



High-molecular weight hyaluronan reduced renal PKC activation in genetically diabetic mice

Giuseppe M. Campo ^{a,*}, Angela Avenoso ^a, Antonio Micali ^b, Giancarlo Nastasi ^a, Francesco Squadrito ^c, Domenica Altavilla ^c, Alessandra Bitto ^c, Francesca Polito ^c, Maria Grazia Rinaldi ^c, Alberto Calatroni ^a, Angela D'Ascola ^a, Salvatore Campo ^a

^a Department of Biochemical, Physiological and Nutritional Sciences, section of Medical Chemistry, School of Medicine, University of Messina, Policlinico Universitario, 98125 – Messina, Italy

^b Department of Biomorphology, School of Medicine, University of Messina, Policlinico Universitario, 98125 – Messina, Italy

^c Department of Clinical and Experimental Medicine and Pharmacology, Section of Pharmacology, School of Medicine, University of Messina, Policlinico Universitario, 98125 – Messina, Italy

ARTICLE INFO

Article history:

Received 30 June 2010

Received in revised form 4 August 2010

Accepted 10 August 2010

Available online 13 August 2010

Keywords:

Hyaluronan

NF- κ B

PKC

Cytokines

Diabetes

CD44

ABSTRACT

The cluster determinant (CD44) seems to play a key role in tissues injured by diabetes type 2. CD44 stimulation activates the protein kinase C (PKC) family which in turn activates the transcriptional nuclear factor kappa B (NF- κ B) responsible for the expression of the inflammation mediators such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), interleukin-18 (IL-18), inducible nitric oxide synthase (iNOS), and matrix metalloproteinases (MMPs). Regulation of CD44 interaction with its ligands depends greatly upon PKC. We investigated the effect of the treatment with high-molecular weight hyaluronan (HA) on diabetic nephropathy in genetically diabetic mice.

BKS.Cg-m+/+Lepr^{db} mice had elevated plasma insulin from 15 days of age and high blood sugar levels at 4 weeks. The severe nephropathy that developed was characterized by a marked increased in CD44 receptors, protein kinase C beta1, beta2, and epsilon (PKC β_1 , PKC β_2 , and PKC ϵ) mRNA expression and the related protein products in kidney tissue. High levels of mRNA and related protein levels were also detected in the damaged kidney for NF- κ B, TNF- α , IL-6, IL-18, MMP-7, and iNOS.

Chronic daily administration of high-molecular mass HA for 2 weeks significantly reduced CD44, PKC β_1 , PKC β_2 , and PKC ϵ gene expression and the related protein production in kidney tissue and TNF- α , IL-6, IL-18, MMP-7, and iNOS expression and levels also decreased. Histological analysis confirmed the biochemical data. However, blood parameters of diabetes were unchanged.

These results suggest that the CD44 and PKC play an important role in diabetes and interaction of high-molecular weight HA with these proteins may reduce inflammation and secondary pathologies due to this disease.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Over the last decade, considerable evidence has demonstrated the molecular and cellular links between metabolism and inflammation, particularly in obesity and diabetes. Diabetic nephropathy is one of the major microvascular complications in diabetes, and it is the most common single cause of end-stage renal disease worldwide [1]. The development of diabetic nephropathy is described as a process progressing from moderate glomerular hyperfiltration and nephro-

megaly to frank proteinuria with severe hypertension and marked renal insufficiency until end-stage renal disease [2]. Chronic inflammation, characterized by elevated circulating levels of inflammatory markers, appears to play a critical role in the pathogenesis of type 2 diabetes and its associated complications, particularly diabetic nephropathy and the consequent cardiovascular risk [3]. Recent evidence indicates that chemokines, interleukins, adhesion molecules, growth factors, and free radicals are deeply involved in the development of diabetic nephropathy [4,5]. The cytokines that seem to play a key role in diabetic renal failure include proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and interleukin-18 (IL-18) [6–8]. Inducible nitric oxide synthase (iNOS) and cytokine-stimulated metalloproteinases (MMPs), especially MMP-7, are also greatly involved in the development of diabetic complications and kidney damage [9,10]. One ROS, nitric oxide (NO),

* Corresponding author. Department of Biochemical, Physiological and Nutritional Sciences, School of Medicine, University of Messina, Policlinico Universitario, Torre Biologica, 5° piano, Via C. Valeria – 98125 – Messina, Italy. Tel.: +39 90 221 3334; fax: +39 90 221 3898.

E-mail address: gcampo@unime.it (G.M. Campo).

is a highly reactive, cytotoxic molecule that is heavily implicated in diabetes [11]. Increased levels of NO promote numerous effects on podocytes, including alteration of the extracellular matrix synthesis [12] and activation of apoptosis [13].

Biologics that inhibit the signalling cascade mediated by proinflammatory cytokines are effective in treating diabetic pathologies by reducing both inflammation and renal failure [6,14,15]. However, blocking IL-18 and/or TNF- α does not lead to total protection from diabetic complications, indicating that other signalling pathways that mediate inflammation and tissue injury have still to be elucidated [6,14,15].

Hyaluronan (HA) is a linear polymer composed of repeating disaccharides of D-glucuronic acid-N-acetyl D-glucosamine [16]. HA can exist as a high-molecular-weight polymer (10^6 D) or in lower-molecular weight forms. Low-molecular mass HA in particular is produced after tissue injury [17]. Several findings have shown that HA functions are determined in part by the size of the molecule. High-molecular weight HA is suggested to play a structural role and to promote tissue integrity, while low-molecular weight HA may be a signal of tissue injury [18–20].

The best-known HA receptor is CD44 [21]; it is a transmembrane glycoprotein which is widely found in T lymphocytes, granulocytes, monocytes, fibroblasts, keratinocytes, and epithelial cells. CD44 stimulation with HA plays a role in various physiological functions, such as cell adhesion, cell-substrate interactions, and lymphocyte recruitment, as well as in pathological processes such as chronic inflammation and metastasis of malignant cells [22]. A role for CD44, as HA receptor, in the regulation of inflammation *in vivo* has been shown by studies in which anti-CD44 treatment reduces not only inflammation in experimental diabetes [23,24] but also leukocyte migration in skin-associated immune disease [25].

