 8800, Agilent Technologies). For RT, the mixtures were incubated at 46°C for 20 min then heated to 95°C for 1 min and finally rapidly cooled at 4°C. Quantitative real-time PCR (QRT-PCR) reactions were performed using Stratagene Mx3005P (Agilent Technologies) with the SsoAdvanced™ Universal SYBER® Green Supermix (Bio-Rad). Specific primers for WNT4 (*WNT4*), WNT10A (*WNT10A*), β-catenin (*CTNNB1*), GSK-3β (*GSK3B*), and GAPDH (*GAPDH*) were designed by Bio-Rad. The housekeeping gene GAPDH (*GAPDH*) was used as a reference gene for quantification. In order to determine the amounts of tested genes expression levels, standard curves for each gene separately were constructed with serially diluted PCR products. PCR products were obtained by amplification of cDNA using specific primers as follows: *WNT4* (qRnoCID0007729, Bio-Rad), *WNT10A* (qRnoCED0004311, Bio-Rad), *CTNNB1* (qRnoCID0053256, Bio-Rad), *GSK3B* (qRnoCID0001683, Bio-Rad), and *GAPDH* (qRnoCID0057018, Bio-Rad). QRT-PCR was carried out in a dublete in a final volume of 10 µL under the following conditions: 2 min polymerase activation at 95°C, 5 s denaturation at 95°C, 30 s annealing at 60°C for 40 cycles. PCR reactions were checked by including no-RT-controls, by omission of templates, and by melting curve to ensure only a single product was amplified. The relative quantification of gene expression was determined by comparison of values of Ct using the ΔΔCt method. All results were normalized to *GAPDH*.

Quantitative analysis

From each animal, twelve (12) sections of adrenal were studied (three sections for each: WNT4, WNT10A, β-catenin, and GSK-3β immunostaining). Five randomly selected microscopic fields (each field 0.785 mm^2, 200× magnification (20× lens and 10× eyepiece)) from each adrenal section were documented using an Olympus DP12 microscope camera. Each digital image of the adrenal section was morphometrically evaluated using NIS Elements AR 3.10 Nikon for microscopic image analysis.

The intensity of the immunohistochemical reaction for WNT4, WNT10A, β-catenin, and GSK-3β was measured in each image and determined using a gray scale level of 0–255, where the value of the completely white or light pixel is 0, while the completely black pixel is 255.

All collected data were statistically analyzed by means of the software computer package Statistica Version 12.0. The corresponding mean values were computed automatically; significant differences were determined by a one-way ANOVA test; $P < 0.05$ was taken as the level of significance.

Results

In Table 1 the mean values of BP for each studied groups of animals are presented. Rats belonging to SHR, 2K1C, and DOCA-salt developed systemic hypertension at the course of experiment.

A positive immunohistochemical reaction to WNT4, WNT10A, β-catenin, and GSK-3β was noted in the adrenal glands of all rats tested, excepting the SHR adrenal glands, where no β-catenin was present. The intensity of the immunoreactions for the individual antibodies used in the study differed to varying degrees between the experimental and control animals (Figs 1, 2, 3, 4 and 5).

Table 1 Mean values of systolic blood pressure (mmHg) of rats in control and hypertensive groups (mean ± S.D.).

Values of BP (mmHg)			
Control groups		Hypertensive groups	
WKY	122.3 ± 2.3	SHR	160.8 ± 3.3[a]
SHAM-operated	120.2 ± 2.2	2K1C	162.6 ± 2.2[a]
UNX	126.0 ± 4.0	DOCA-salt	180.0 ± 13.0[a]

[a]$P < 0.05$ control group vs hypertensive group.

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The location and intensity of WNT4 immunoreactivity were similar in the adrenal glands of all control rats (Fig. 1A, B, C, D, E, and F). In the glomerular layer of the adrenal cortex, the intensity of the reaction was moderate, while in the fasciculata and reticular layers, the reaction was much weaker (Fig. 1A, B, and C). In the cytoplasm of glandular cells of the adrenal medulla of control animals, small, only a few WNT4-immunopositive granules were observed (Fig. 1D, E, and F).

In SHR- and 2K1C-hypertensive animals, the intensity of the reaction showing WNT4 was attenuated, almost undetectable (Fig. 1G, J and H, K), whereas the adrenal glands of the DOCA-salt rats showed a slight increase in WNT4 immunoreactivity (Fig. 1I and L).

The adrenal glands of the WKY rats (Fig. 2A and D) and UNX (Fig. 2C and F) showed intense WNT10A immunoreaction in the glomerular layer, weak in the fasciculata and reticular layers of the adrenal cortex, and moderate to strong in some cells of the adrenal medulla (Fig. 2D and F). Whereas, in the adrenal glands of sham-operated rats (Fig. 2B and E), a significantly lower immunoreactivity of WNT10A was observed as compared to the remaining control groups.

