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The expression of p73 in the organum vasculosum of the lamina terminalis and choroid plexus of spontaneously hypertensive rats

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Summary. The p73 proteins are present in different kinds of cells of the central nervous system, such as the choroid plexus, circumventricular structures and neuroepithelium. It has been reported that spontaneously hypertensive rats show ventricular dilation, changes in cerebrospinal fluid proteins and variations in the circumventricular structures such as the organum vasculosum of the lamina terminalis and the choroid plexus, which are altered in ventricular dilation. The aim of the present work is to study p73 expression in the organum vasculosum of the lamina terminalis and the choroid plexus and its variations in high blood pressure. Brains from control Wistar-Kyoto rats and spontaneously hypertensive rats were used. The organum vasculosum of the lamina terminalis and the choroid plexus were processed by immunohistochemistry and western blot with anti-TAp73. We found weaker markings in the organum vasculosum of the lamina terminalis and stronger markings in the choroid plexus of the hypertensive than the control rats. Therefore, hypertension in the spontaneously hypertensive rats produces alterations in choroid plexus protein p73 expression that is similar to that described for other circumventricular organs, but it is different in the organum vasculosum of the lamina terminalis. We can conclude that the functional balance between p73, organum vasculosum of the lamina terminalis and choroid plexus, which is probably necessary to maintain the normal functioning of these structures, is altered by the hypertension found in these rats.

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Key words: p73, Organum vasculosum of the lamina terminalis, Choroid plexus, Hypertensive rats

Introduction

Protein p73 has two isoforms: the transactivating isoform of p73 (TAp73), which is similar to p53 acting as a transcription factor that induces cellular apoptosis and the N-terminal truncated isoform (ΔNp73) which can inhibit the transcriptional function of p53 and TAp73 (Pozniak et al., 2000). Protein p73 is significant in central nervous system development, since p73 knock-out mice present hippocampal dysgenesis, cortical hypoplasia and hydrocephalus, meaning that p73 plays an important role in the different parameters that regulate brain development (Meyer et al., 2004).

The organum vasculosum of the lamina terminalis (OVLT), located in the anteroventral region of the third ventricle (AV3V), contains angiotensin II (AGII) and catecholamines, and like the subfornical organ is characterized by the absence of a blood-brain barrier. The AV3V, with the OVLT, plays a critical role in the regulation of body fluid volume and cardiovascular function. The OVLT has an important role during plasma AGII increase, facilitating higher blood pressure, but is not involved with baseline effects of endogenous AGII (Vieira et al., 2010). The AV3V is richly innervated by noradrenergic nerve terminals coming from the brainstem (Saper et al., 1983; Castañeyra-Perdomo et at., 1992; Lenkei et al., 1995) and this innervation is essential for both angiotensin II-induced pressor and drinking responses, as well as for vasopressin release (Ushigome et al., 2004; Adams et al., 2009).

The choroid plexus (ChP) are also considered as

circumventricular organs located in the lateral, third and fourth ventricles. The ChP and blood to CSF barrier are altered in arterial hypertension and hydrocephalus (Rodríguez et al., 1992; Johanson et al., 2008; González-Marrero et al., 2012). Furthermore, ventricular dilation and alterations of the circumventricular structures are also observed in hypertensive rats, alterations that are also present in hydrocephalus (Ritter et al., 1988; Rodriguez et al., 1992; Castañeyra-Perdomo et al., 1998; Martínez de la Peña y Valenzuela et al., 2006; González-Marrero et al., 2007; Carmona-Calero et al., 2012).

It has been reported that TAp73 is present in the subcommissural organ, subfornical organ, area postrema and CSF, where it could play an important role in the maintenance of the ventricular wall and in the development of neuroepithelium proliferation, and equilibrium between both the TAp73 and ΔNp73 isoforms is necessary for normal neuronal development and maintenance (Cabrera-Socorro et al., 2006; Tomasini et al., 2008; Carmona-Calero et al., 2009, 2012; Fujitani et al., 2010). Taking into consideration that p73 is implicated in the normal development of the neuroepithelium, that the OVLT and the ChP are mainly formed by neuroepithelium and that SHRs present ventricular dilation as well as alterations in several circumventricular organs (CVO), the aim of the present work is to study p73 expression in the OVLT and ChP in arterial hypertension structures closely connected with cardiovascular regulation.