A number of reports have shown PKC members to be greatly involved in the mediation of inflammation [26]. The regulation of CD44 interaction with its ligands depends heavily on PKC which modulates the phosphorylation state of CD44 [27] and the various anchoring proteins [28]. Interestingly, PKC activation leads to the redistribution of CD44 receptors in membrane ruffles located at the leading edge of glioma cells [29]; a key feature of inflammation and invading cells. PKC therefore has the potential to modulate both the affinity of CD44 for HA [30] as well as the localization of the receptor over the cell surface [29]. It has also been reported that the activation of the CD44 receptor by HA produces PKC activation [31], thus establishing a concatenated mechanism between the CD44 receptor and PKC. In addition, these processes are to a large extent mediated by the increased expression of proinflammatory cytokines and other detrimental molecules such as NO and MMPs, which are in turn controlled by the activation of key transcriptional nuclear factor kappa B (NF- κ B) [32].

As previous investigations reported that the inhibition of PKC isoforms such as PKC α , PKC β_1 and PKC β_{11} reduces diabetic complications, especially diabetic nephropathy [33–35], and since we demonstrated in a previous study that high-molecular mass HA is able to reduce phorbol-12-myristate-13-acetate (PMA)-induced inflammation in mouse chondrocytes by interacting with PKC [19], the aim of this study was to investigate whether high-molecular weight HA administration, at different concentrations, influences CD44 and PKC function in congenic diabetic BKS.Cg-m+/+Lepr^{db} mice.

2. Materials and methods

2.1. Animals

Male congenic diabetic BKS.Cg-m+/+Lepr^{db} mice 15 weeks old with a mean weight of 25–30 g were used in our study. Mice, purchased from The Jackson Laboratory (Bar Harbor, ME, USA) were maintained under climate-controlled conditions with a 12-h light/dark cycle. The animals were fed standard rodent chow and provided water *ad libitum*.

The health status of the animal colony was monitored in accordance with Italian Veterinary Board guidelines. Mice were divided into the following groups: 1) control ($n=12$), 2) control + HA (30 mg/kg) ($n=12$), 3) diabetic ($n=17$), 4) diabetic + HA (7.5 mg/kg) ($n=17$), 5) diabetic + HA (15 mg/kg) ($n=17$), 6) diabetic + HA (30 mg/kg) ($n=17$).

2.2. Materials

High molecular weight HA (4,000,000 Da) was purchased from Sigma-Aldrich Srl, (Milan, Italy). Mouse TNF- α (cat. IB49688), IL-6 (cat. IB49686), IL-18 (cat. IB49697) commercial ELISA kits were provided by Immuno-Biological laboratories Inc. (Minneapolis, MN, USA). Mouse MMP-7 (cat. E0102Mu) and iNOS (cat. E0837Mu) commercial ELISA kits were obtained from USCN Life Science Inc. Wuhan (Wuhan, China). Mouse CD44 monoclonal antibodies were supplied by Millipore (Billerica, MA, USA), mouse PKC α monoclonal antibodies were supplied by Abcam plc (Cambridge, UK), mouse PKC β_{11} polyclonal antibodies were supplied by Sigma-Aldrich (Milan, Italy), and PKC β_1 polyclonal antibodies and horseradish peroxidase-labeled goat anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents used were purchased from Fluka (division of Sigma-Aldrich Srl, Milan, Italy).

2.3. HA treatment

HA was dissolved in saline solution (0.9% NaCl) at three different concentrations (7.5, 15, and 30 mg/kg) and administered intraperitoneally at a volume of 1.0 ml/kg body weight, once a day starting from day 1 up to day 15.

2.4. Plasma glucose and insulin

Glucose was evaluated in plasma samples (100 μ l) at the end of the experiment. Concentrations were assayed using commercial clinical test kits (cat. GAGO20) obtained from Sigma-Aldrich (Milan, Italy). Insulin levels were also measured in plasma samples (10 μ l) at the end of the experiment using an ELISA commercial kit (cat. EZRMI-13 K) provided by Millipore (Billerica, MA, USA).

2.5. RNA isolation, cDNA synthesis, and real-time quantitative PCR amplification

Total RNA was isolated from renal tissue for reverse-PCR real-time analysis of CD44, PKC β_1 , PKC β_{11} , PKC α , TNF- α , IL-6, IL-18, MMP-7, and iNOS (RealTime PCR system, Mod. 7500; Applied Biosystems, USA) using an Omniscript Reagent Kit (Euroclone, West York, UK). The first strand of cDNA was synthesized from 1.0 μ g total RNA using a high-capacity cDNA Archive kit (Applied Biosystems, USA). β -Actin mRNA was used as an endogenous control to allow the relative quantification of CD44, PKC β_1 , PKC β_{11} , PKC α , TNF- α , IL-6, IL-18, MMP-7, and iNOS. Real-time PCR was performed by means of ready-to-use assays (Assays on demand; Applied Biosystems) on both targets and endogenous controls. The amplified PCR products were quantified by measuring the calculated cycle thresholds (C_T) of CD44, PKC β_1 , PKC β_{11} , PKC α , TNF- α , IL-6, IL-18, MMP-7, iNOS, and β -actin mRNA. The amounts of specific mRNA in samples were calculated using the $\Delta\Delta C_T$ method. The mean value of normal cartilage target levels became the calibrator (one per sample), and the results are expressed as the n -fold difference relative to normal controls (relative expression levels).

2.6. Western blot assay of CD44, PKC β_1 , PKC β_{11} , and PKC α proteins

For SDS-PAGE and Western blotting, the kidney tissue samples were washed twice in ice-cold PBS and subsequently dissolved in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% wt./vol. SDS, 10%

glycerol, 50 mM dithiothreitol, 0.01% wt./vol. bromophenol blue). Aliquots of whole cell protein extract (10–25 μ l/well) were separated on a mini gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences) using a semidry apparatus (Bio-Rad). The membranes were then incubated overnight in a roller bottle with the specific diluted (1:1000) primary antibody in 5% bovine serum albumin, 1x PBS, and 0.1% Tween 20 at 4 °C. After being washed in three stages in wash buffer (1x PBS, 0.1% Tween 20), the blots were incubated with the diluted (1:2500) secondary polyclonal antibody (goat anti-rabbit conjugated with peroxidase), in TBS/Tween-20 buffer, containing 5% nonfat dried milk. After 45 min of gentle shaking, the blots were washed five times in wash buffer, and the proteins, after coloration with DAB liquid substrate (Sigma-Aldrich, Milan, Italy) were made visible using a UV/visible transilluminator (EuroClone, Milan, Italy) and Kodak BioMax MR films. A densitometric analysis was also run to quantify each band.

