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Original Article

Acid-sensing ion channel 2 (asic 2) and trkb interrelationships within the intervertebral disc

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Abstract: The cells of the intervertebral disc (IVD) have an unusual acidic and hyperosmotic microenvironment. They express acid-sensing ion channels (ASICs), gated by extracellular protons and mechanical forces, as well as neurotrophins and their signalling receptors. In the nervous tissues some neurotrophins regulate the expression of ASICs. The expression of ASIC2 and TrkB in human normal and degenerated IVD was assessed using quantitative-PCR, Western blot, and immunohistochemistry. Moreover, we investigated immunohistochemically the expression of ASIC2 in the IVD of TrkB-deficient mice. ASIC2 and TrkB mRNAs were found in normal human IVD and both increased significantly in degenerated IVD. ASIC2 and TrkB proteins were also found co-localized in a variable percentage of cells, being significantly higher in degenerated IVD than in controls. The murine IVD displayed ASIC2 immunoreactivity which was absent in the IVD of TrkB-deficient mice. Present results demonstrate the occurrence of ASIC2 and TrkB in the human IVD, and the increased expression of both in pathological IVD suggest their involvement in IVD degeneration. These data also suggest that TrkB-ligands might be involved in the regulation of ASIC2 expression, and therefore in mechanisms by which the IVD cells accommodate to low pH and hypertonicity.

Keywords: Acid-sensing ion channel 2, neurotrophin receptor TrkB, human intervertebral disc, degenerate intervertebral disc, TrkB-deficient mice

Introduction

The intervertebral disc (IVD) is a cartilaginous joint placed between two consecutive vertebrae consisting of a central gelatinous nucleus surrounded by an outer fibrous structure called nucleus pulposus (NP) annulus fibrosus (AF), respectively. The adult human IVD contains different types of cells similar to fibroblasts in AF, and similar to chondrocytes in NP, distributed alone or in groups within the extracellular matrix (ECM) [1, 2]. The cells in IVD have an unusual microenvironment because ECM is rich in negatively charged proteoglycans, which attract cations creating a high extracellular osmolarity. Furthermore, the IVD cells have an active anaerobic metabolism leading to the accumulation of lactate and low values of pH [3]. Among the molecular mechanisms involved in maintaining cell survival in these adverse conditions are different families of ion channels [4]. In cells similar to those of the NP these ion channels participate not only in preservation of cells against adverse pH and pressure, but also in regulating the production of ECM compounds [5-7] and cell survival [8, 9].

Acid-sensing ion channels (ASICs), are a family of cation-selective channels gated by extracellular protons, which mediate an increase in intracellular Ca^{2+} upon exposure to acidic conditions and can be gated by mechanical forces [10, 11]. Articular chondrocytes [12-15] as well as IVD cells express different ASIC proteins [16-18], which are required for cell survival in a low pH and hyperosmotic medium.

On the other hand, members of the neurotrophin (NT) family of growth factors, as well as their signalling receptors, have been detected in IVD where presumably regulate the density of innervation and are involved in the genesis of

the discogenic pain [19-24]. In particular, the brain-derived neurotrophic factor (BDNF) and its signalling receptor TrkB are present in native chondrocytes of human IVD, thus suggesting an autocrine or paracrine role of BDNF in regulation of IVD cell biology [19-23]. Interestingly, NTs influence the expression of ASICs, and ASICs co-localize with NT receptors in the peripheral nervous system [see for a review 24]. Recently, it was also demonstrated that the pan-neurotrophin receptor $p75^{NTR}$ participates in the regulation of ASIC3 basal promoter activity in the IVD cells [16], and TrkB co-localizes with ASIC3 in cells isolated from degenerated IVD [22].

Based on the above data, we have designed this study to determine the occurrence and distribution of ASIC2 and TrkB in the human normal and degenerate IVD using quantitative-PCR, Western blot and immunohistochemistry. Moreover, we used IVD from TrkB-deficient mice to analyze the immunohistochemical expression of ASIC2 in IVD in absence of this NT receptor. The study was undertaken to investigate whether or not NTs regulate proteins involved in the mechanisms by which IVD cells accommodate to the low pH and hypertonicity, and the involvement of ASIC2 and TrkB in IVD degeneration.