Material and methods

Brains from twelve male control Wistar-Kyoto rats (WKY) and twelve male hypertensive rats (SHR) from Charles River Laboratories España S.A. (Barcelona, Spain) of 12 months of age were used. Rats were kept under lighting conditions of 12:12, and food and water were provided ad libitum. Before sacrifice, the body weight and blood pressure were taken. The mean of body weigh was: 432±17 grams in WKY rats and 401±24 grams in SHRs. The mean tail cuff of blood pressure was: in WKY rats systolic 120±8 mmHg and diastolic 66±7 mmHg; in SHRs systolic 160±14 and diastolic 73±15. The rats were anesthetized with chloral hydrate (200 μl/100 g of body weight at 160 mg/ml). Four rats from each group were fixed by intracardiac perfusion with Bruin's fluid, dehydrated and embedded in paraffin under standard conditions. Brains were cut into four serial coronal and sagittal sections of ten micrometers thick. One of the serial coronal sections was stained by the Klüver-Barrera method. The ethical committee of the University of La Laguna approved the study.

Immunohistochemistry

The following primary antibodies were used: a polyclonal antibody against amino acids 1–15 at the N-terminus of human TAp73 (Ab-5, Neomarkers, Fremont, CA, USA) and a polyclonal antibody against an epitope within amino acids 1–62 of TAp73 (Ab14430, Abcam, Cambridge, UK). The sections at the same anatomical level of the OVLT and ChP of the third ventricle from the WKY and SHRs were simultaneously incubated: firstly, containing the anti-TAp73(1-15) 1:500 for 24h followed by "DAKO" StreptABCcomplex/HRP Duet, Mouse/Rabbit procedure and the peroxidase reaction product was visualized using diaminobenzidine intensified with nickel at 0.5% in order to get grey-blue immunostaining; and secondly they were incubated with anti-TAp73(1-62) 1:1000 for 24 h at room temperature, followed by "DAKO" StreptABCcomplex/HRP Duet, Mouse/Rabbit procedure, and the peroxidase reaction product was visualized using diaminobenzidine reaction to get a brilliant brown immunostaining. The primary antibodies were omitted to validate the control method specificity.

Western blots

The OVLT and ChP of third ventricle were identified and extracted from the same anatomical level with the aid of Paxinos and Watson atlas (1998) and by Evans-blue injection (del Valle et al., 2008). The extracts (30μl of extracts volume) from eight rats from each group were processed by protein electrophoresis according to Laemmli (1970), the proteins were then transferred from gel to nitrocellulose membrane. The membranes with the blotted proteins were incubated in tris-saline (TBS) non-fat milk 5% for 60 minutes and then incubated in both primary anti-TAp73 antibodies (1-14, 1-62) 1:1000 overnight. Anti-mouse IgG labelled with peroxidase (PIERCE) was used as the secondary antibody at a dilution of 1:80000 for 1.45 h at room temperature. The peroxidase reaction products from western blot were visualized by Chemoluminescence (PIERCE). The primary antibody was omitted to validate the control method specificity.

Three rostrocaudal levels of the immunohistochemistry slides were converted to digital images by using an LEICA DMRB photomicroscope with an LEICA DC 300 F camera (Germany). Image analysis was completed in Image J (v. 1.43 u, NIH, Bethesda, MD, USA). The 'Mean Gray Value' was measured from the selected nuclei for all stained tissue and membranes. This value gives the average stain intensity in grayscale units for all threshold pixels. A single-factor analysis of variance (ANOVA) and post Hoc test Tukey was used for the immunohistochemistry statistical study, which was conducted using IBM SPSS statistic 19 software.

Results

The antibody (1-62) anti-TAp73 was observed by immunohistochemistry in the cytoplasm of ependymal cell of the OVLT of the lamina terminalis and neurons located surrounding the OVLT, specifically in the preoptic medial area (PMA) of WKY rats (Fig. 1A,B).

