Erectile tissue molecular alterations with aging—differential activation of the p42/44 MAP Kinase pathway

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Abstract Erectile dysfunction (ED) is a common problem in aged men; however, the molecular events involved in aging ED remain unclear. To better characterize the effects of aging in the penis, we evaluated cavernosal tissue remodeling capability and the downstream activation of the intracellular signaling mediator mitogen-activated protein p42/44 kinase (p42/44 MAPK). We used male Wistar rats, which were divided in groups of 2, 6, 12, 18, and 24 months old. Penile tissues were harvested and processed for protein isolation and immunohistochemical analysis. Cavernosal viability was assessed by TUNEL assay, and proliferation was analyzed by immunohistochemical detection of proliferating cell nuclear antigen

(PCNA). Immunolocalization of the activated form of p42/44 MAPK was evaluated by immunofluorescence, and changes in its phosphorylation status were quantified by western blotting. p42/44 phosphorylation profile was also assessed in situ in human young and elderly cavernosal samples. With the advancement of age, experimental cavernosal tissue remodeling was affected by an age-dependent unbalance between the rate of apoptosis and proliferation, in all erectile components. Moreover, this turnover alteration was accompanied by significant modifications in the activation profile of the downstream effector p42/44 MAPK. In the youngest corporeal samples, p42/44 was mostly activated at perivascular sites, potentially mediating cell survival/proliferation. However, in elderly experimental erectile tissue, p42/44 phosphorylation shifted to trabecular fibroblasts, indicating a potential role in extracellular matrix (ECM) production. More importantly, the same differential pattern of p42/44 activation was observed in human young and aged cavernosal fragments, suggesting a distinct function of this protein with aging. We provided evidence for the first time that with the advancement of age, there is a differential activation of p42/44 MAPK in cavernosal tissue, which may promote ECM expansion and fibrosis, therefore compromising erectile function in the elderly.

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Introduction

Erectile dysfunction (ED) is a public health problem affecting seriously the quality of life of men and their partners (Travison et al. 2007). The advancement of age has been associated with the decline in erectile function, which occurs particularly in men older than 50 years (Diokno et al. 1990; Feldman et al. 2000). It is thought that the structural organization of penile erectile components, which play a key role in erection mechanisms, is affected by the aging process. Cavernosal perivascular smooth muscle cells (SMCs) and sinusoidal lining endothelium build up the vascular component (Goldstein and Padma-Nathan 1990), which is embedded in an extracellular matrix (ECM) mostly composed by collagen and elastic fibers (Wespes et al. 1991). With the advancement of age, several modifications in the composition and organization of these components were reported and suggested to alter elderly erectile capability. Aged cavernosal tissue presents variations in SMC content and function (Bakircioglu et al. 2001; Wespes 2002), changes in vascular angioarchitecture (Costa and Vendeira 2008), increase in collagen production (Lin et al. 2001), and degeneration of elastic fibers (Calabrò et al. 1996). These modifications affect penile hemodynamics by impairing cavernosal SMC relaxation, reducing penile elasticity, compliance, and promoting fibrosis (Montorsi et al. 2003; Moreland 2000; Siroky and Azadzoï 2003). However, the downstream molecular mechanisms underlying these corporeal changes in the elderly remain to be elucidated. It was suggested that altered SMC biology was due to a decreased expression of anti-apoptotic molecules in aged corpus cavernosum (CC; Yamanaka et al. 2002). In fact, with aging, events controlling tissue remodeling, such as the ratio apoptosis/proliferation, become unbalanced ensuing in loss of homeostasis. Molecularly, alterations in the serine/threonine protein kinase, mitogen-activated protein p42/44 kinase (p42/44 MAPK), has been suggested to occur in aged tissues affecting remodeling status (Ikeyama et al. 2002). This important downstream effector kinase regulates a multitude of cellular activities, including cell proliferation, survival, and ECM expansion (Papakrivopoulou et al. 2004; Pearson et al. 2001). However, a thorough evaluation of CC remodeling alterations and the activation of the aforementioned kinase pathway have never been

addressed in aging ED. Therefore, we investigated aging erectile tissue remodeling status and characterized alterations in the activation pattern of the p42/44 signaling kinase.