Material and methods

Human material and treatment of the tissues

Lumbar human IVDs were obtained from subjects of both sexes (21 males, 17 females; age range 46 to 73 years) during surgical procedures to remove IVD for treatment of low back pain due to IVD degeneration from the Servicio de Neurocirugía, Complejo Asistencial Universitario de León (Leon, Spain) and Hospital Universitario Central de Asturias (HUCA, Oviedo, Spain). The material corresponded to grades II (n = 8), III (n = 8), IV (n = 12) and V (n = 10) of the Thompson scale of IVD degeneration [25]. Moreover, freshly lumbar IVD were obtained from subjects free of low back pain history, who had died in traffic accidents (n = 8, males; age range 38-52 years) during removal of organs for transplantation (HUCA); they were considered grade I of the Thompson scale. The collected material was divided into several segments containing both AF and NP tissue. Some specimens (2 blocks 1 cm^3 approximately from each subject) were fixed in 4% formaldehyde in 0.1 M phosphate buffer saline (pH 7.4) for 24 h, dehydrated and embedded in paraffin, sectioned 10 μm thick, and the sections mounted on gelatine-coated microscope slides. Fresh samples (4 blocks 0.5 cm^3 approximately from each subject) were quickly frozen and stored at -80°C until used. All the tissues used in the present study were obtained in compliance with Spanish Law and the guidelines of the Helsinki Declaration II.

Mice material and treatment of the tissues

The spines used in the present study were kindly provided by Dr. I. Silos-Santiago, and were obtained from mice described elsewhere [26]. Wild type (n = 4, TrkB +/+), and homozygous TrkB-deficient mice (n = 5, TrkB -/-) were studied at 15 days postnatally which is at the limit of the survival. Animals were euthanized with an overdose of chloral hydrate and were perfussed transcardially with a cold solution of 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Then, the lumbar spine was removed and placed into the same perfusion fixative. The samples were processed for routine paraffin embedding, sectioned at 10 μm thick, and the sections mounted on gelatin-coated microscope slides.

Quantitative PCR

The technical procedure was described in detail previously [17]. The sequences of the oligonucleotide primers were based upon the published sequences for *Homo sapiens TrkB* (GeneBank accession number NM_001007097.1) and *Homo sapiens* ASIC2 (GeneBank accession number NM_001094), and were: for TrkB forward: 5'-gatcctgagaacatcccga-3', reverse: 5'-caccaggatcagttcagac-3'; for ASIC2 forward: 5'-accaccaatgacctgtacca-3', reverse: 5'-gccctttgaacttgcagtag-3'. The homemade TaqMan probes were labelled at the 50 with 60FAM fluorochromes for the ASIC2 and TrkB, and VIC fluorochrome for β-actin, while the 30 ends were labelled with the Minor Grow Binder (MGB) quencher. The assays were performed in triplicate using a 7500 PCR real-time System (Applied Biosystems), and quantification was calculated using the 2-DDCt algorithm. The average value in grade I IVD was considered as the standard, and the final results were expressed as ± n-fold difference versus standard (relative expression). Statistical differences

among experimental groups with respect to the standard were performed using analysis of variance (ANOVA). *P*-values less than 0.05 were considered significant.

Western blot

Western blot analysis was performed in frozen IVD samples containing both AF and NP tissue as described earlier [17]. The anti-TrkB antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; TrkB [794]: *sc-12*) was raised in rabbit and used diluted 1:100 in the blocking buffer. This antibody is directed against the residues 794-808 of the intracytoplasmatic domain of human TrkB. The anti-ASIC2 antibody (Lifespan Biosciences, Seattle, WA, USA; LS-B156/12883) was raised in rabbit and used diluted 1:200. It was raised against a synthetic peptide from the extracellular domain of mouse ASIC2 conjugated to an immunogenic carrier protein.

Single immunohistochemistry

Sections from human and mice IVD were processed for detection ASIC2 and TrkB using the EnVision antibody complex detection kit (Dako, Copenhagen, Denmark), following supplier's instructions, as reported earlier [17]. The sections were incubated overnight at 4°C with primary antibodies described above both used diluted 1:100.