2.7. NF- κ B p50/65 transcription factor assay

NF- κ B p50/65 DNA binding activity in nuclear extracts of kidney tissue samples was evaluated in order to measure the degree of NF- κ B activation. Analysis was performed in line with the manufacturer's protocol for a commercial kit (NF- κ B p50/65 Transcription Factor Assay Colorimetric, cat. no. SGT510; Chemicon International, USA). In brief, one of the kidneys was removed from the animals at the end of the experimental phase, washed in ice-cold 10 mM Tris-HCl, pH 7.4, and blotted on an absorbent paper. Samples were then plotted, using an automatic plotter, to isolate renal cells. Cytosolic and nuclear extraction was performed by lysing the cell membrane with an appropriate hypotonic lysis buffer containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. After centrifugation at 8000 \times g, the supernatant containing the cytosolic fraction was discarded, while the pellet containing the nuclear portion was then resuspended in the apposite extraction buffer, and the nuclei

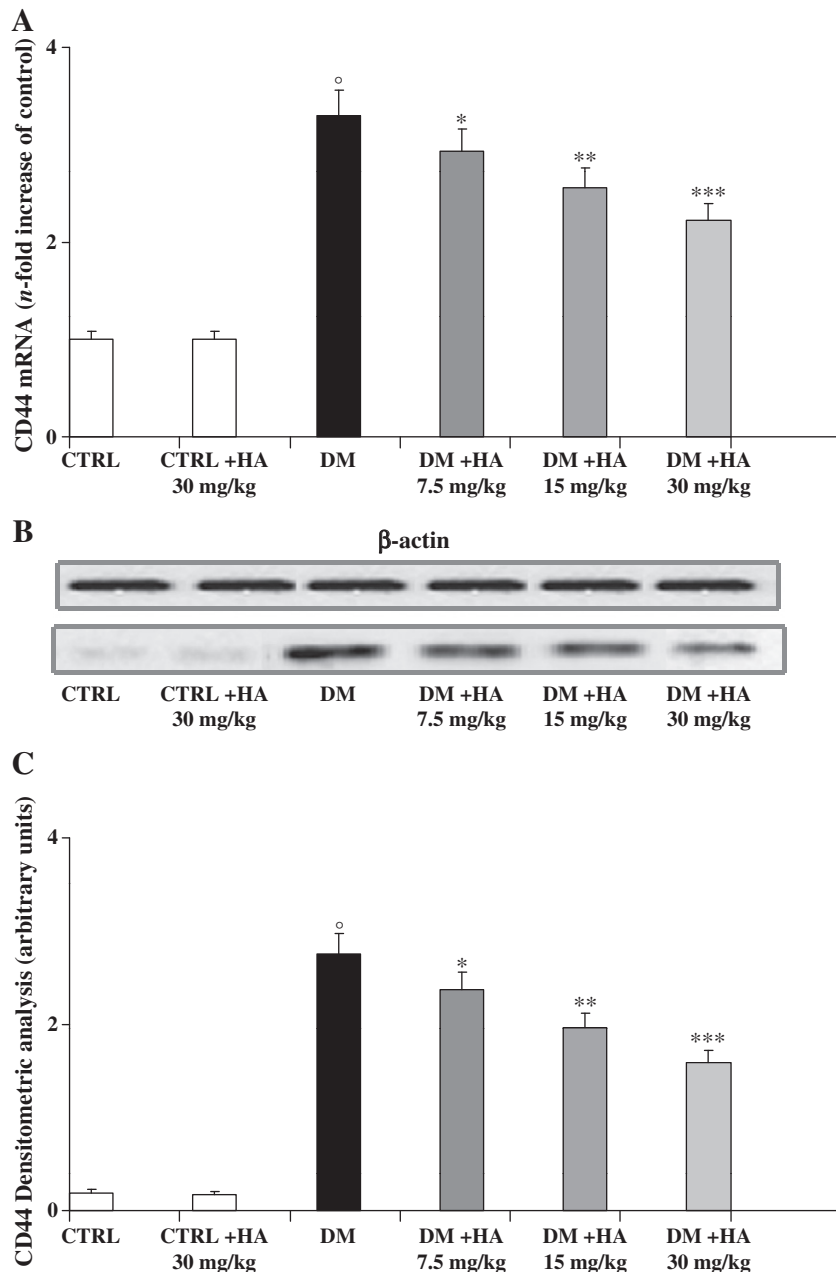


Fig. 1. Effect of HA treatment on CD44 mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for CD44 protein levels. ^o*p*<0.001 vs control; **p*<0.01, ***p*<0.005, and ****p*<0.001 vs diabetic.

were disrupted by a series of drawing and ejecting actions. The nuclear suspension was then centrifuged at $16,000 \times g$. The supernatant fraction was the nuclear extract. After determination of protein concentration and adjustment to a final concentration of approximately 4.0 mg/ml, this extract was stored in aliquots at -80°C for the subsequent NF- κ B assay. After incubation with primary and secondary antibodies, colour development was observed following the addition of the substrate TMB/E. Finally, the absorbance of the samples was measured using a spectrophotometric microplate reader set at λ 450 nm. Values are expressed as relative optical density (OD) per mg protein.