Materials and methods

Animals

All experimental procedures complied with European Union regulations for the Care and Use of Laboratory Animals. Male Wistar rats (Charles River Laboratories, Barcelona, Spain) were divided into five groups: 2, 6, 12, 18, and 24 months old ($n=5$). After sacrifice, the penises were excised: one half was processed for western blotting and the other penile half was fixed in 10% buffered formaldehyde and paraffin embedded. Five-micrometer cross-sections of equivalent penile regions were placed in 0.1% poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA)-covered slides.

Human samples

All patients gave informed consent for the removal of cavernous tissue, and this study was approved by the local ethics committee. Nine aged patients with ED (mean age $\pm$ standard error (SE), 74.1 $\pm$ 1.08) and five young non-ED individuals (mean age, 27.5 $\pm$ 4.2) were enrolled in this study. Elderly individuals underwent penile implant surgery, and younger men had procedures for penile enlargement and/or lengthening. During the surgical interventions, cavernosal fragments with approximately 5–7.5-mm length and 2.5-mm width were cut with an 11 surgical knife and immediately collected and fixed in formaldehyde. Human corporeal tissue was processed and sectioned as described above for rat penile samples.

TUNEL assay and apoptotic cell density (ACD) quantification

Apoptosis was evaluated by in situ fluorescein labeling of DNA fragments by TUNEL (Terminal Transferase dUTP Nick End Labeling) assay, as modified by Costa et al. (2009). Sections were observed under a fluorescence microscope (Imager. Z1, Carl Zeiss MicroImaging GmbH, Jena, Germany). Each TUNEL experiment was performed in duplicate.

Apoptotic cell density (ACD) quantification was performed as described by Costa et al. (2009), and ACD results were expressed as mean number of apoptotic nuclei per square millimeter of cavernosal surface area.

Immunohistochemical detection of proliferating cell nuclear antigen (PCNA)

Immunohistochemistry was performed using the streptavidin–biotin peroxidase complex method, as modified by Costa et al. (2009) and Costa and Vendeira (2008). Slides were incubated with the primary antibody, a cross-reactive mouse anti-human proliferating cell nuclear antigen (PCNA; 1:200; Chemicon International, Harrow, UK), and after with the respective secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by the avidin–biotin complex reagents (DAKO, Glostrup, Denmark). Negative controls were performed by omission of the primary antibody. The reaction was developed using diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA), and sections were counterstained with hematoxylin. Slides were observed under the Olympus AH3-RFCA microscope (Olympus Imaging Europa GmbH, Hamburg, Germany), and images were captured using the Olympus C-35AD-4 camera (Olympus Imaging Europa GmbH, Hamburg, Germany). Each PCNA immunostaining experiment was performed in duplicate.

Quantification of density of proliferating cells (DPC)

PCNA-expressing cells were counted throughout each CC samples, using the $\times 20$ objective of the Olympus AH3-RFCA microscope (Olympus Imaging Europa GmbH, Hamburg, Germany). Erectile tissue areas were calculated as described by Costa et al. (2009), and the results were expressed as DPC, mean number of PCNA-positive cells per square millimeter of cavernosal surface area (mean PCNA-positive cells per square millimeter).

Dual immunolabeling detection of phosphorylated (phospho)-p42/44 and α -smooth muscle actin (α -SMA)

The activation pattern of p42/44 and the detection of SMCs were assessed by double immunofluorescence,

using specific antibodies anti-phospho-p42/44 and anti- α -SMA, as modified by Costa et al. (2009) and Costa and Vendeira (2008). Briefly, after re-hydration, rat penile and human cavernosal sections were incubated with a mixture of the primary antibodies: a cross-reactive rabbit anti-human phosphop42/44 (1:150 dilution; Thr202/Tyr204; D13.14.4E Cell Signaling Technology, USA) and a cross-reactive mouse anti-human β -SMA monoclonal antibody (dilution 1:500; clone ASM; Chemicon International, UK). Next, slides were incubated with the following secondary antibodies: a donkey anti-rabbit conjugated with a red fluorochrome (dilution 1:1,000; Alexa 594, Lot 44787A; Invitrogen, Carlsbad, CA, USA) and a donkey anti-mouse conjugated with a green fluorochrome (dilution 1:1,000; Alexa 488, Lot 49728A; Invitrogen, Carlsbad, CA, USA). Negative controls were performed in adjacent tissue sections, by omission of the primary antibodies. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; Invitrogen, Carlsbad, CA, USA), and sections were mounted and observed under the fluorescence microscope (Imager.Z1, Zeiss, Germany). Each immunostaining experiment was performed in duplicate.