Double immunohistochemistry

To investigate whether or not ASIC2 and TrkB co-localized, and to establish the percent of chondrocytes expressing each protein, IVD sections were processed for alternative detection of ASIC2 and TrkB. Non-specific binding was reduced by incubation for 30 minutes with a solution of 1% bovine serum albumin in Tris buffer solution (TBS). The sections were then processed as follow: in the first step were incubated overnight at 4°C in a humid chamber with anti-ASIC2 antibody diluted 1:200, then rinsed with TBS, and incubated for 1 hour with Alexafluor 546-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000 in TBS, then rinsed again; in a the second step sections were incubated overnight at 4°C in a humid chamber with anti-TrkB antibody diluted 1:100, then rinsed with TBS, and incubated for 1 hour with Alexafluor 488-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000. Both steps were performed at room temperature in a dark humid chamber. Moreover the sections were incubated with DAPI (diluted in glycerol medium, 10 ng/ml) to ascertain that immunoreactivity occurred in the cells and not in ECM. Fluorescence were detected using a Leica DMR-XA microscope coupled with a Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany) and the images captured were processed using the software Image J version 1.43 g Master Biophotonics Facility, Mac Master University Ontario (www.macbiophotonics.ca).

For control purposes sections were processed in the same way as described above using specifically preabsorbed antisera (5 μg/ml of the ASIC2 blocking peptide-Lifespan Biosciences LS-PB156 in the working solution; 10 μg/ml of the TrkB blocking peptide-sc-12P, Santa Cruz Biotechnology- in the working solution). Under these conditions no positive immunostaining was observed (data not shown).

Quantitative study

The percentage of cells in AF and NP displaying immunoreactivity for ASIC2 or TrkB was calculated automatically in 5 randomly selected fields per section, 10 sections per specimen 100 μm apart to avoid count a cell twice. Counts were carried out using an automatic image analysis system (Quantimet 550, Leika, QWIN Program, Servicio de Análisis de Imágenes, University of Oviedo). The total number of cells was established by counting number of DAPI positive nuclei, and the percentage of the cells immunoreactive determined on the basis of the association of ASIC2 or TrkB immunoreactivity and DAPI in the same cell. The percentage of ASIC cells was calculated automatically and the data expressed as mean ± S.E.M. The data were tested for normality using the Shapiro-Wilke W method of analysis, and as they did not follow a normal distribution, a two-sided Mann-Whitney U test was carried out to determine differences between the percentage of ASIC2 or TrkB positive cells in the normal and degenerate IVDs. A Wilcoxin test was used to identify significance between the different regions of the IVD (NP and AF). $P < 0.05$ was considered significant. Values of $P < 0.05$ were considered as significant.

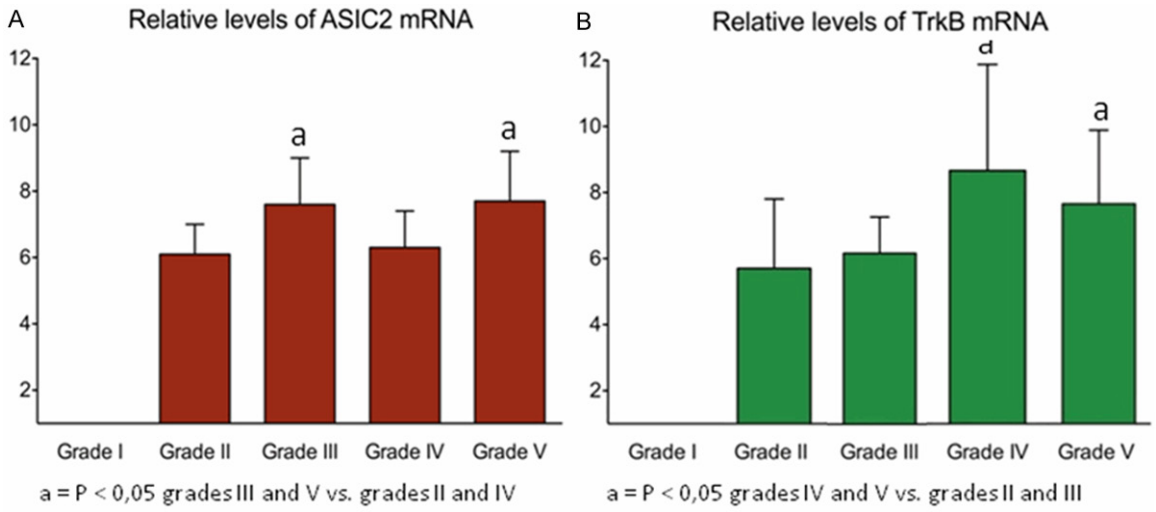

Figure 1. Relative mRNA levels of ASIC2 (A) and TrkB (B) in degenerated human intervertebral disc in relation to the grade I of Thompson's.