Immunoreactive material for both TAp73 antibodies, (1-15) and (1-62), was also identified in different PMA neurons (Fig. 1A-D) and even in diverse sub cellular compartments of the same neuron, with a considerable overlap in the cytoplasm (Fig. 1E-G). The presence of the (1-15) TAp73 in the ChP and OVLT was almost undetectable. Therefore, the comparative study between hypertensive and control rats was performed using anti-

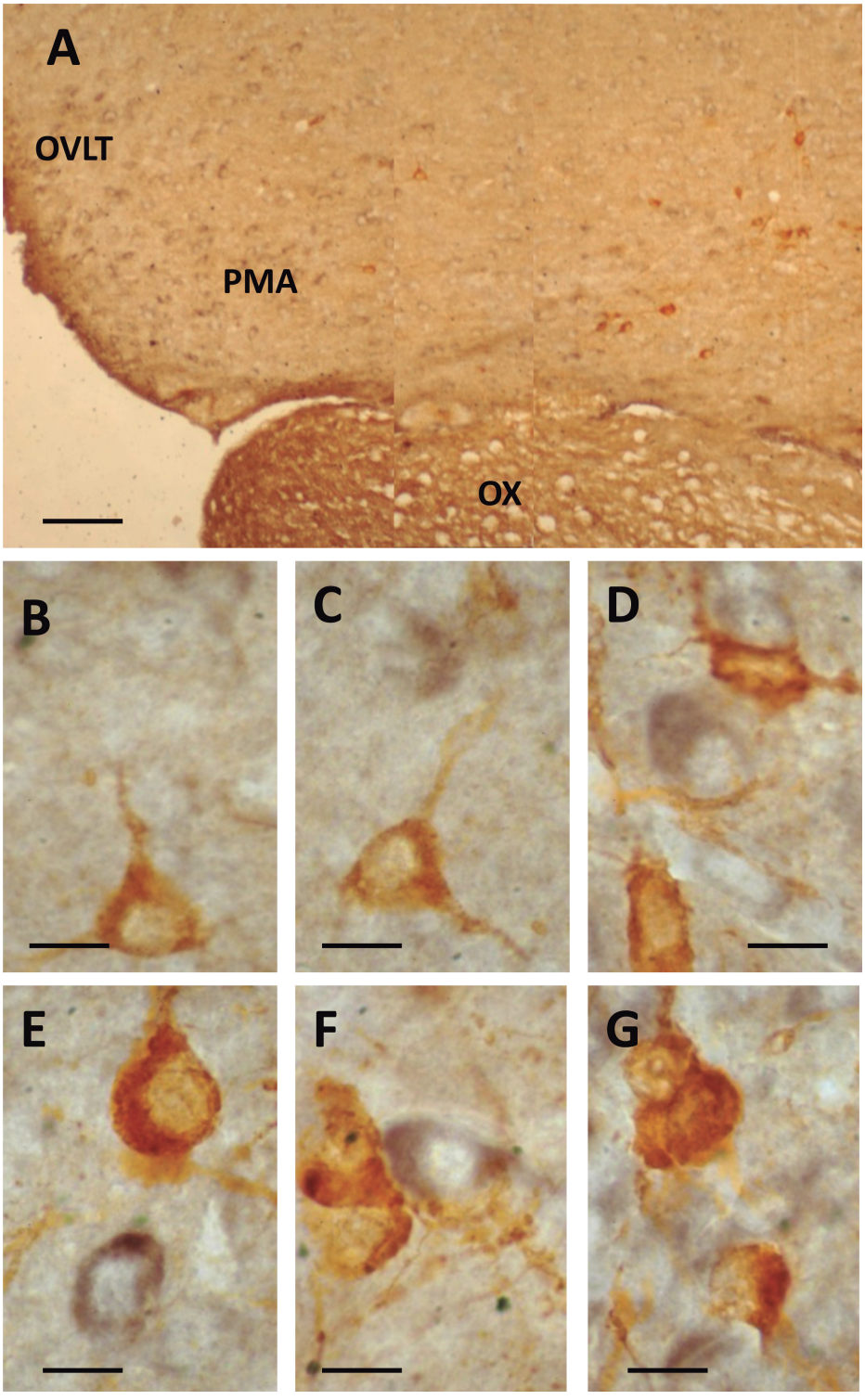

Fig. 1. Shows photographs of the rat PMA and OVLT immuno-stained with anti-TAp73 in WKY rats. **A.** Sagittal view of the PMA and OVLT; **B, C, D, E, F, G** neurons of the PMA marked with anti-TAp73(1-62) brown and anti-TAp73(1-15) grey-blue. OVLT, organum vasculosum of the lamina terminalis; PMA, preoptic medial area; OX, optic chiasma. Bar: A, 150 μm; B-G, 25 μm