Western blotting (WB)

One half of the rat penises were processed for total protein extraction, by mechanical homogenization in cold lysis buffer, phosphate buffer solution, and 0.1% Triton in the presence of phosphatase inhibitors (phosphatase inhibitor cocktail 1 and 2; P5726 and P2850 Sigma-Aldrich, USA) and protease inhibitors (protease inhibitor cocktail; P8340, Sigma-Aldrich, USA). After centrifugation (1,600 rpm, 5 min), supernatants were collected, and proteins were quantified using the NanoDrop 1000 (ThermoScientific, Wilmington, DE, USA). Eight micrograms of each sample was separated by electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel. After transfer, the membranes were incubated with the primary antibody: a cross-reactive rabbit anti-human phospho-p42/44 (1:1,000 dilution; Thr202/Tyr204; D13.14.4E Cell Signaling Technology, USA). Antibody binding was detected after incubation with the respective secondary horseradish-peroxidase coupled antibody (1:2,000; Santa Cruz Biotechnology, CA, USA) and using enhanced chemiluminescence (ECL

kit; Amersham Biosciences, UK). Membranes were stripped and re-probed with a rabbit anti-human p42/44 and goat anti-human β -actin antibodies (1:1,000; Santa Cruz Biotechnology, USA), as described above. Age-related changes in phosphorylation were detected by densitometry (Vision Works LS Software; UVP Inc, Upland, CA, USA). Results were expressed as mean relative intensity and calculated by comparing the intensity of phospho-p42/44 with the intensity of total p42/44 protein.

Statistical analysis

Data is presented as mean $\pm$ SE. Statistical analyses were performed using the Student–Newman–Keuls test, and $P<0.05$ was considered to be statistically significant. Significant differences between animal age groups were determined using the one-way analysis of variance; a value of $P<0.05$ was considered statistically significant.

Results

Age-dependent increase in cavernosal tissue apoptotic cell density (ACD)

To evaluate cavernosal tissue remodeling with aging, we began by assessing corporeal apoptosis, by TUNEL assay. As illustrated in Fig. 1a, 2-month-old CC presented few apoptotic cells scattered throughout the tissue (Fig. 1a, arrowheads). Most sinusoidal endothelial cells (ECs) appeared to have their integrity maintained (Fig. 1a). With aging, there was a marked increase in cells undergoing programmed death (Fig. 1b–e). A significant increase in apoptotic cells was observed in 6-month CC, as compared to younger CC (Fig. 1b). In the elderly 12-, 18-, and 24-month-old samples, the number of apoptotic cells augmented in all corporeal components, in particular, in lining ECs (Fig. 1c–e, arrowheads). Additionally, with aging, it was noticeable that the number of DAPI-labeled nuclei had decreased (Fig. 1a–e). To express the differences in apoptosis with aging, we established an ACD index (mean number of apoptotic cells/tissue area; square millimeter $\pm$ SE), as previously described (Costa et al. 2009). The results, compiled in Fig. 1f, demonstrate that 2-month-old

CC had an ACD of 13.55 ± 1.64 , which significantly increased ($p<0.001$) in an age-dependent fashion to 47.67 ± 1.34 at 6 months and to 65.54 ± 6.38 , 66.48 ± 4.96 , and 76.76 ± 8.19 in the remaining aged groups. This significant increase in ACD, allied with the observation that with aging there was a reduction in the total number of DAPI-stained nuclei, led us to hypothesize that cell proliferation could also be deregulated.