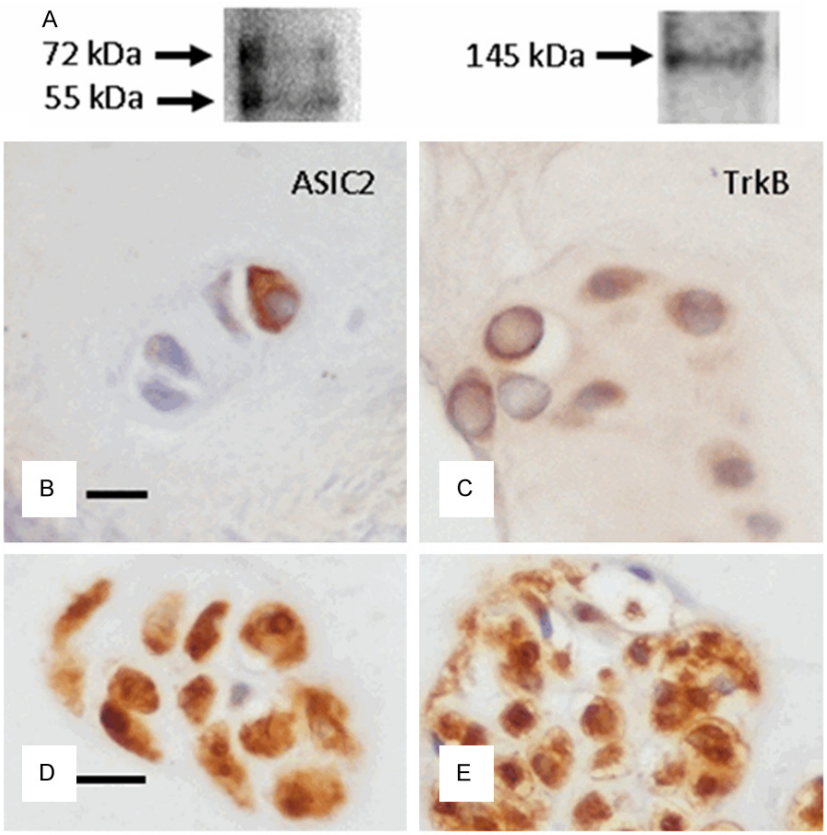

Figure 2. Western blot detection of TrkB and ASIC2 in homogenates of normal human intervertebral disc (A). The anti-TrkB antibody used recognizes a single protein band of 145 kDa consistent with full-length TrkB. The anti-ASIC2 antibody used labels two protein bands of estimated molecular weights of 72 kDa and 55 kDa. By immunohistochemistry a variable number of cells in normal (B, C) and degenerated (D, E, Thompson's III grade) IVD, in both the nucleus pulposus and the annulus fibrosus, displayed ASIC2 (B, D) and TrkB (C, E). Scale bar = 15 μm for (B, C); 10 μm for (D, E).

Results

TrkB and ASIC2 mRNAs are present in normal and degenerated human IVD

The expression of *ASIC2* was analyzed in samples of human IVD, graded from I to V in the Thompson's scale. Considering grade I as the standard, there was a significant increase of *ASIC2* expression in all grades of degenerated IVD, but this increase was not linearly correlated with the grade of degeneration (**Figure 1A**). Regarding the expression of TrkB, and considering also as a standard the levels obtained in grade I, there was a significant increase in the *TrkB* expression in all groups of degenerated IVD, reaching maximal values in grades IV and V (**Figure 1B**).