In animals with SHR hypertension (Fig. 2G and J) and DOCA-salt (Fig. 2I and L), immunoreactivity of WNT10A

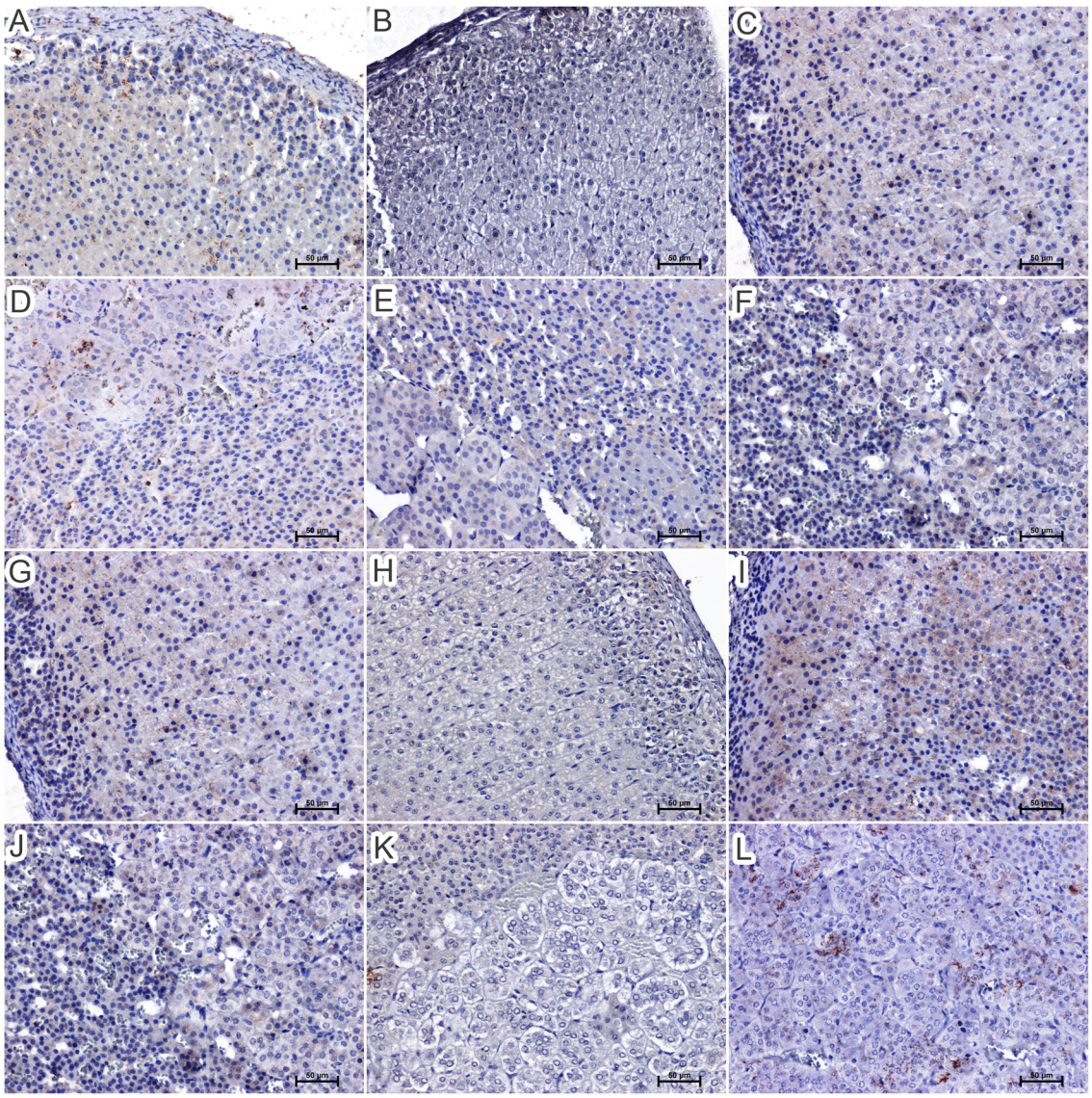

Figure 1
Immunodetection of WNT4 in adrenal glands of normotensive (A, B, C, D, E, and F) and hypertensive (G, H, I, J, K, and L) rats. Adrenal cortex and medulla of WKY (A and D), sham (B and E), UNX (C and F); adrenal cortex and medulla of SHR (G and J); 2K1C (H and K), DOCA-salt (I and L).