Erectile tissue proliferating cell density (PCD) declines with aging

We further analyzed CC cell turnover by immunohistochemistry for PCNA. As shown in Fig. 2a, 2-month-old CC presented an elevated number of PCNA-positive cells, dispersed throughout. Perivascular SMCs and ECs expressing PCNA were commonly observed in the youngest group (Fig. 2a, arrows). With aging, there was a clear age-dependent reduction in the number of corporeal proliferating cells on 6-, 12-, 18-, and 24-month-old samples (Fig. 2b–e). To quantify our PCNA findings, we used the same approach as for apoptosis evaluation, establishing a PCD index (mean number of proliferating cells per area; square millimeter $\pm$ SE). As shown in Fig. 2f, 2-month-old CC presented a statistically significant ($p<0.001$) PCD index (42.26 ± 4.97), as compared to all aging groups. Younger CC had approximately a fivefold higher PCD than 6-month-old samples (8.23 ± 3.80). This difference increased considerably when comparing the youngest group with the elderly 12, 18, and 24 months old, with PCDs of 4.11 ± 0.47 , 3.53 ± 0.78 , and 2.36 ± 0.70 , respectively. The overall apoptosis and proliferation results indicate that erectile tissue remodeling with aging is altered due to unbalanced apoptosis/proliferation. Since the p42/44 downstream effector is involved in the regulation of these mechanisms, we further investigated this kinase activation status and its potential relationship with aging corporeal turnover alterations.

Age-related alterations in the activation profile of p42/44 in rat erectile tissue

Alterations in the activation pattern of the p42/44 protein were analyzed by immunofluorescence and quantified by WB. Upon stimuli, p42/44 kinase is

Fig. 1 Evaluation and quantification of erectile tissue apoptosis with aging. Cavernosal viability was assessed by terminal transferase dUTP nick end labeling (TUNEL) assay: (a) 2 months old, (b) 6 months old, (c) 12 months old, (d) 18 months old, and (e) 24 months old. Scale bar=100 μ m. TUNEL fluorescein labeling of apoptotic nuclei (green labeling); DAPI DNA intercalator, allows nuclear identification (blue labeling); SML smooth muscle layer; T trabeculae; VS

vascular space. (f) Apoptotic cell density (ACD) quantification. A significant increase in cavernosal ACD was observed with aging. * $p < 0.001$, 2 months vs. 6, 12, 18, and 24 months; § $p < 0.0001$; 6 months vs. 12, 18, and 24 months. Data presented as mean ACD (mean number of cells in apoptosis per tissue area square millimeter) $\pm$ standard error. 199 $\times$ 180 mm (150 $\times$ 150 DPI)

Fig. 2 Immunohistochemical detection of PCNA and quantification of erectile tissue proliferation density: (a) 2 months old, (b) 6 months old, (c) 12 months old, (d) 18 months old, and (e) 24 months old. Scale bar=100 μ m. SML smooth muscle layer, T trabeculae, VS vascular space. Arrows: black, SMC mitosis; nucleus positive for PCNA; blue, EC nucleus labeled with PCNA; brown, PCNA-positive SM nucleus. (f) Proliferating

cell density (PCD) evaluation. A significant decrease in cavernosal PCD was observed with the advancement of age. * $p<0.001$, 2 months vs. 6, 12, 18, and 24 months. Data presented as mean PCD (mean number of proliferating cells per tissue area square millimeter) $\pm$ standard error. 184 $\times$ 185 mm (150 $\times$ 150 DPI)

phosphorylated in the cytoplasm and migrates to the nucleus where it regulates the transcription of several genes, ensuing in increased cell proliferation, survival, and/or ECM expansion (Papakrivopoulou et al.

2004; Pearson et al. 2001). In 2-month-old CC, phospho-p42/44 was mostly expressed in SMCs and ECs, as detected by double immunofluorescence for phospho-p42/44 and α -SMA (Fig. 3a). The same

technique revealed that in 6-month-old tissues, activated p42/44 was present particularly in SMCs with some activation in trabecular fibroblasts (Fig. 3b, arrowhead). As quantified by WB, cavernosal activation of p42/44 did not significantly change among the younger animal groups (Fig. 3f, g). With aging, there were significant changes in the activation pattern of the p42/44 protein in corporeal tissue. As illustrated in Fig. 3c–e, in CC of elderly 12, 18, and 24 months old, p42/44 phosphorylation occurred almost exclusively in trabecular fibroblasts, with a significant increase in protein activation in the 18- and 24-month groups, as quantified by WB (Fig. 3f, g). This shift in corporeal phospho-p42/44 pattern led us to assume that this kinase could play different roles in erectile tissue with the advancement of age. Its perivascular activation in younger CC suggested that p42/44 could mediate intracellular activation of survival/proliferation pathways, corroborating the results on low apoptosis and increased proliferation (Figs. 1a and 2a). With age progression, activated p42/44 significantly increased in trabecular fibroblasts, suggesting that this protein was instead playing a potential role in ECM deposition. To verify if this p42/44 distinct activation profile was not restricted to animal corporeal tissue, we analyzed in situ the phosphorylation of this MAPK in human cavernosal samples.