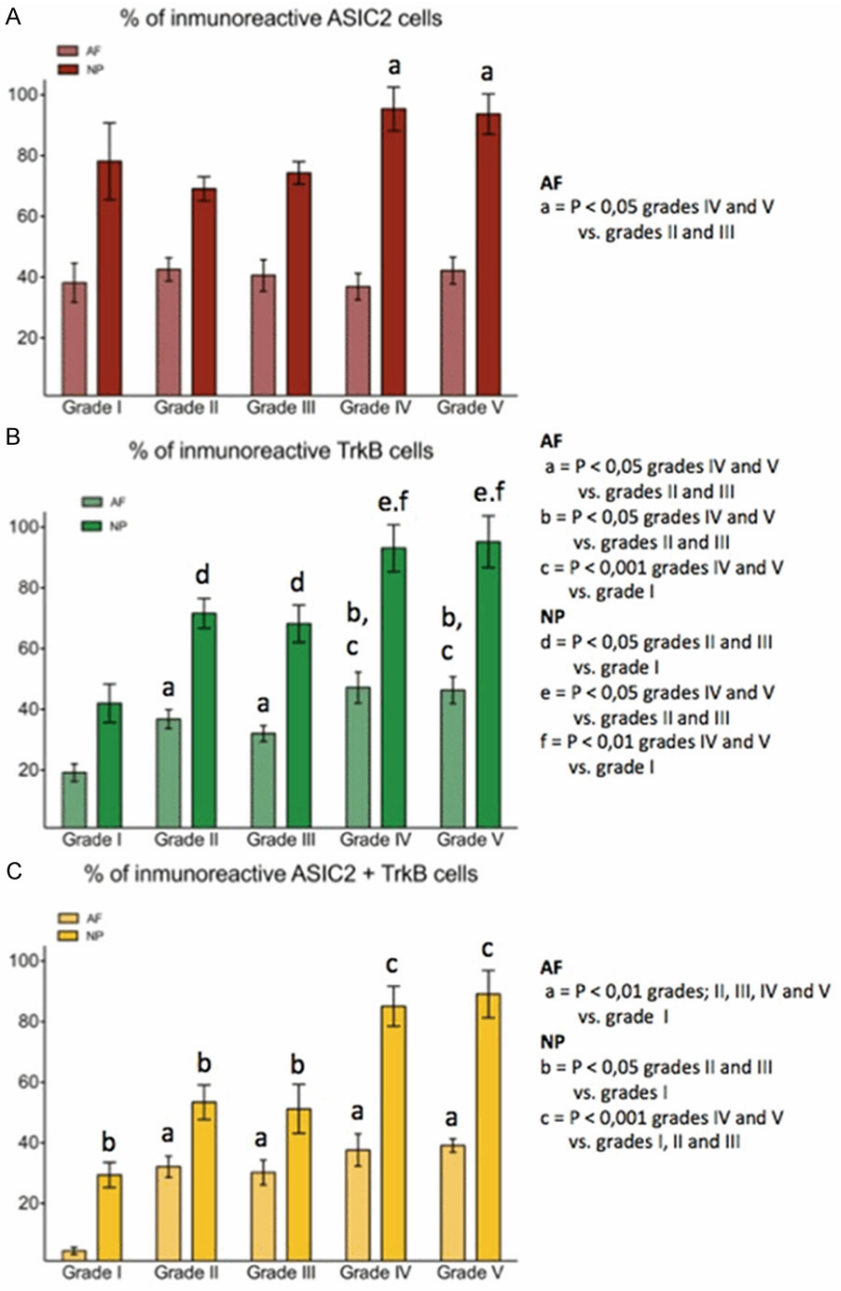

Figure 3. Percentage of cells displaying ASIC2 (A) and TrkB (B), and co-localization of TrkB-ASIC2 (C) in the anullus fibrosus (AF) and nucleus pulposus of grade I and degenerated human intervertebral disc. Degeneration was graded following the Thompson's scale.

TrkB and ASIC2 proteins are present in normal and degenerate human IVD

Western blot analysis of ASIC2 in homogenates of normal and degenerated human IVD indicates the presence of two protein bands whose estimated molecular weight were of 72 kDa and 55 kDa, respectively (**Figure 2A**). Regarding TrkB, Western blot in homogenates of normal human IVD shows the existence of a single protein band with an estimated molecular weight of 145 kDa, which is the expected for the full-length isoform of TrkB (**Figure 2A**).

Consistently, in both normal and degenerated IVD positive immunoreactivity was observed in sections processed for detection of TrkB and ASIC2, and in both cases cells displaying immunostaining were identified as chondrocyte-like cells of the NP and as fibroblast-like cells of the AF (**Figures 2B-E**, **3**).

The percentage of ASIC2 positive cells in the grade I was significantly greater ($P < 0.05$) in the NP (78.1 ± 12.6) compared with AF (38.2 ± 6.4). In the samples from degenerated IVD the density of ASIC2 positive cells did no changed significantly in AF for none of the groups whereas there were non-significant changes in the NP of stages II and III, and significant increases in stages IV and V (**Figure 4A**). From II to V stages significant differences in the percent of immunoreactive cells between AF and NP were observed.