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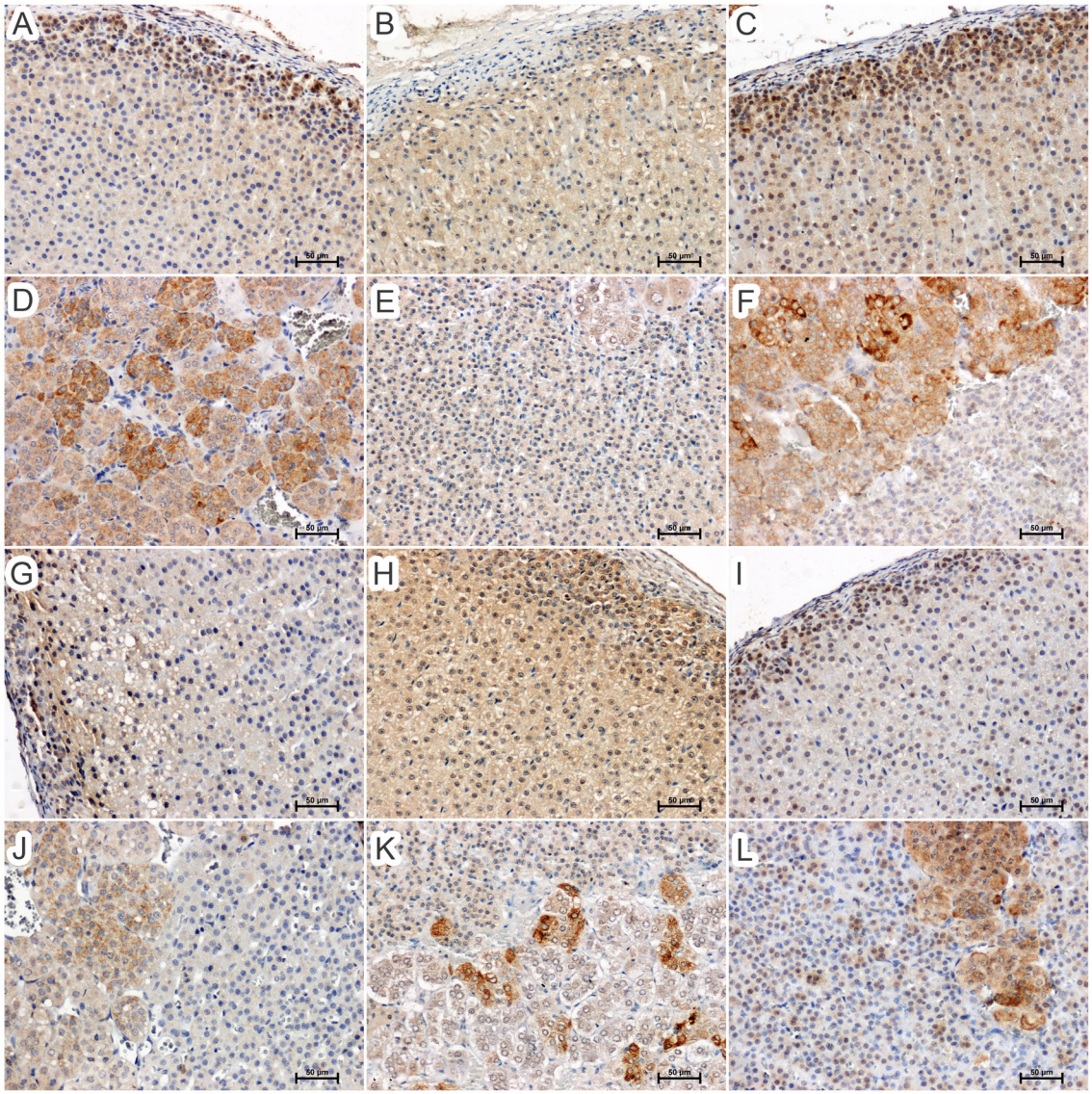

Figure 2
Immunolabeling of WNT10A in adrenals of normotensive (A, B, C, D, E, and F) and hypertensive (G, H, I, J, K, and L) rats. Adrenal cortex and medulla of WKY (A and D), sham-operated (B and E), UNX (C and F); Adrenal cortex and medulla of SHR (G and J); 2K1C (H and K), DOCA-salt (I and L).

was weaker in both the cortex and medulla of adrenal glands, compared to the corresponding control group. While the immunoreactivity of WNT10A in the adrenal glands of the 2K1C rats (Fig. 2H and K) was significantly stronger compared to the sham-operated animals.

Immunostaining against β-catenin in the adrenal glands of WKY rats showed a very slight reaction only in the glomerular layer of the cortex (Fig. 3A). In the adrenal medulla of these animals, weak to moderate β-catenin immunoreactivity was found (Fig. 3D). In turn, the adrenal glands of rats in the remaining control groups (sham operated (Fig. 3B and E) and UNX (Fig. 3C and F)) showed a strong immune signal of β-catenin in the glomerular zone, weak or very weak immune response in the fasciculata and reticular layers of the cortex, and a slight or moderate staining in the gland medulla (Fig. 3E and F).

The performed immunohistochemical reaction did not show the presence of β-catenin in the adrenal glands of SHR rats (Fig. 3G and J). The adrenal glands of the 2K1C rats (Fig. 3H and K) showed a reduction in the intensity of the reaction compared to that observed in sham-operated animals (Fig. 3B and E). In rats with DOCA-salt, hypertension (Fig. 3I and L) stronger immunoreactivity against β-catenin was found only in the glomerular layer, in the remaining cortical layers, and in the adrenal medulla, the intensity of the reaction was similar to that observed in UNX rats (Fig. 3C and F).