Differential phosphorylation of p42/44 kinase in human cavernosal tissue

We assessed in a total of 14 human corporeal samples, by dual immunofluorescence for phospho-p42/44 and α -SMA, if the activation profile of p42/44 with aging was similar to what was observed in rat tissue. As illustrated in Fig. 4, phospho-p42/44 was strongly expressed in the cytoplasm of SMC layers in all five younger human cavernosal fragments analyzed (Fig. 4a, b). In certain vascular spaces, phospho-p42/44 was clearly identified lining the sinusoids (Fig. 4b, arrowhead). In contrast, corporeal tissue from older individuals ($n=9$) had few activation of this kinase in the SMC counterpart. Instead, elderly samples presented p42/44 activation mostly in areas of collagen deposition, a characteristic associated with aging erectile tissue (Fig. 4c, d). Phospho-p42/44 immunodetection in the ECM component was particularly evident in elderly tissue sections, which presented a vast expansion of fibroblasts, associated with an

increase in collagen deposition (Fig. 4c, d). This marked phosphorylation of p42/44, specifically immunolocalized to the ECM in older cavernosal fragments, corroborated the observations carried out in animal penile tissue. In fact, in younger versus elderly human CC, there seems to occur a differential activation of the p42/44 kinase, suggesting a distinct function for this protein during adulthood.

Discussion

ED, defined as the constant inability to attain and maintain a penile erection sufficient for a satisfactory sexual performance, presents a high prevalence in the population (Travison et al. 2007). Epidemiologic studies have shown an association between aging and male ED, demonstrating that the percentage of potent men decreases from 60% to 33% between 40 and 70 years of age (Diokno et al. 1990; Feldman et al. 2000). Besides the decrease in erectile responses, the progression of age affects the composition and organization of cavernosal structures, having an impact on penile hemodynamic status (Bakircioglu et al. 2001; Calabrò et al. 1996; Costa and Vendeira 2008; Lin et al. 2001; Montorsi et al. 2003; Moreland 2000; Wespes 2002). Variations in SMC content, integrity and function (Bakircioglu et al. 2001; Wespes 2002), changes in cavernosal angioarchitecture (Costa and Vendeira 2008), increased trabecular deposition of collagen and decreased concentration of elastic fibers were reported with aging (Calabrò et al. 1996; Lin et al. 2001; Moreland 2000). These changes reduce the elasticity and compliance of the elderly penis, contributing to erectile failure (Montorsi et al. 2003). These age-related CC structural modifications are thought to be the result of molecular alterations associated to cavernosal remodeling. Overall, tissue remodeling is characterized by molecular and subsequent structural changes leading to altered tissue functionality. In the penis, physiologic structural rearrangements occur during embryogenesis and tissue growth, whereas a “pathologic remodeling” is due to occur as consequence of aging, altering tissue morphological homeostasis and contributing to ED. Tissue remodeling involves cellular and molecular events such as apoptosis, proliferation, and ECM synthesis, regulated by multiple signaling pathways, including the p42/44 MAPK (Papakrivopoulou et al.

Fig. 3 Alterations in the phosphorylation pattern of p42/44 in experimental aging corpus cavernosum, quantification by western blotting: (a) 2 months old, (b) 6 months old, (c) 12 months old, (d) 18 months old, and (e) 24 months old. With aging, the cellular location of phosphorylated p42/44 shifts from perivascular cells in younger animals to trabecular fibroblasts in elderly groups. *P-p42/44* phosphorylated-p42/44; *SMA* smooth muscle actin; *DAPI* DNA intercalator, allows nuclear identification (blue labeling). Scale bar=100 μ m. (f) Quantification of p42/44 activation status by western blotting and (g) densitometry and mean relative intensity quantification, by comparing the intensity of phospho-p42/44 with the intensity of total p42/44 protein. Data presented as mean relative intensity $\pm$ standard error. * $p<0.001$; 12 months vs. 18 and 24 months. 216 $\times$ 170mm (150 $\times$ 150 DPI)

2004; Pearson et al. 2001). However, there are no reports of a throughout evaluation of the aforementioned intracellular mechanisms in aging cavernosal tissue.