In grade I the percentage of TrkB positive cells was significantly greater (in the NP (42.01 ± 6.3) compared with AF (19.21 ± 2.9). In the sections from degenerated IVD the density of TrkB positive cells markedly increased in both AF and NP, which progressively increased with the

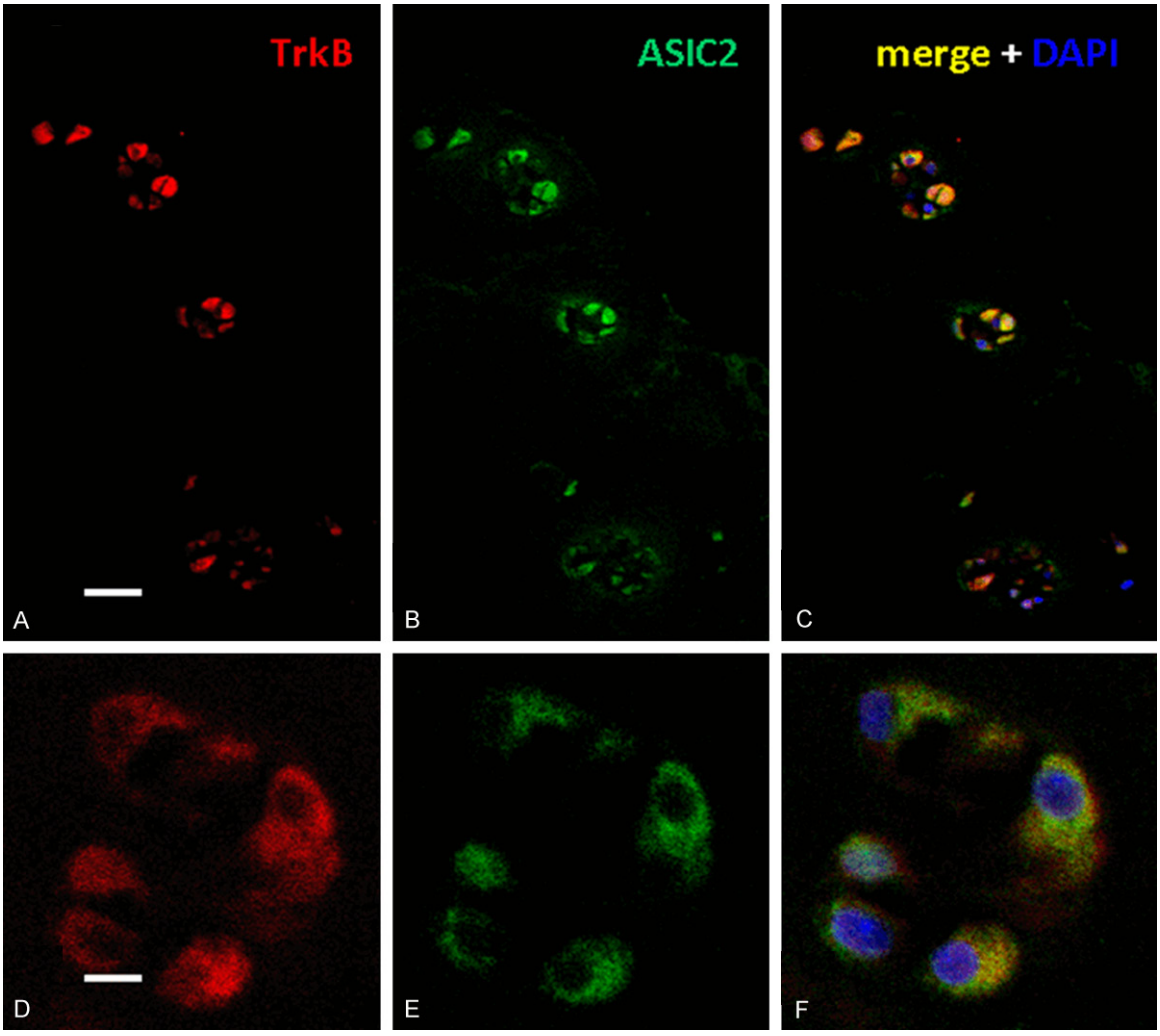

Figure 4. Immunohistochemical detection of ASIC2 and TrkB in cells of the nucleus pulposus (6) of normal human IVD. Co-localization of both proteins was evident in the nucleus pulposus cells. Scale bar: 10 μm.

grade of Thompson's scale. All the differences were statistically significant with respect to the grade I in both AF and NP, and also between stages II and III vs. IV and V (**Figure 4B**). From II to V stages significant differences in the percent of immunoreactive cells between AF and NP were observed.