2.8. TNF- α , IL-6, IL-18, MMP-7, and iNOS ELISA assay

Tissue samples obtained from mice kidneys in the presence of 1.0 nM PMSF and protease inhibitor cocktail were first lysed using a

suitable hypotonic lysis buffer and then centrifuged at 13,000 rpm for 10 min at 4°C . The analysis of TNF- α , IL-6, and IL-18 was carried out using a specific commercial kit. Briefly, 50 μl of standards, samples, and controls were added to each well of the coated microplate. Then, 50 μl of each specific biotin-conjugate antibody was then added to each well. After 120 min of incubation at $20\text{--}22^\circ\text{C}$, the liquid from the wells was discarded, the wells were washed three times and 100 μl of Streptavidin-HRP was added. After further incubation for 60 min and having washed the wells once more, 100 μl of a substrate chromogen solution was added. After 10 min of incubation and the addition of 100 μl of stop solution, the absorbance of each well was read spectrophotometrically at λ 450 nm. TNF- α , IL-6, and IL-18 values are expressed as picograms per milligram of protein. For MMP-7 and iNOS, 100 μl of previously diluted samples, standards, and controls were added to each well of the coated microplate. After incubation for 2 hours at 37°C , the liquid from

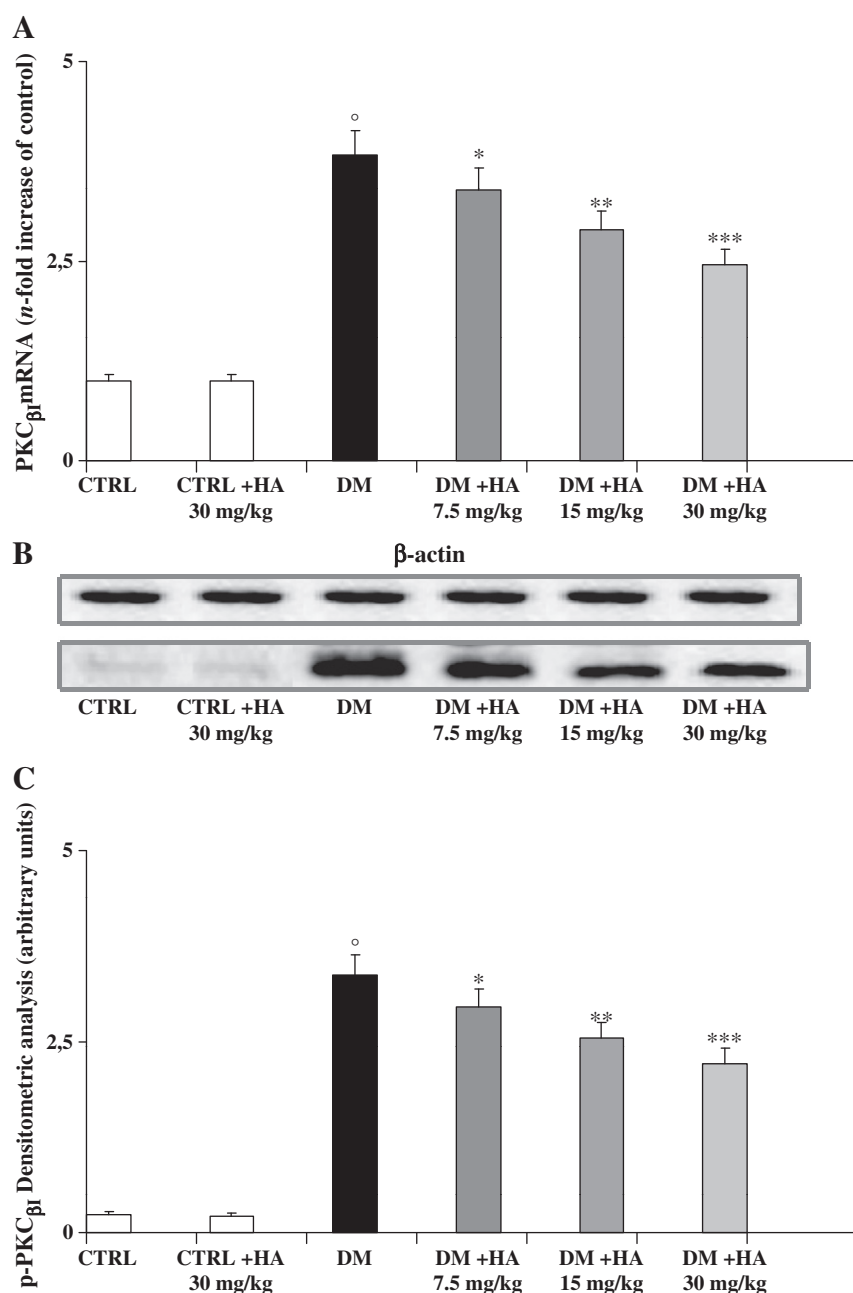


Fig. 2. Effect of HA treatment on PKC β I mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the n -fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for PKC β I protein levels. $^\circ p < 0.001$ vs control; $^* p < 0.01$, $^{**} p < 0.005$, and $^{***} p < 0.001$ vs diabetic.

each well was discarded. After adding 100 μ l of the detection reagent A and incubation at 37 °C for 1 hour, the liquid was aspirated, and wells were washed with wash buffer. At this point, 100 μ l of detection reagent B was added to each well, and after a further incubation for 1 hour at 37 °C, the liquid of wells was first aspirated then wells were washed with wash buffer. Then, 90 μ l of the substrate solution was added, and after incubation at room temperature for 30 minutes, 50 μ l of stop solution was added. Within 30 minutes, the absorbance of each well was read spectrophotometrically at λ 450 nm. MMP-7 values are expressed as picograms per milligram of protein, while iNOS values are expressed as units per milligram of protein.

2.9. Histology

At the end of the experimental phase, the right kidney was first excised and then perfused with 4% paraformaldehyde (PFA)/0.1 M phosphate buffer (PB), decapsulated, and immersed in 4% PFA/0.1 M PB. Then

kidneys were dehydrated in graded ethanol and embedded in a special tissue embedding media (Paraplast, McCormick Scientific, Richmond, VA, USA). Sections of 4 μ m thickness were cut with a microtome (mod. RM 2125 RT; Leica Camera AG, Solms, Germany), stained with hematoxylin–eosin technique and viewed and photographed with a light microscope (mod. BH2; Olympus America Inc., Melville, USA).

2.10. Protein analysis

The amount of protein was determined using the Bio-Rad protein assay system (Bio-Rad Lab., Richmond, CA, USA) with bovine serum albumin as a standard in accordance with the published method [36].