The present study investigated the molecular mechanisms underlying aging cavernosal remodeling

alterations. Using rat penile tissue with 2, 6, 12, 18, and 24 months old, we started by evaluating CC apoptosis by TUNEL assay, followed by the establishment of an ACD index, which allowed us to quantify the number of apoptotic cells (Costa et al. 2009). We showed that corporeal ACD was significantly increased as age progressed. Even though the number of apoptotic cells augmented in all erectile components, it seemed that perivascular SM and sinusoidal ECs were affected in elderly groups. Although an extensive study of CC programmed cell death in different aging groups has never been performed, it has been reported that aged cavernosal SM content is reduced, due to a decreased expression of anti-apoptotic genes, thus prospecting an apoptotic increase (Yamanaka et al. 2002). These results come in agreement with the present findings and also with our previous data, showing an aged-associated reduction of corporeal SMC layers in the same exact

Fig. 4 Differential activation of p42/44 in human young and aged erectile tissue. (a, b) Younger cavernosal tissues. (c, d) Older corporeal samples. In younger CC, p42/44 is mostly phosphorylated in SMCs (a, b). In certain vascular spaces, activated p42/44 was clearly identified lining the sinusoids (b,

arrowhead). Elderly erectile tissue presented increased phospho-p42/44 expression in areas of collagen deposition (c, d). *P-p42/44* phosphorylated-p42/44; *SMA* smooth muscle actin; *DAPI* DNA intercalator, allows nuclear identification (blue labeling). Scale bar=100 μ m. 119 $\times$ 89 mm (150 $\times$ 150 DPI)

groups of aged animals (Costa and Vendeira 2008). In addition, in elderly groups, the observed augment of EC death was potentially caused by increased oxidative stress, decreased androgen levels, and impaired expression of growth factors, mechanisms altered during aging and reported to affect endothelial viability (Costa and Virag 2009; Mirone et al. 2009; Rajasekaran et al. 2003; Shabsigh et al. 1998). However, a recent study carried out in aged patients with ED showed a nonstatistical increase in corporeal apoptosis with aging (Tarhan et al. 2009). This discrepancy is most certainly due to the use of a different methodology for apoptosis quantification, allied to the reduced number of samples analyzed. In addition to the marked increase in apoptosis with age progression, we also observed that the total number of CC nuclei was clearly less, indicating that cell proliferation could be impaired.

It was reported that aged tissues lose some remodeling capability due to altered proliferative activity (Tanno et al. 1996). However, and regarding ED, only a study has reported that aged erectile tissue presents low percentage of proliferating cells (Ferrini et al. 2007). Nonetheless, CC proliferation with age progression was never evaluated and quantified. Through PCNA immunostaining and by establishing a PDC index, we showed that with aging, all erectile components presented a significant decrease in PCNA-positive cells. Younger 2-month-old CC had the highest number of PCNA-labeled cells, potentially due to the action of growth factors and androgens, whose expression is elevated in youngsters and reported to induce cell proliferation (Mirone et al. 2009; Rajasekaran et al. 2003; Shabsigh et al. 1998). Growth factors and hormonal levels decrease with aging, consequently restraining penile tissue proliferative capacity. Overall, these results demonstrate that younger CC is undergoing a physiological structural process reaching a maturity stage at 6 months old. In aged groups, there are significant pathophysiological alterations, which may contribute to tissue loss of functionality.

As previously mentioned, remodeling events are regulated by intracellular pathways, including the p42/44 MAPK. However, and up to date, there was only one publication where the activation of this kinase was evaluated in a heterogeneous group of cavernosal tissue samples (Sommer et al. 2002). Nonetheless, no conclusions regarding p42/44 phos-

phorylation pattern and function in remodeling were attained. Moreover, there is no available data concerning p42/44 phosphorylation and its potential function in aging CC. We first analyzed *in situ*, in all aging animal cavernosal samples, the phosphorylation profile of p42/44 and quantified its activation levels by WB. We showed that p42/44 was activated in all aging groups; however, its distribution pattern was altered in an age-dependent fashion. In younger rat CC, phospho-p42/44 was mostly detected in perivascular areas, whereas in older CC, its distribution was shifted to trabecular fibroblasts. The phosphorylation levels of p42/44 were not statistically different between 2- and 12-month-old corporeal tissues, but significantly increased in elderly 18 and 24 months. Although this result at 12 months of age seemed unexpected, it might be related to the remodeling status of the cavernous tissue. We considered that a physiological remodeling is occurring in younger samples, 2 and 6 months, however, at 12 months old, CC may be undergoing a pathophysiological structural process.