Co-localization of ASIC2 and TrkB in normal and degenerated human IVD

The co-localization of TrkB and ASIC2 paralleled the data reported for ASIC and TrkB individually. Merge ASIC2-TrkB immunoreactivity results in yellow in the whole cytoplasm or in a segment of the cytoplasm. AF of normal IVD showed a low percentage of cells displaying ASIC2-TrkB co-localization (**Figure 4C**), while in NP almost all the ASIC2 positive cells also were TrkB positive (**Figure 4C**; $P < 0.001$). Regarding the degenerated IVD, co-localization of TrkB and ASIC2 was observed in a variable percentage of cells. In AF significant differences ($P < 0.001$) were found in stages II to V with respect to the stage I, thus almost all TrkB positive cells were also ASIC2 positive. In NP the percentage of cells showing ASIC2-TrkB co-localization ranged from around 60% in grades IV and V to around 35% in grades II and III of the Thompson's scale (**Figure 4C**), and all the differences were statistically significant. From I to V stages significant differences in the percent of immunoreactive cells between AF and NP were observed.

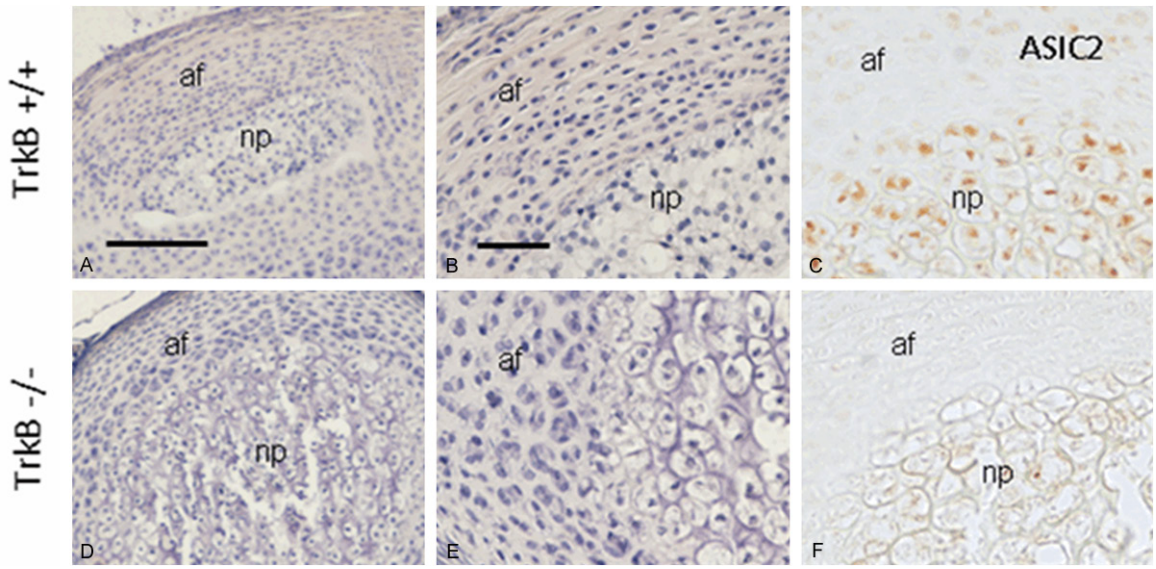

Figure 5. Structure and immunohistochemical detection of ASIC2 in the lumbar IVD of wild-type (A-C) and TrkB-deficient mice (D-F). The absence of TrkB abolished the expression of ASIC2 by the annulus fibrosus (AF) and nucleus pulposus (np) cells. Scale bar: 1 mm for (A and D); 20 μm for (B, C, E and F).

ASIC2 expression in mouse IVD of TrkB deficient mice

To elucidate whether TrkB and their ligands might regulate the expression of ASIC2 in cells of IVD, we have investigated immunohistochemically the occurrence of ASIC2 in IVD of wild-type and TrkB deficient mice. In wild-type animals, two weeks old, there was faint ASIC2 immunostaining in the AF cells, which was more evident in the chondrocyte-like cells of NP. Conversely in the IV from functionally deficient TrkB mice there was a complete absence of ASIC2 immunostaining (**Figure 5**).