2.11. Statistical analysis

Data are expressed as means \pm SD of no less than seven experiments for each test. Statistical analysis was performed by one-way analysis of

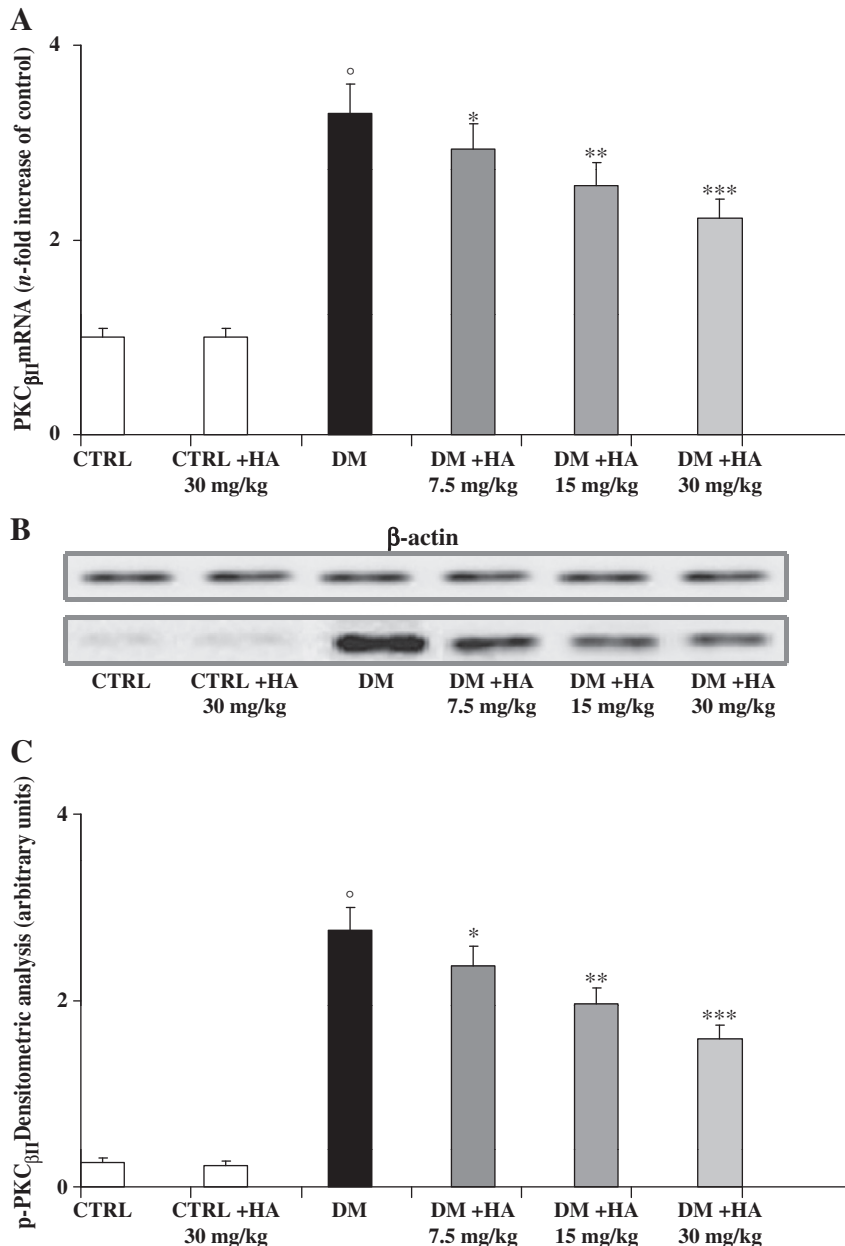


Fig. 3. Effect of HA treatment on PKC $_{\beta III}$ mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for PKC $_{\beta III}$ protein levels. ^o*p*<0.001 vs control; ^{*}*p*<0.01, ^{**}*p*<0.005, and ^{***}*p*<0.001 vs diabetic.

variance (ANOVA) followed by the Student–Newman–Keuls test. The statistical significance of differences was set at $p < 0.05$.

2.12. Statement of animal care

The studies reported in this article were carried out in accordance with the Helsinki declaration and the NIH guidelines for the Care and Use of Laboratory Animals.

3. Results

3.1. Effects of HA treatment on plasma glucose and insulin

At the age of 4 weeks, the acute expression of the *db/db* mutation commonly induces noticeable alterations in body phenotype, tissue

indexes, and the endocrine-related parameters of BKS.Cg-m+/+Lepr^{db} mice relative to controls [37]. Increased body weight in these mice is related to concomitant elevations in blood glucose and insulin levels when compared with control values [37]. The 15-day HA treatment period, at all doses, had no effect on plasma glucose and insulin in both diabetic and control mice (data not shown).

3.2. CD44, PKC β_1 , PKC β_{II} , and PKC α mRNA expression and Western blot analysis

CD44, PKC β_1 , PKC β_{II} , and PKC α (Figs. 1, 2, 3, and 4) mRNA evaluation (panels A of each figure) and Western blot analysis with densitometric evaluation (Figs. 1, 2, 3, and 4, panels B and C of each figure) were assayed to estimate the degree of CD44 activation and the consequent cell signalling pathway booster that culminates in NF-