As mentioned, p42/44 kinase controls a multitude of functional activities in different cell types, including activation of proliferation, inhibition of apoptosis, and stimulation of ECM production (Papakrivopoulou et al. 2004; Pearson et al. 2001). By comparing the p42/44 activation profile with the apoptosis and proliferation results, it is suggested that in the youngest CC, this kinase was potentially mediating intracellular anti-apoptotic and/or proliferative signals. In contrast, in older corporeal tissue, activated p42/44 was mostly present in trabecular fibroblasts. This phosphorylated p42/44 shift in cell localization occurred at the age of 12 months and significantly increased in the elderly groups. This observation might indicate a different cell-specific role for this kinase in aging CC. In order to verify if this p42/44 particular activation was not restricted to rat CC, we have performed an evaluation in younger and aged human cavernosal tissue. Interestingly, and corroborating our initial observation, p42/44 phosphorylation in younger CC immunolocalized at SMC layers, whereas in aged human tissue, it was mostly activated in the expanded ECM counterpart. In fact, aged cavernosal trabeculae are characterized by an increased collagen deposition (Lin et al. 2001). This feature correlated with the activation pattern of p42/44, both in animal and human corporeal samples,

suggesting that this kinase could be mediating ECM expansion in the elderly CC. Furthermore, it was reported that p42/44 kinase is a downstream effector of the transforming growth factor-beta 1 (TGF- β 1) signaling pathway (He et al. 2010). TGF- β 1 expression is increased in aging trabecular fibroblasts and reported to be the main stimulator of collagen synthesis, inducing cavernosal fibrosis and promoting ED (Ferrini et al. 2007; Ryu et al. 2004). We hypothesize that increased p42/44 activation in aged ECM component may be a downstream event of the TGF- β 1 pathway, adding a new piece in the complex puzzle of aging CC signaling events. The observed molecular alterations, involved with ECM expansion with aging, come in agreement with previous data relating increased collagen content with loss of erectile function with the advancement of age, as assessed by intracavernous pressure measurements (Bakircioglu et al. 2001). In fact, these remodeling modifications were reported to have a direct effect in corporeal functionality, by impairing tissue elasticity, compliance, and vasorelaxation mechanisms, consequently, resulting in reduced penile hemodynamics.

In conclusion, our results support a differential role for the p42/44 pathway in aging cavernosal remodeling. This kinase may play a dual function in the penis with the progression of age, mediating survival and proliferating signals in younger tissue and promoting ECM deposition and fibrosis in aged corporeal tissue.