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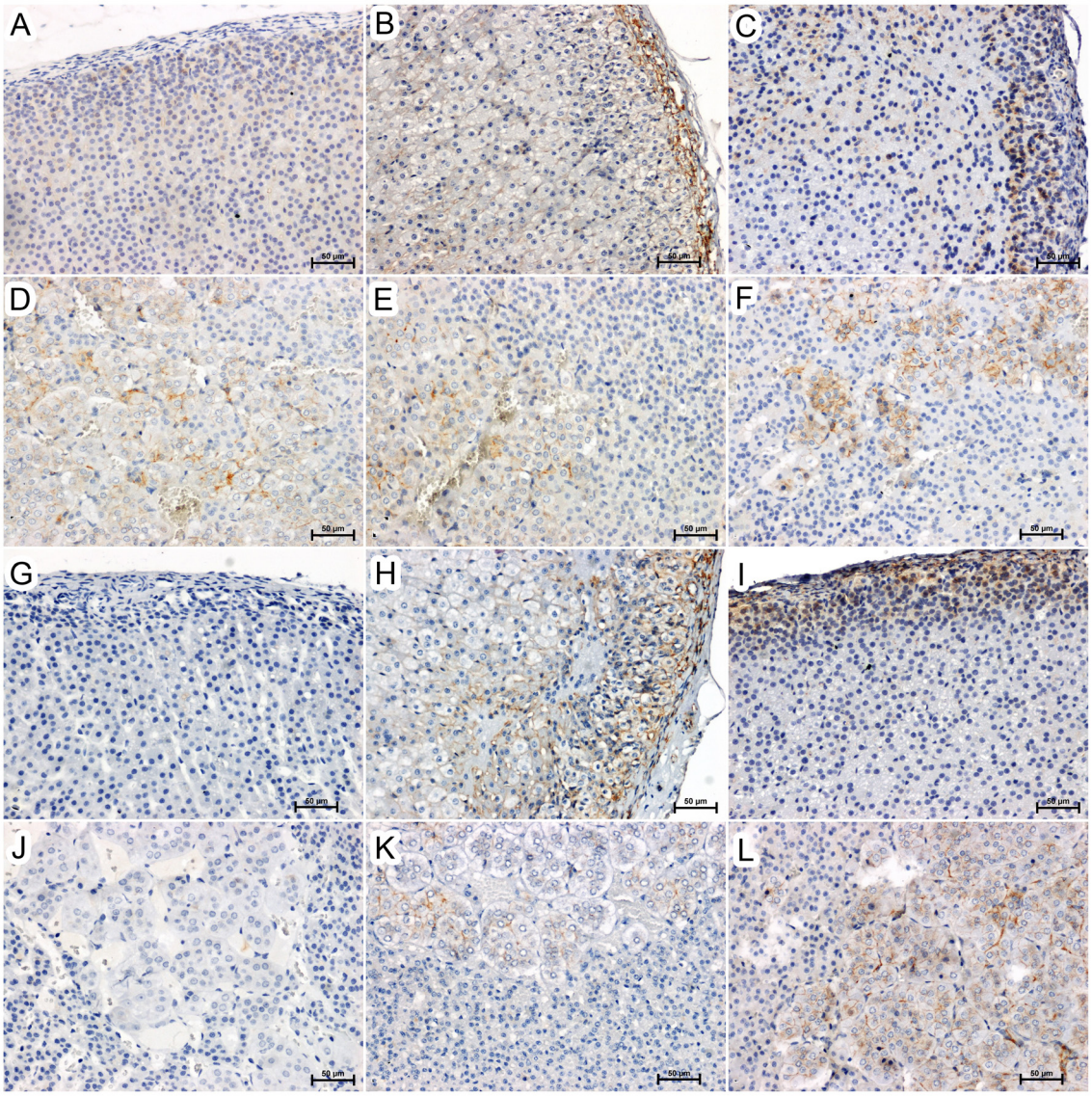

Figure 3
Immunodetection of β-catenin in adrenals of normotensive (A, B, C, D, E, and F) and hypertensive (G, H, I, J, K, and L) rats. Adrenal cortex and medulla of WKY (A and D), sham (B and E), UNX (C and F); Adrenal cortex and medulla of SHR (G and J); 2K1C (H and K), DOCA-salt (I and L).

The immunoidentification of GSK-3β in the adrenal glands of rats with normal arterial pressure (WKY, sham, and UNX) gave a positive result of moderate intensity in both the cortical and the medullary parts, slightly stronger staining, compared to other control groups, was found in the glands of WKY rats (Fig. 4A, B, C, D, E, and F).

The intensity of the anti-GSK-3β reaction in the adrenal glands of SHR rats (Fig. 4G and J) was significantly reduced compared to WKY animals (Fig. 4A and D). The adrenal glands of 2K1C rats (Fig. 4H and K) showed increased GSK-3β immunoreactivity compared to sham-operated animals (Fig. 4B and E), except for the glomerular layer, where immunoreactivity was similar to that observed in normal pressure rats. The location and intensity of the reaction with the anti-GSK-3β antibody in the adrenal glands of rats with the hypertension DOCA-salt (Fig. 4I and L) and UNX (Fig. 4C and F) were very similar.

The results of densimetric studies confirmed the visual differences in the intensity of the immunohistochemical reactions against WNT4, WNT10A, β-catenin, and GSK-3β in the adrenal glands of control and hypertensive rats (Tables 2 and 3).

QRT-PCR analysis showed a significant decrease in WNT4 expression in adrenals of SHR and 2K1C rats compared to the respective normotensive rats, whereas