(1-62) TAp73. In the OVLT, TAp73-ir was expressed for the whole of the parenchyma in some neurons and in the ependymal layer, the intensity of the reaction was qualitatively quite low in the OVLT of the SHRs (Figs. 2A-D, 4B). TAp73-ir was also observed in the cytoplasm of the ChP cells and other brain cells. The reaction intensity of TAp73 in the ChP cells of the SHR rats was higher than in the WKY group (Figs. 3A-D, 4B).

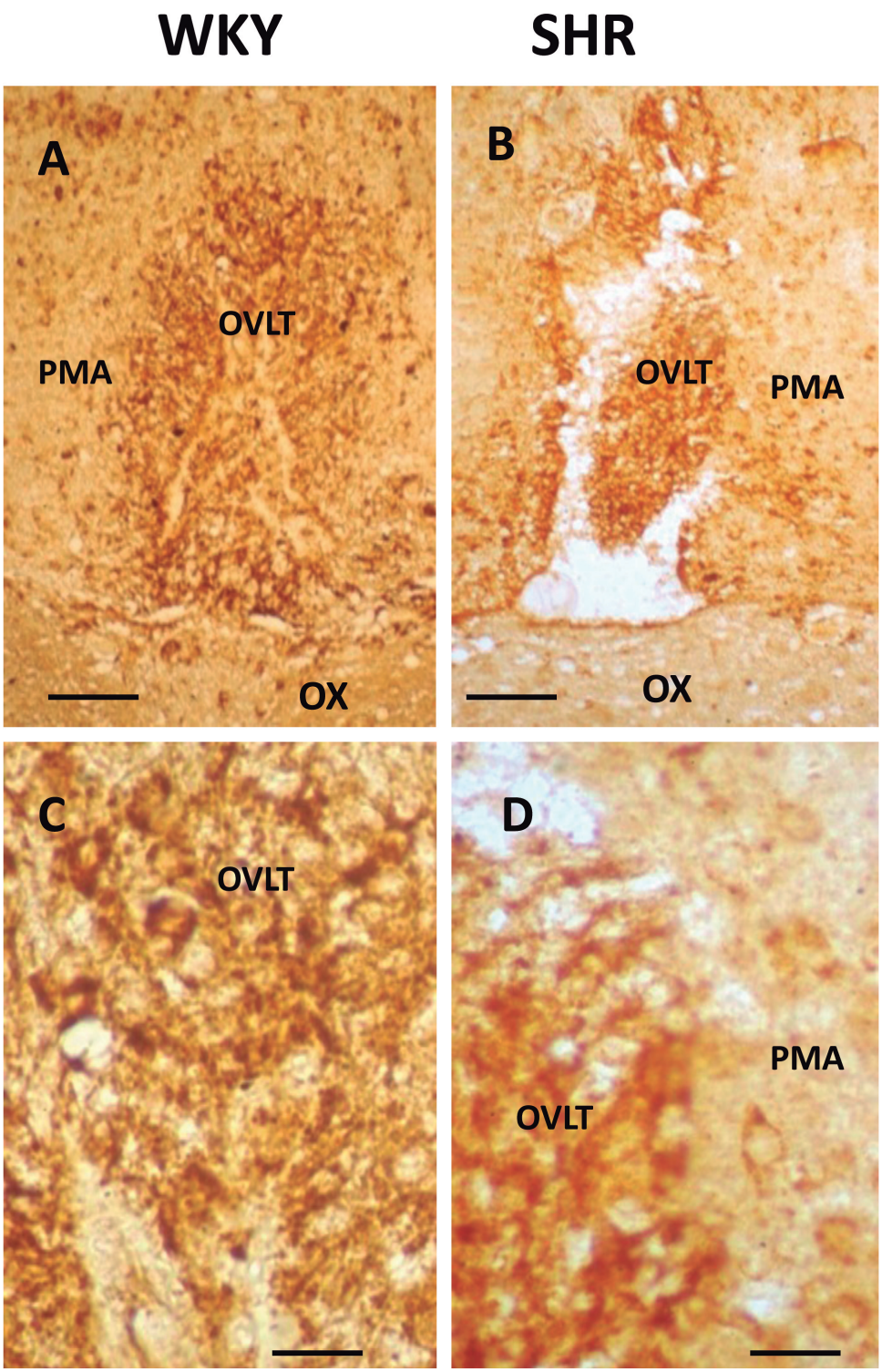

Fig. 2. Shows photographs of the rat OVLT immunostained with anti-TAp73 (1-62). Coronal view of the OVLT; **A, C** WKY rats, **B,D** SHR rats. OVLT, organum vasculosum of the lamina terminalis; PMA, preoptic medial area; OX, optic chiasma. Bar: A, C, 160 μm; B, D, 60 μm

The immunoblotting of OVLT and ChP extracts using Ab14430 (1-62) and Ab-5 (1-15) TAp73 antibodies showed the same 90 and 65 kDa bands, and thus recognized the same protein. The anti-TAp73 (1-62) reactions in the immunoblotting of both bands of 90 and 65 kDa in OVLT extracts were quantitatively less intense