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References

- Bakircioglu ME, Sievert KD, Nunes L, Lau A, Lin CS, Lue TF (2001) Decreased trabecular smooth muscle and caveolin-1 expression in the penile tissue of aged rats. *J Urol* 166:734–738
- Calabrò A, Italiano G, Pescatori ES, Marin A, Gaetano O, Abatangelo G, Abatangelo G, Pagano F (1996) Physiological aging and penile erectile function: a study in the rat. *Eur Urol* 29:240–244
- Costa C, Vendeira P (2008) Does erectile tissue angioarchitecture modify with aging? An immunohistological and morphometric approach. *J Sex Med* 5:833–840
- Costa C, Virag R (2009) The endothelial-erectile dysfunction connection: an essential update. *J Sex Med* 6:2390–2404
- Costa C, Soares R, Castela A, Adães S, Hastert V, Vendeira P, Virag R (2009) Increased endothelial apoptotic cell density in human diabetic erectile tissue—comparison with clinical data. *J Sex Med* 6:826–835
- Diokno AC, Brown MB, Herzog AR (1990) Sexual function in the elderly. *Arch Intern Med* 150:197–200
- Feldman HA, Johannes CB, Derby CA, Kleinman KP, Mohr BA, Araujo AB, McKinlay JB (2000) Erectile dysfunction and coronary risk factors: prospective results from the Massachusetts Male Aging Study. *Prev Med* 30:328–338
- Ferrini MG, Kovanez I, Sanchez S, Vernet D, Davila HH, Rajfer J, Gonzalez-Cadavid NF (2007) Long-term continuous treatment with sildenafil ameliorates aging-related erectile dysfunction and the underlying corporal fibrosis in the rat. *Biol Reprod* 76:915–923
- Goldstein AM, Padma-Nathan H (1990) The microarchitecture of the intracavernosal smooth muscle and the cavernosal fibrous skeleton. *J Urol* 144:1144–1146
- He S, Liu X, Yang Y, Huang W, Xu S, Yang S, Zhang X, Roberts MS (2010) Mechanisms of MAPK pathway mediating TGF- β 1/Smad signal in keloid fibroblasts. *Br J Dermatol* 162:538–546
- Ikeyama S, Kokkonen G, Shack S, Wang XT, Holbrook NJ (2002) Loss in oxidative stress tolerance with aging linked to reduced extracellular signal-regulated kinase and Akt kinase activities. *FASEB J* 16:114–116
- Lin JS, Tsai YS, Lin YM, Lin CS, Chow NH (2001) Age-associated changes in collagen content and its subtypes within rat corpora cavernosa with computerized histomorphometric analysis. *Urology* 57:837–842
- Mirone V, Imbimbo C, Fusco F, Verze P, Creta M, Tajana G (2009) Androgens and morphologic remodeling at penile and cardiovascular levels: a common piece in complicated puzzles? *Eur Urol* 56:309–316
- Montorsi F, Briganti A, Salonia A, Deho' F, Zanni G, Cestari A, Guazzoni G, Rigatti P, Stief C (2003) The ageing male and erectile dysfunction. *BJU Int* 92:516–520
- Moreland RB (2000) Pathophysiology of erectile dysfunction: the contributions of trabecular structure to function and the role of functional antagonism. *Int J Impot Res* 12(Suppl 4):S39–S46
- Papakrivopoulou J, Lindahl GE, Bishop JE, Laurent GJ (2004) Differential roles of extracellular signal-regulated kinase 1/2 and p38MAPK in mechanical load-induced procollagen α 1(I) gene expression in cardiac fibroblasts. *Cardiovasc Res* 61:736–744
- Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22:153–183
- Rajasekaran M, Kasyan A, Allilain W, Monga M (2003) Ex vivo expression of angiogenic growth factors and their receptors in human penile cavernosal cells. *J Androl* 24:85–90
- Ryu JK, Han JY, Chu YC, Song SU, Lee KH, Yoon SM, Suh JK, Kim SJ (2004) Expression of cavernous transforming growth factor- β 1 and its type II receptor in patients with erectile dysfunction. *Int J Androl* 27:42–49
- Shabsigh R, Raymond JF, Olsson CA, O'Toole K, Buttyan R (1998) Androgen induction of DNA synthesis in the rat penis. *Urology* 52:723–728
- Siroky MB, Azadzi KM (2003) Vasculogenic erectile dysfunction: newer therapeutic strategies. *J Urol* 170:S24–S29
- Sommer F, Klotz T, Steinritz D, Schmidt A, Addicks K, Engelmann U, Bloch W (2002) MAP kinase 1/2 (Erk 1/2)

- and serine/threonine specific protein kinase Akt/PKB expression and activity in the human corpus cavernosum. *Int J Impot Res* 14(217):225
- Tanno M, Ogihara M, Taguchi T (1996) Age-related changes in proliferating cell nuclear antigen levels. *Mech Ageing Dev* 92:53–66
- Tarhan F, Demirel GY, Kuyumcuoglu U, Faydaci G, Eryildirim B (2009) Apoptosis of corpus cavernosum in patients with organic erectile dysfunction. *World J Urol* 27:235–240
- Travison TG, Shabsigh R, Araujo AB, Kupelian V, O'Donnell AB, McKinlay JB (2007) The natural progression and remission of erectile dysfunction: results from the Massachusetts Male Aging Study. *J Urol* 177:241–246
- Wespes E (2002) Smooth muscle pathology and erectile dysfunction. *Int J Impot Res* 14(Suppl 1):S17–S21
- Wespes E, Goes PM, Schifflmann S, Depierreux M, Vanderhaeghen JJ, Schulman CC (1991) Computerized analysis of smooth muscle fibers in potent and impotent patients. *J Urol* 146:1015–1017
- Yamanaka M, Shirai M, Shiina H, Shirai M, Tanaka Y, Fujime M, Okuyama A, Dahiya R (2002) Loss of anti-apoptotic genes in aging rat crura. *J Urol* 168:2296–2300