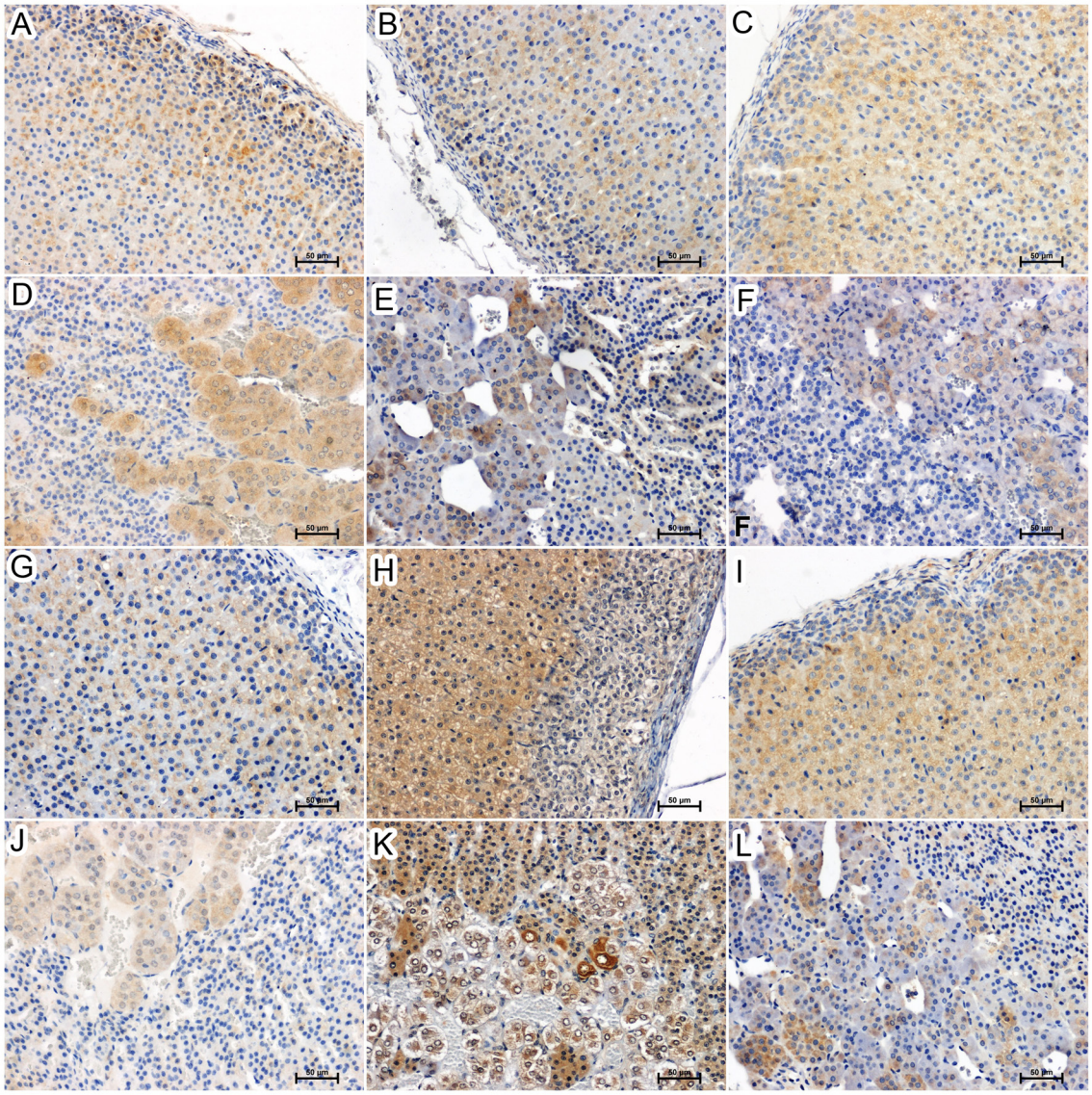

Figure 4
Immunodetection of GSK-3β in adrenals of normotensive (A, B, C, D, E, and F) and hypertensive (G, H, I, J, K, and L) rats. Adrenal cortex and medulla of WKY (A and D), sham (B and E), UNX (C and F); Adrenal cortex and medulla of SHR (G and J); 2K1C (H and K), DOCA-salt (I and L).

the expression of the gene encoding WNT4 in the adrenal glands of DOCA-salt rats was significantly higher compared to the UNX group. The expression of WNT10A in the adrenal glands of the SHR and DOCA-salt rats was lower than in the control groups, while in the 2K1C rats it was higher compared to the sham-operated animals. The analysis of the results showed a significant decrease in the expression of the gene encoding β-catenin in the SHR and 2K1C rats, and a significant increase in the expression of the tested gene in the DOCA-salt rats compared to the control groups. GSK-3β expression was lower in the adrenal glands of the SHR rats and slightly higher in the 2K1C and DOCA-salt animals compared to the corresponding normotensive rats (Fig. 5 and Table 4).

Discussion

Hypertension is a complex disease, in which pathogenesis involves many mechanisms. One of the pathomechanisms is dysfunction of the endocrine glands, especially the adrenal glands, whose hormones play a key role in maintaining the body's homeostasis. In 95% of cases, the pathology of secondary arterial hypertension is caused

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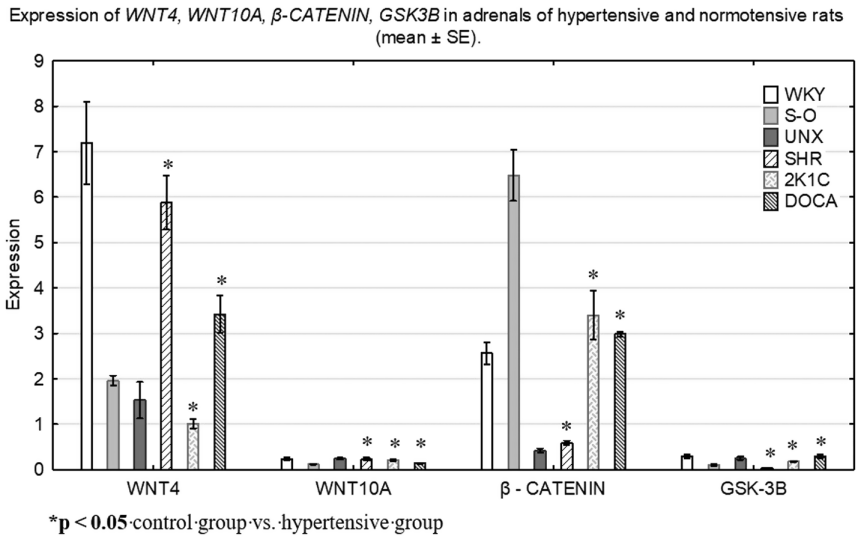