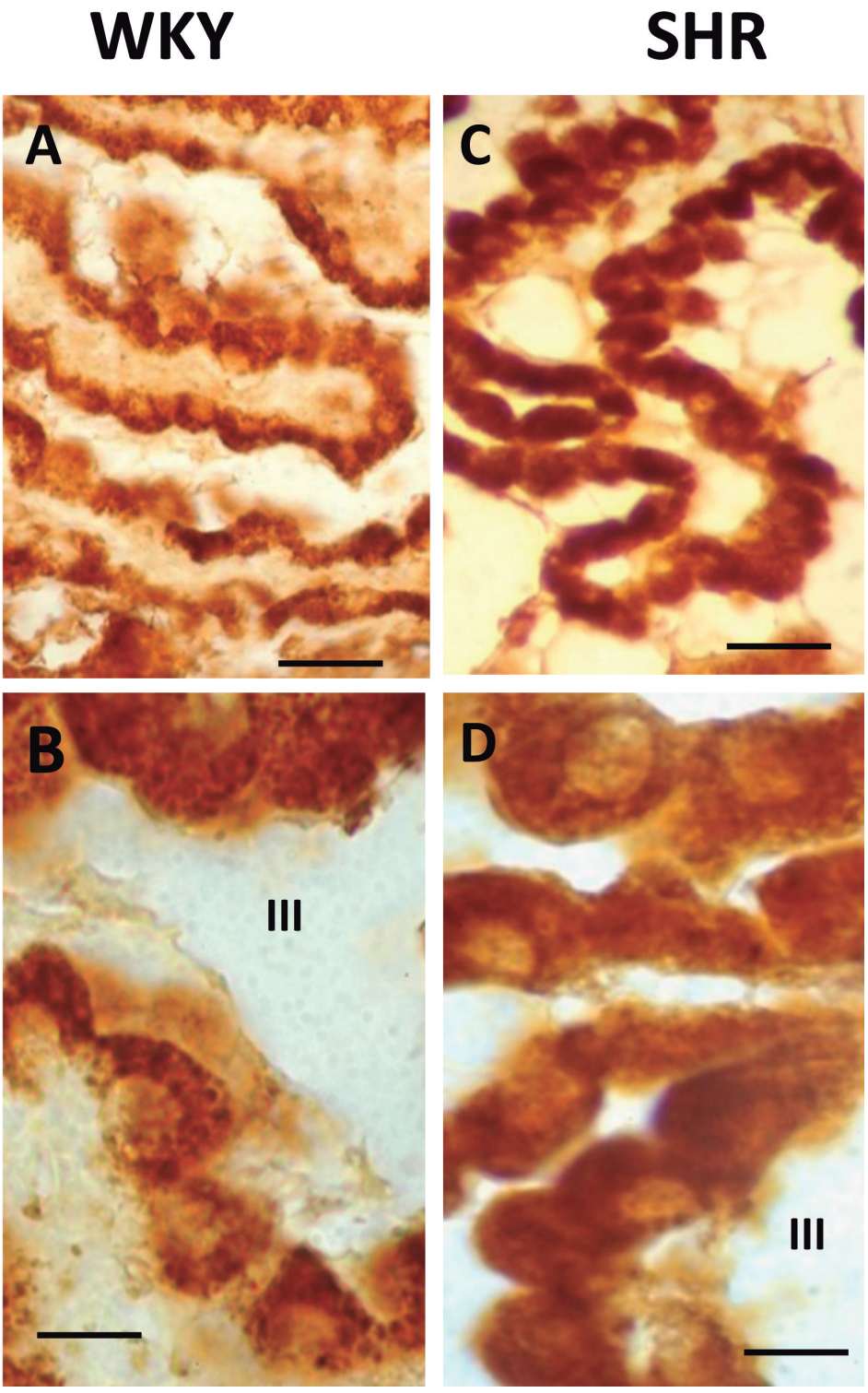

Fig. 3. Shows photographs of the rat choroid plexus immuno-stained with anti-TAp73 (1-62). Coronal view of the ChP; **A, C** WKY rats, **B, D** SHR rats. III, third venticle. Bar: A, C, 90 μm; B, D, 30 μm

in the SHRs (F_{1-10} =214,809 P<0.05) with respect to WKY rats. There were also two bands of the same molecular weight in the ChP extracts and the intensity of the reaction was quantitatively greater in the ChP of the SHRs than in the WKY rats (Fig. 4A,B).

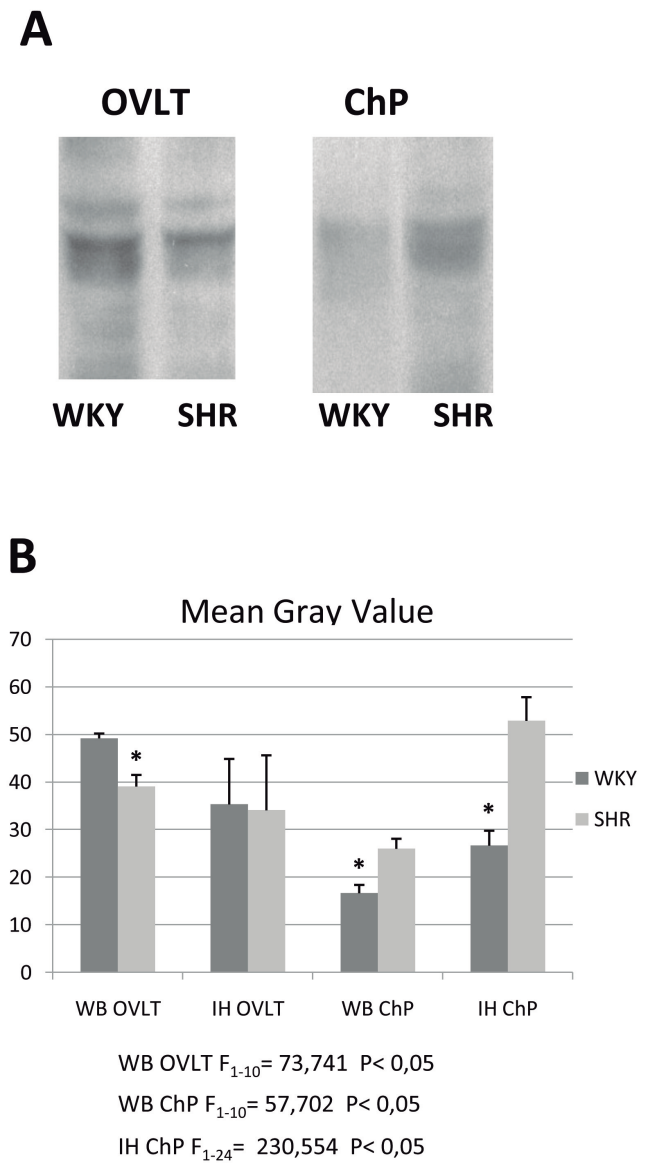

Fig. 4. Shows TAp73(1-62) Western blot and Densitometry of the OVLT and ChP of WKY and SHR rats. **A.** Western blot OVLT and ChP. **B.** Densitometry of the 'Mean Gray Value of the immunostained (IH) slides and Western blot (WB). IH OVLT: densitometry of the OVLT immunostained slides. WB OVLT densitometry of the OVLT Western blot, *significant difference F_{1-10}= 73,741 P<0,05; IH ChP: densitometry of the ChP immunostained slides *significant difference F_{1-24}= 230,554 P<0,05. WB ChP densitometry of the ChP Western blot, *significant difference F_{1-10}= 57,702 P<0,05 ChP: choroid plexus; OVLT: organum vasculosum of the lamina terminalis.