Discussion

The cells of the human IVD are embedded in an unusual microenvironment characterized by low pH and a high extracellular osmolarity [3]. Moreover, they are exposed to a wide range of mechanical loads, mainly hydrostatic pressure in NP and tensile strain in AF [see for a review 27]. Consequently, the cells in IVD have developed different strategies for survival including the expression of different families of ion channels [4]. ASICs are cation selective channels that work as acid-sensors and mechanosensors [10, 12, 13, 15], and they have been detected in the IVD cells [16, 17, 28]. On the other hand, it is well documented that NTs influence expression of ASIC2 in the sensory neurons of the peripheral nervous system [29-32], and of ASIC3 in cultures of IVD cells [16].

Based on these data we designed the present study to investigate the occurrence of ASIC2 and TrkB in the normal and degenerated human IVD, and whether or not the ligands of TrkB, i.e. BDNF and/or NT-4, may regulate the expression of ASIC2 in the IVD cells. The changes in the expression of these proteins, and its mRNAs, in degenerated human IVD were also investigated, as well as the absence of TrkB in the expression of ASIC2 by the IVD cells.

Our results demonstrate ASIC2 at the mRNA and protein levels in normal IVD, the density of ASIC2 positive cells being greater in NP than in AF. These data are in total agreement with a previous study from our group [17], and add new data about the presence of members of ASIC family in the IVD [16, 18, 28]. TrkB mRNA and TrkB full-length protein as well as TrkB positive cells were also found in normal human IVD, being greater the percentage of cells in the NP than in the AF. These findings are in good agreement with previous studies reporting the occurrence of TrkB in a subpopulation of IVD cells [20-23]. Interestingly co-localization of ASIC2 and TrkB was detected in IVD cells in both the AF and NP; similarly, co-localization of ASIC3-TrkB in cultured NP cells has been shown [22].

In degenerated IVD, we observed increased levels of ASIC2 mRNA and higher density of cells showing ASIC2 immunoreactivity with respect to the controls, as previously reported [17]. These findings suggest that high levels of ASIC2 seem to be related to IVD degeneration. At present is difficult to link changes in ASIC2 expression with IVD degeneration. Presumably, ASIC2 has a similar role as in articular chondrocytes [6-9, 13-15, 33-35] altering ECM composition and cell survival that occur in IVD degradation. However, a definitive role of ASIC2 in cells of IVD remains to be established.

The TrkB mRNA and the number of cells displaying TrkB immunoreactivity also increased in degenerated human IVD, especially in the more advanced grades. Globally our results are in good agreement with previous studies by Purmessur et al. [21] although we have observed that the increase in TrkB correlates with the grade of IVD degeneration, while these authors do not. Recently increased levels of BDNF and TrkB have been found in degenerated [23] and painful whole body vibration [36] human IVD.

In our study, the increased expression of ASIC2 and TrkB in degenerated IVD were basically parallel, thus leading support to a close relationship between them within the IVD, and to a possible regulation of ASIC2 expression by the TrkB ligands, as it occurs in the peripheral nervous system. Interestingly, elevated values of BDNF were found in degenerated IVD [21, 22, 37]. Additional support for a control of ASIC2 for TrkB and their ligands was provided by our results in TrkB deficient mice, since ASIC2 positive cells is absent from IVD in these animals whereas it is present in the wild-type ones.

The results reported here expands our knowledge about the role of TrkB and their ligands in the human IVD although further studies are necessaries to clarify the networks involved in the regulation of IVD cell survival in normal conditions and the changes when IVD degenerate. Although the occurrence of TrkB and BDNF in human IVD suggest that autocrinia and/or paracrinia exists in this organ, and therefore, other molecules than NTs surely control the expression of ASICs in the IVD. It is know that NTs are expressed in non-neuronal tissues, including the IVD [24] but few studies have examined the function of NTs in non-neural cells.

In our study we have regularly found that NP cells were more affected that AF cells in IVD degeneration. Probably these differences reflects the different degeneration manner of the each IVD components [2, 38] and the different sensitivity of AF and NP cells to mechanical forces, changing the biological responses according the cell type and the properties of loading [27]. This might explain at least in part the differences in the expression of ASICs found between AF and NP during IVD degeneration.

As summary, all together data from the present study indirectly argues for a functional interaction between TrkB and ASIC2, but in the absence of further experimental procedures it remains to be definitively demonstrated. Experiments are in progress, in our laboratory, to elucidate the mechanisms throughout this regulation occur. Finally and overall, we report unprecedented data about expression of ASIC2 in IVD.

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Disclosure of conflict of interest

None.

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