Figure 5
The expression of genes coding WNT4, WNT10A, β-CATENIN, GSK-3β in adrenal glands of hypertensive and normotensive rats (mean ± S.E.).

by abnormal aldosterone secretion by the cells of the glomerular layer of the adrenal glands (30).

Taking into account the important role of adrenal hormones in blood pressure regulation and the latest reports indicating the involvement of the Wnt/β-catenin pathway in the development and physiology of the adrenal glands, in our research, we assessed the WNT/β-catenin signaling in the adrenal glands of hypertensive rats of various etiology.

Our studies showed reduced immunoreactivity and expression of WNT4, WNT10A, β-catenin, and GSK-3β in the adrenal glands of SHR compared to control rats. In the adrenal glands of 2K1C rats, the intensity of the immunohistochemical reaction and expression of WNT4 and β-catenin were lower, while the immunoreactivity and expression of WNT10A and GSK-3β were higher compared to animals with normal pressure. Whereas, the adrenal glands of DOCA-salt rats showed much stronger immunoreactivity and expression of WNT4, β-catenin, and GSK-3β, but weaker immunoreactivity and expression of WNT10A compared to control rats.

The localization of proteins of the canonical pathway in the adrenal glands of rats, demonstrated in our studies, is consistent with the reports of Drelon *et al.* (2), Pignatti *et al.* (5), Kim *et al.* (7) and Berthon *et al.* (9) who described the presence of selected WNT and β-catenin proteins in the adrenal glands of mice. We observed the strongest immunoreactivity of WNT4, WNT10A, β-catenin, and

Table 2 The intensity of immunoreaction determining WNT4 and WNT10A in adrenals of hypertensive and normotensive rats (mean ± S.E.).

	Intensity of immunohistochemical reaction in rat adrenals, scale from 0 (white pixel) **to 255** (black pixel)					
	Control groups			Hypertensive groups		
	WKY	SHAM-operated	UNX	SHR	2K1C	DOCA-salt
WNT4						
Zona glomerulosa of adrenal cortex	128.6 ± 4.45	100.6 ± 3.00	102.0 ± 3.54	83.5 ± 2.38[a,c]	76.3 ± 2.22[a,c]	113.4 ± 2.19[a,b]
Zona fasciculata of adrenal cortex	87.7 ± 3.67	81.9 ± 3.10	82.5 ± 2.15	76.4 ± 2.04[a,c]	69.6 ± 1.41[a,c]	99.6 ± 2.08[a,b]
Zona reticularis of adrenal cortex	78.2 ± 2.53	77.9 ± 2.24	80.1 ± 3.03	74.7 ± 1.72	69.0 ± 1.28[a,c]	98.4 ± 3.06[a,b]
Adrenal medulla	83.6 ± 5.76	63.7 ± 1.83	66.4 ± 2.40	64.9 ± 2.17[a,c]	52.7 ± 2.01[a,c]	81.7 ± 4.36[a,b]
WNT10A						
Zona glomerulosa of adrenal cortex	114.1 ± 4.29	71.3 ± 1.63	129.8 ± 4.21	98.2 ± 2.57[a,c]	106.9 ± 1.91[a,b]	95.8 ± 1.87[a,c]
Zona fasciculata of adrenal cortex	62.3 ± 1.16	66.0 ± 1.09	71.1 ± 1.34	55.4 ± 1.61[a,c]	70.5 ± 1.40[a,b]	55.0 ± 1.14[a,c]
Zona reticularis of adrenal cortex	42.5 ± 0.95	43.9 ± 0.67	43.2 ±1.14	43.4 ± 0.78	43.7 ± 0.94	46.0 ± 1.39
Adrenal medulla	102.8 ± 3.35	57.7 ± 2.00	112.4 ± 4.67	71.8 ± 1.89[a,c]	85.8 ± 5.07[a,b]	100.2 ± 3.76[a,c]

[a]$P < 0.05$ control group vs hypertensive group; [b]Intensification of immunohistochemical reaction; [c]Weakening of immunohistochemical reaction.

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Table 3 The intensity of immunoreaction determining β-catenin and GSK-3β in adrenals of hypertensive and normotensive rats (mean ± S.E.).