Discussion

The expression of p73 in ChP (Yang et al., 2000; Swetloff and Ferretti, 2005; Cabrera-Socorro et al., 2006) and other CVO (Cabrera-Socorro et al., 2006; Carmona-Calero et al., 2009, 2012) has been described. Thus, p73 expression that has been found in CVO and in CSF was defined by immunohistochemistry and western blot using 1-62-TAp73 as primary antibody (Cabrera-Socorro et al., 2006, 2007; Carmona-Calero et al., 2009, 2012). In the results presented here, the expression of p73 in OVLT ChP cells has mainly been found by using 1-62-TAp73, since immunoreactive reaction with 1-15-TAp73 in OVLT and ChP was scarce or undetectable. In concordance with previous works, the choroid plexus epithelium presented an intense cytoplasm staining with 1-62-TAp73, but another isoform, ΔNp73, only marked nuclear membrane (Cabrera-Socorro et al., 2006, 2007). The discrepancy between the different antibody stainings suggests that the widespread expression of TAp73 in the brain may not be due to the full-length protein TAp73 but to other splice variants whose distribution in the brain is still unknown. The p73 in the CSF is not the result of diffusion from plasma since it is not present in plasma, therefore the presence of TAp73 in the CSF suggests that it is secreted by the CVO, such as the OVLT and ChP in the CSF of the ventricular system (Cabrera-Socorro et al., 2006; Carmona-Calero et al., 2012).

An increase of anti-p73 expression in the subcommissural organ, subfornical organ, area postrema and CSF has been described in SHRs rats with respect to WKY rats in previous works (Carmona-Calero et al., 2009, 2012), and these findings are similar to the results of the present work, where hypertension produced an increase of anti-p73 (1-62) reaction in the SHRs with respect to WKY rats in cytoplasm of the ChP cells, but a decrease in anti-p73 (1-62) reaction in the cytoplasm of the ependyma and in several neurons of the OVLT. The SHRs present an increase in blood pressure from 10 weeks of age and a progressive increase of ventricular size from 4 to 56 weeks of age (Ritter and Dinh, 1986). The variations of anti-p73 reaction in the OVLT and ChP of the SHRs with respect to WKY rats could be due to the fact that hypertension and ventricular dilation induce alterations of the ependymal layer and the neuroepithelium, since one of the principal components of the OVLT and ChP are ependymal cells. On the other hand, previous studies have demonstrated that, in general, the CVO and specifically the OVLT are involved in osmoregulation (McKinley et al., 2004), and activation of the aldosterone-mineralocorticoid receptor system is intensified by aging in SHRs (Pinto et al., 2012). Thus, there is a relationship between OVLT, Na metabolism, CSF and hypertension, since the increases of Na in CSF caused by a high-salt diet lead to increased levels of endogenous ouabain-like substance, which is an inhibitor of the Na pump and binds to the Na^+-K^+-ATPase, and produces a pressor response. This suggests

an enhancement of the brain renin-angiotensin system activity that may be mediated by the increased brain angiotensin converting enzyme located in the OVLT (Hou et al., 2009) and by the increase of vasopressin in the ChP (Carmona-Calero et al., 2012). Furthermore, mice deficient in TAp73 isoform have alterations in the dentate gyrus of the hippocampus (Tomasini et al., 2008) and TAp73 ensures normal adult neurogenesis, raising the long-term maintenance of neural stem cells (Fujitani et al., 2010).

Arterial hypertension in the SHRs produces variations of TAp73 expression in the CVO and CSF; moreover, TAp73 in the CSF plays an important role in normal adult neurogenesis, maintenance of the adult ependyma and ventricular wall. We may conclude that the functional equilibrium and correlation between the p73 proteins, OVLT and ChP, which probably maintains the normal functioning of these structures, is altered by the hypertension present in these kinds of rats.

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