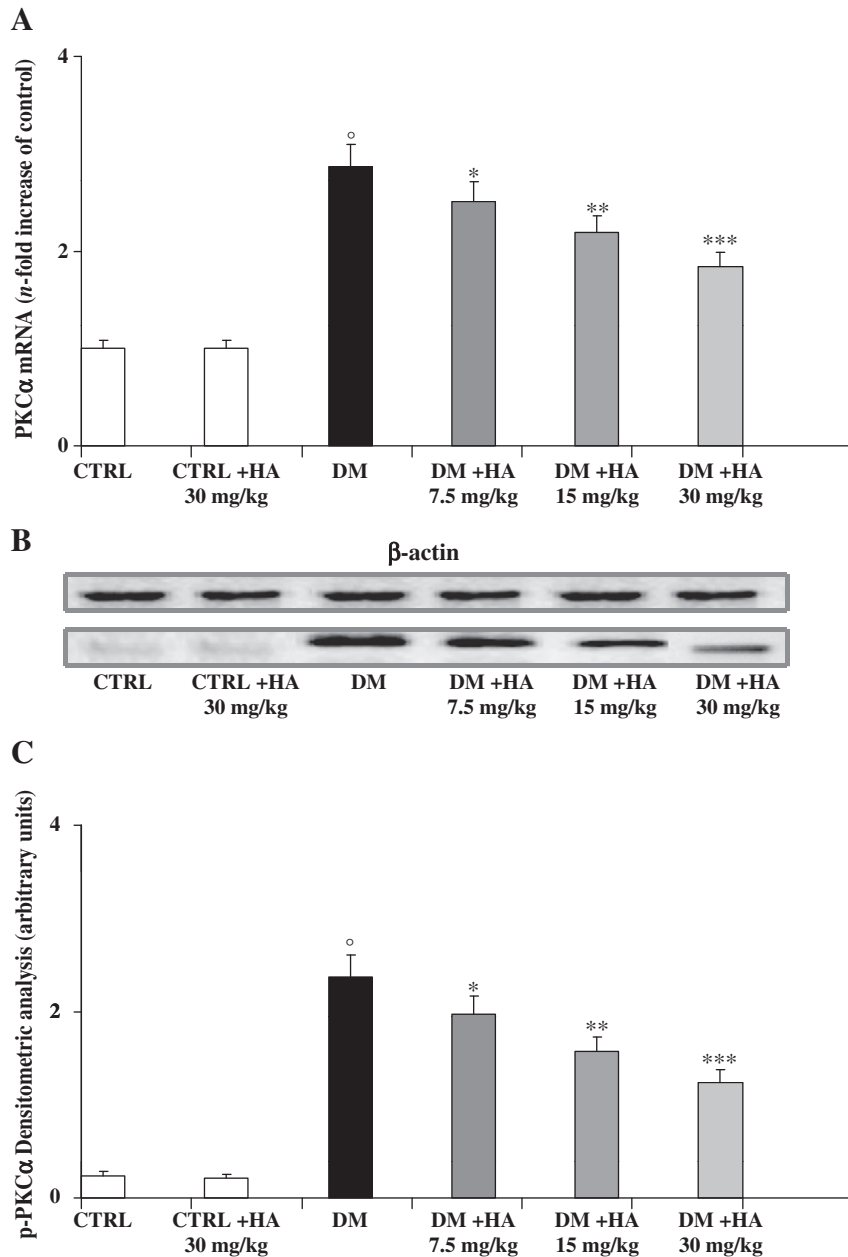


Fig. 4. Effect of HA treatment on PKC α mRNA expression (A) and related protein production (B, C) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for PKC α protein levels. $^{\circ}p < 0.001$ vs control; * $p < 0.01$, ** $p < 0.005$, and *** $p < 0.001$ vs diabetic.

κ B factor activation. Very low gene expression and the related protein production of CD44, PKC $_{\beta I}$, PKC $_{\beta II}$, and PKC $_{\alpha}$ were obtained in nondiabetic mice (CTRL and CTRL + HA). In contrast, a marked increase in the expression and protein synthesis of the CD44 receptor and their signal mediators PKC $_{\beta I}$, PKC $_{\beta II}$, and PKC $_{\alpha}$ was found in the kidney tissue of diabetic mice. This means that CD44 receptor and the PKC pathway are involved in the inflammatory mechanism consequent to this pathology.

The treatment with high-molecular weight HA for 15 days significantly reduced CD44 receptor activation and its signal mediator levels in diabetic mice. As reported in Figs. 1, 2, 3, and 4, HA was able to lower CD44, PKC $_{\beta I}$, PKC $_{\beta II}$, and PKC $_{\alpha}$ expression and protein synthesis at all concentrations in a dose-dependent manner.

3.3. NF- κ B activation

Fig. 5 shows the changes in the NF- κ B p50/p65 heterodimer translocation over the course of the experiment. NF- κ B DNA binding was present at very low levels in the renal tissue of nondiabetic mice. In contrast, diabetic complications induced massive NF- κ B translocation into the nucleus of the damaged podocytes; the treatment of diabetic mice with HA at different concentrations reduced NF- κ B activation in a dose-dependent manner, thereby confirming the effect on CD44, PKC $_{\beta I}$, PKC $_{\beta II}$, and PKC $_{\alpha}$ parameters.

3.4. TNF- α , IL-6, IL-18, MMP-7, and iNOS mRNA expression and ELISA assay

TNF- α , IL-6, IL-18, MMP-7, and iNOS were assayed because of their active role in inflammation and diabetic nephropathy (Figs. 6, 7, 8, 9, and 10). Their mRNA evaluation (panel A of each figure) and protein concentration (panel B of each figure) showed that the mRNA of these detrimental mediators was not stimulated in nondiabetic mice and protein level was very low. However, in diabetic mice, TNF- α , IL-6, IL-18, MMP-7, and iNOS expression and the related protein concentra-

tion were significantly upregulated. The administration of high-molecular weight HA was able to reduce this increment in mRNA expression at all doses. As reported (Figs. 6, 7, 8, 9, and 10; panels A and B of each figure), the increase in mRNA expression of TNF- α , IL-6, IL-18, MMP-7 and iNOS in diabetic mice correlated well with the increment in protein synthesis. This correlation was also maintained in mice treated with HA. In fact, the reduction in mRNA expression resulted in a similar diminution in protein formation.

3.5. Histological analysis

Glomerular injury in the diabetic mice can be early characterized by glomerular hyperplasia and mesangial matrix expansion. Fig. 11 demonstrates a representative kidney histopathology of all the experimental groups. Panels A and B show a normal kidney tissue section from nondiabetic control mice untreated or treated with HA. In contrast, as reported in Fig. 11, panel C, the glomerular hyperplasia and mainly the mesangial cell increment is clearly evident. In panels D, E, and F, a representative selection of kidney tissue sections from diabetic mice receiving HA treatment shows that a gradual reduction in glomerular hyperplasia and mesangial matrix expansion occurred. As indicated by the intensive coloration, the number of mesangial cells increased dramatically.

4. Discussion

Diabetes is a chronic disease in which patients develop dramatic and devastating complications. The most severe complication of diabetes is nephropathy, which frequently causes death in diabetic patients and accounts for a large number of cases of end-stage renal disease.