	Intensity of immunohistochemical reaction in rat adrenals, scale from 0 (white pixel) **to 255** (black pixel)					
	Control groups			Hypertensive groups		
	WKY	SHAM-operated	UNX	SHR	2K1C	DOCA-salt
β-catenin						
Zona glomerulosa of adrenal cortex	55.3 ± 1.42	85.1 ± 4.32	92.8 ± 3.59	ND[a,c]	67.5 ± 2.49[a,c]	108.5 ± 3.56[a,b]
Zona fasciculata of adrenal cortex	42.1 ± 0.91	68.9 ± 2.38	39.2 ± 1.62	ND[a,c]	43.0 ± 2.19a,c	40.4 ± 1.11
Zona reticularis of adrenal cortex	32.9 ± 1.12	31.5 ± 0.78	31.5 ± 0.59	ND[a,c]	33.9 ± 1.71	31.8 ± 1.76
Adrenal medulla	51.1 ± 2.89	71.2 ± 3.09	71.6 ± 2.60	ND[a,c]	46.9 ± 2.47[a,c]	69.3 ± 2.30
GSK-3β						
Zona glomerulosa of adrenal cortex	67.7 ± 2.15	68.1 ± 1.10	59.6 ± 1.26	44.1 ± 1.88[a,c]	66.9 ± 2.13	59.6 ± 2.75
Zona fasciculata of adrenal cortex	62.7 ± 1.89	63.0 ± 1.42	68.9 ± 1.67	54.0 ± 1.48[a,c]	90.4 ± 1.21[a,b]	67.1 ± 1.54
Zona reticularis of adrenal cortex	40.8 ± 1.53	57.7 ± 1.47	40.8 ± 1.71	39.1 ± 0.98	90.8 ± 2.79[a,b]	41.7 ± 1.26
Adrenal medulla	71.7 ± 1.03	74.3 ± 1.92	73.4 ± 2.90	48.5 ± 1.02[a,c]	92.3 ± 2.62[a,b]	75.3 ± 2.62

[a]$P < 0.05$ control group vs hypertensive group; [b]Intensification of immunohistochemical reaction; [c]Weakening of immunohistochemical reaction.
ND, not detected.

GSK-3β in the glomerular zone of the adrenal cortex of rats, a similar result is presented by the authors in the adrenal glands of mice (7).

In our studies, we also demonstrated the presence of WNT4, WNT10A, β-catenin, and GSK-3β in the rat adrenal medulla. The immunoreactivity of β-catenin in the adrenal medulla of mice is confirmed by the studies by Kim *et al.* (7).

This is the first study describing the effect of hypertension on Wnt/β-catenin signaling in the adrenal glands. So far, the localization of the Wnt/β-catenin pathway components in the adrenal glands of mice under physiological conditions as well as constitutive activation and targeted inactivation of the β-catenin gene has been defined (2, 5, 7, 9). Due to the lack of reports in the literature on changes in the Wnt/β-catenin pathway in the adrenal glands under conditions of elevated arterial pressure, the discussion of the results obtained in our studies is quite difficult and may only be a hypothetical consideration.

Wnt/β-catenin signaling is necessary for proper differentiation, glandular cells organization, hormonal activity, and regeneration of the adrenal cortex (2, 3, 4, 5, 6, 7, 8, 9, 10).

The changes in the Wnt/β-catenin pathway in the adrenal glands of SHR, 2K1C, and DOCA rats suggested in our studies indicate an important role of this signaling pathway in the regulation of adrenal function in hypertension.

Some experimental data indicate on the impact of Wnt/β-catenin signaling on aldosterone secretion (8, 12). In studies on the human adrenocortical cell line H295R, Berthon *et al.* (12) showed that a reduction in β-catenin expression resulted in a reduction of angiotensin II-stimulated basal aldosterone production. The authors

Table 4 The expression of genes coding WNT4, WNT10A, β-CATENIN, and GSK-3B in adrenal glands of hypertensive and normotensive rats (mean ± S.E.).

	The expression of genes coding WNT4, WNT10A, β-CATENIN, and GSK-3B in the adrenals of hypertensive and normotensive rats (mean ± S.E.)					
	Control groups			Hypertensive groups		
	WKY	SHAM-operated	UNX	SHR	2K1C	DOCA-salt
WNT4	7.19 ± 1.58	1.96 ± 0.19	1.53 ± 0.69	5.88 ± 1.02[a,c]	1.01 ± 0.17[a,c]	3.42 ± 0.72[a,b]
WNT10A	0.23 ± 0.06	0.12 ± 0.02	0.24 ± 0.04	0.10 ± 0.01[a,c]	0.21 ± 0.04[a,b]	0.14 ± 0.02[a,c]
β-catenin	2.56 ± 0.42	6.40 ± 0.98	0.42 ± 0.08	0.59 ± 0.08[a,c]	3.40 ± 0.93[a,c]	2.98 ± 0.09[a,b]
GSK-3β	0.29 ± 0.08	0.10 ± 0.03	0.25 ± 0.06	0.04 ± 0.01[a,c]	0.18 ± 0.02[b]	0.29 ± 0.08[b]

[a]$P < 0.05$ control group vs hypertensive group; [b]Increase in gene expression; [c]Decrease in gene expression.
ND, not detected.

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made the same observations when cultured adrenocortical cells were treated with an inhibitor (PKF115-584) of β-catenin transcriptional activity (12). Heikkila *et al.* (8) stated that mice with Wnt-4 deficiency have lower serum level of aldosterone compared to WT animals.

Earlier experimental studies showed an increase in aldosterone secretion by the adrenal glands of rats with spontaneous and renal-vascular hypertension, while in animals with DOCA-salt hypertension, the production of this hormone was significantly reduced (22, 24, 31, 32, 33). The present study showed an inhibition of the Wnt/β-catenin pathway in the adrenals of SHR, rather than a weakening of the expression of this pathway in the adrenal glands of 2K1C rats, and an increase in the activity of the Wnt/β-catenin pathway in the adrenal glands of DOCA-salt rats. As there are no existing publications on the role of the Wnt/β-catenin signaling pathway in the regulation of aldosterone secretion in hypertension, further research is needed to explore this aspect.

There is evidence of a correlation between Wnt/β-catenin signaling and the renin-angiotensin system (RAS), which plays a fundamental role in blood pressure control and body fluid balance (34, 35). Experimental studies showed that chronic angiotensin II infusion significantly induced the expression of WNT and β-catenin proteins in rat kidneys and in cultured rat kidney fibroblast (NRK-49F) (35). Induced overexpression of the Wnt/β-catenin pathway has also been shown to increase the expression of RAS genes in the human renal tubular epithelial cell line (HKC-8) (34).

Surprisingly, in our studies, we observed a lowered expression of the Wnt/β-catenin pathway in the adrenals of SHR and a rather decreased activity of this pathway in the adrenal glands of 2K1C rats, although according to the literature (36), in spontaneous and renal-vascular hypertension the activity of the RA system is increased. In rats with DOCA-salt hypertension, a significantly stronger expression of the Wnt/β-catenin pathway was observed, although in this hypertensive model the activity of the RA system is reduced (36). Obtained results might suggest the occurrence of some other mechanism, beyond the RA system, regulating the expression of Wnt/β-catenin pathway in adrenals of hypertensive rats. However, due to lack of literature this aspect is still not fully explained. Our study provides strong immunohistochemical and molecular evidence that in various types of arterial hypertension, Wnt/β-catenin signaling is incorrectly activated, which, according to the current knowledge, maybe a significant molecular change in the pathology of hypertension.

An interesting fact is that we did not immunohistochemically detect β-catenin in adrenals of SHR rats. The immunohistochemical reaction showing β-catenin in the adrenal glands of SHR rats was weakened compared to control rats to such an extent that it was very poorly detectable. Whereas using the QRT-PCR method we stated the expression of β-catenin in the glands of these rats, which was lowered compared to normotensive control. Possibly, the observed herein absence of β-catenin immunosignal in adrenals of the SHR suggests that β-catenin peptide produced in adrenal glandular cells is immediately degraded under the state of SHR. In our study, we found weaker GSK-3β immunoreactivity and reduced expression of GSK-3β in adrenals of SHR rats. GSK-3β is one of the main kinases that participate in the phosphorylation and degradation of β-catenin (1). Considering the above, further research should be carried out in order to better understand the processes related to the regulation of degradation and level of β-catenin in adrenal glandular cells in the condition of arterial hypertension.

In our previous study, we demonstrated a dysregulation of Wnt/β-catenin signaling in the kidneys of rats subjected to relevant three models of experimental hypertension (28). The changes in WNT4, WNT10A, β-catenin, and GSK-3β expression observed in the adrenal glands of SHR, 2K1C, and DOCA-salt rats are different from those previously found in the kidneys of these animals (28). Reduced immunoreactivity and expression of WNT4, WNT10A, β-catenin, and GSK-3β were observed in the adrenal glands of SHR rats, while in the kidneys of essential hypertensive rats, immunoreactivity and expression of WNT4, WNT10A and β-catenin were increased compared to control rats. The adrenal glands of 2K1C rats showed weaker immunoreactivity and expression of WNT4, β-catenin, as well as stronger immunoreaction and expression of WNT10A, GSK-3β, while in kidneys of rats with renovascular hypertension was stated an increase in immunosignalization and expression of WNT4, WNT10A, and β-catenin compared to normotensive animals. In the adrenal glands of DOCA-salt rats, the immunoreactivity and expression of WNT4, β-catenin, and GSK-3β were increased, but immunostaining and expression of WNT10A were lowered, compared to control rats. In contrast, kidneys of DOCA-salt rats showed reduced immunoreactivity and expression of WNT4, β-catenin, and higher immunoreactivity and expression of WNT10A and GSK-3β compared to rats with normal blood pressure (28).

The presented research and our previous study by Kasacka *et al.* (28) are innovative because there are no other reports describing changes in the Wnt/β-catenin

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pathway in arterial hypertension. Further studies should be undertaken to better understand these differences in the regulation of the Wnt/β-catenin pathway in the adrenal glands and kidneys of hypertensive rats.

In summary, our research suggests alteration of Wnt/β-catenin signaling in the adrenal glands of the SHR, 2K1C, and DOCA-salt hypertension rats. However, it should be noted that the intensity of changes in Wnt/β-catenin pathway closely depends on the etiology of the disease. This report may provide new information on the pathophysiologic mechanisms leading to dysregulation of adrenal homeostasis in the state of elevated blood pressure. A more detailed understanding of the role of the WNT/β-catenin pathway in hypertension-associated disturbances of adrenal functioning requires further investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conceptualization: I K. Methodology: I K, N D, A L. Supervision: I K. Funding acquistion: I K. Writing – Review and Editing: I K. Formal analysis: Z P, N D. Visualization: N D, A L. Resources: A L. Writing – Original draft: Z P. Approval of final manuscript: all authors.

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