In the present study, we investigated the effects of high-molecular weight HA, at different concentrations, on CD44 receptor modulation in congenitally diabetic mice. This study suggests that highly polymerized HA may reduce diabetes-induced renal injury by

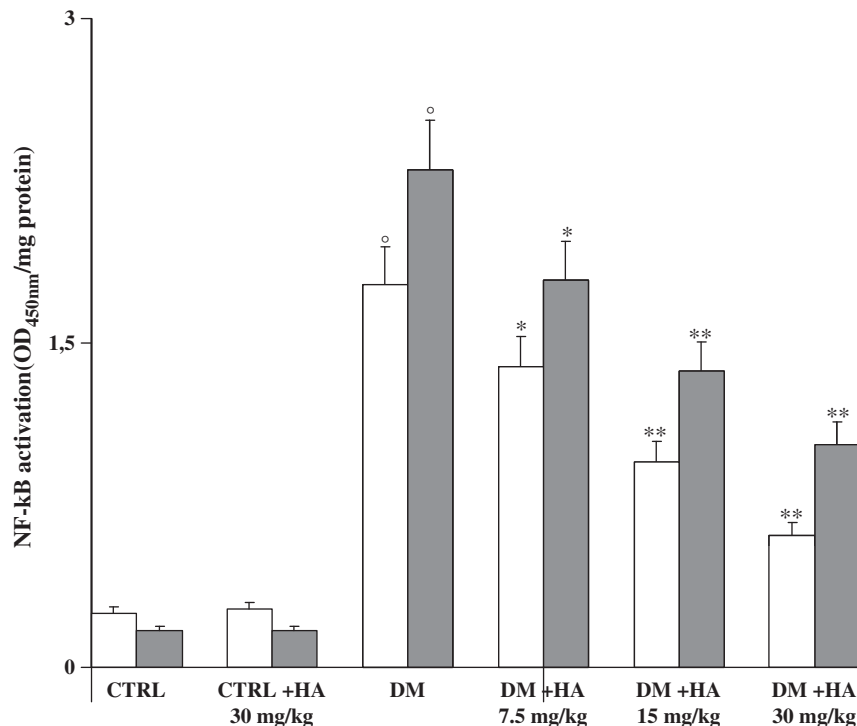


Fig. 5. Effect of HA treatment on NF- κ B p50/p65 transcription factor DNA binding activity in kidney tissue of diabetic mice. White bars represent the p/50 subunit, black bars represent the p/65 subunit. Values are the mean \pm SD of seven experiments and are expressed as optical density at λ 450 nm/mg protein of nuclear extract. $^{\circ}$ p < 0.001 vs control; * p < 0.005 and ** p < 0.001 vs diabetic.

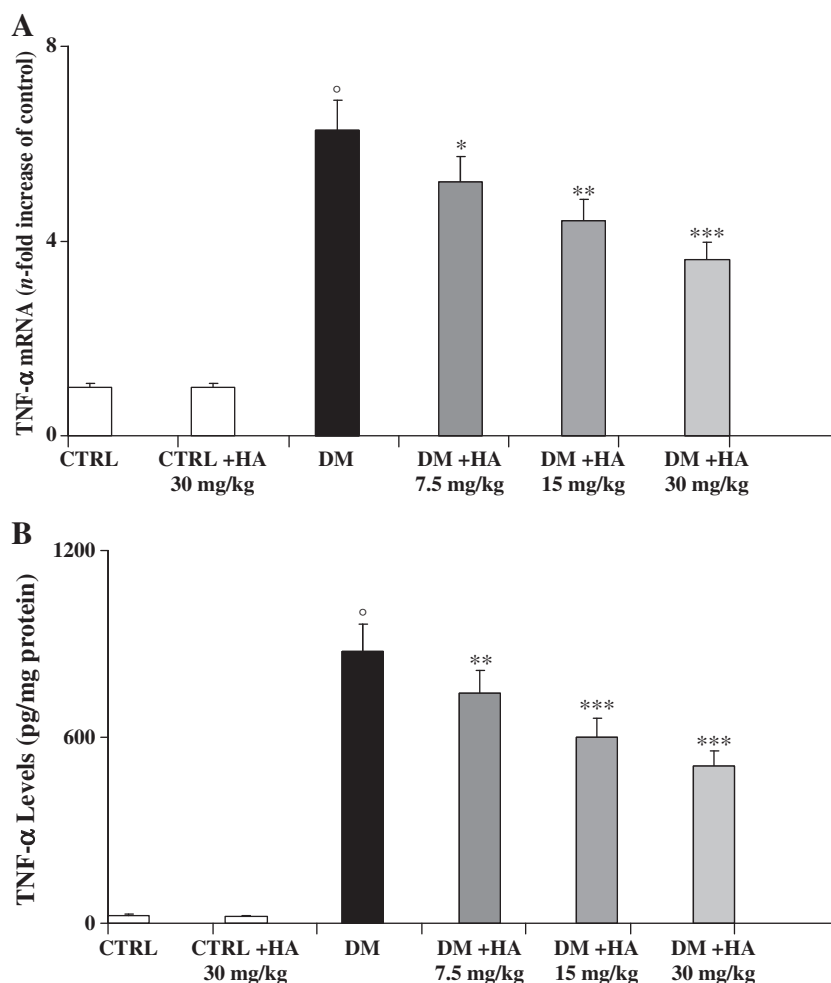


Fig. 6. Effect of HA treatment on TNF- α mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as picograms per milligram of protein (B) for the TNF- α protein levels. * $p < 0.001$ vs control; * $p < 0.01$, ** $p < 0.005$, and *** $p < 0.001$ vs diabetic.

modulating CD44 signalling, in contrast with low-molecular weight HA which may exert the opposite effects as previously demonstrated [17–20]. HA effects were dose dependent and were able to reduce not only the expression of CD44 receptor and of PKC β_1 , PKC β_2 , and PKC α but also NF- κ B activation and the increment of inflammatory cytokines, such as IL-6 and IL-18, and of MMP-7 and iNOS that were upregulated in diabetic mice. Biochemical results were further confirmed by the reduction in glomerulosclerosis as revealed by the histological analysis.

HA possesses an extraordinarily rapid half-life. It is quickly eliminated by means of CD44 receptors on liver endothelium. At a normal plasma concentration, the half-life is approximately 4–6 minutes, while at higher plasma concentrations, the HA half-life is considerable prolonged to 18–43 minutes [38]. The intraperitoneal HA half-life is unknown but is probably also very rapid, and also in this case, an increase in the HA dose administered may result in a prolonged HA half-life with a consequent major bioavailability to different organs. Therefore, we might expect a linear progression of effect by increasing the HA range concentrations administered. However, due to its viscous properties, it is really difficult to administer concentrations higher than 30 mg/kg.

The interaction between cell surface CD44 and extracellular HA are implicated in a variety of physiological and pathological processes, such as the uptake and degradation of HA, T-cell activation, leukocyte extravasation at inflammation sites, and tumor metastasis [39]. CD44

expression in the renal tissue correlates with the degree of the nephropathy in diabetic rats [40] and at high levels in several other form of nephropathy stimulates the increment in inflammatory mediators that are the main cause of renal damage [41,42].

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases that consists of a number of isoforms [43]. The differences between the PKC isoforms dependent on their substrate specificity suggest that a particular PKC isoform within a single cell may regulate specific physiological responses. PKCs regulate a variety of biological functions including ion channel activity, cell differentiation, and other biochemical and physiological responses to various stimuli [44,45]. The PKC/PKC family exerts numerous effects on cellular pathways that are important in inflammation and has previously been considered as a potential therapeutic target in diabetes [33–35,46]. PKC is able to modify the phosphorylation state of the CD44 receptor and the interaction with HA [27], and PKC in turn may be activated through different pathways, among these by CD44 stimulation [31]. Therefore, CD44 and PKC are interdependent and greatly involved in the inflammation cascade. NF- κ B promotes the transcription of several genes including cytokines and other inflammation products [32]. CD44 stimulation activates members of the PKC family, such as PKC α and PKC β [47,48], which in turn activate the NF- κ B responsible for the expression of inflammation mediators such as TNF- α , IL-6, IL-18, iNOS, its dangerous free radical product, NO, and MMPs responsible for diabetic nephropathy [5–13,49–51].

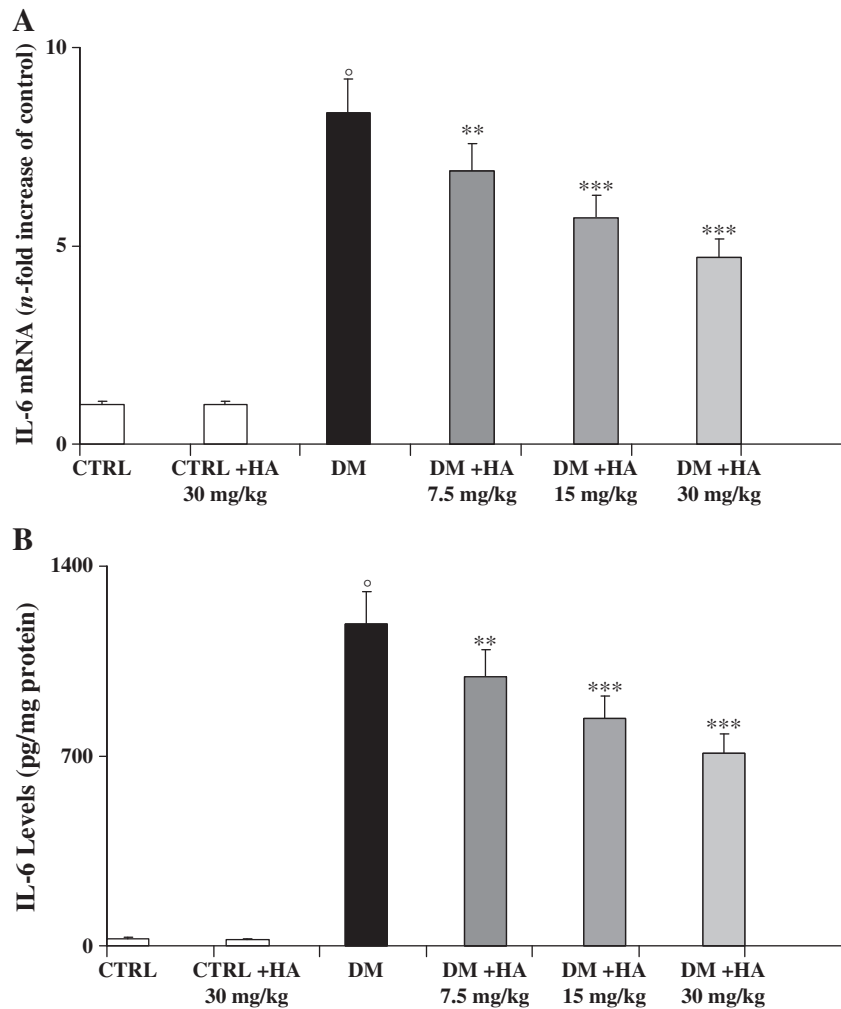


Fig. 7. Effect of HA treatment on IL-6 mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as picograms per milligram of protein (B) for IL-6 protein levels. $^{\circ}p < 0.001$ vs control; $^{\ast}p < 0.01$, $^{\ast\ast}p < 0.005$, and $^{\ast\ast\ast}p < 0.001$ vs diabetic.

Cell interaction with the surrounding extracellular matrix is fundamental in many physiological and pathological mechanisms. Proteoglycans (PGs) may influence cell behaviour through binding events mediated by their glycosaminoglycan (GAG) chains. The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide an optimal charge and surface with the protein [52]. The interaction of HA degradation products with CD44 provides signals to initiate inflammation [22,23,25]. Other data support the idea that a balance between low-molecular weight HA and high-molecular weight HA may control the activation of inflammation [53]. In fact, these preliminary findings suggest that high-molecular weight HA is inactive for CD44 modulation, while low-molecular weight HA originating from depolymerised HA at the inflammation site is active. [17,18,54]. We previously reported that high-molecular weight HA is able to reduce inflammation stimulated by PMA in mouse chondrocytes. By using a specific CD44-blocking antibody, it was demonstrated that PKC and not CD44 receptor is the target for HA action [19]. We suggest the same mechanism to explain the findings of the present study, in which chronic daily administration of high-molecular mass HA for 2 weeks significantly reduced CD44, PKC $_{\beta I}$, PKC $_{\beta II}$, and PKC $_{\alpha}$ gene expression and the related protein production in kidney tissue and TNF- α , IL-6, IL-18, MMP-7, and iNOS expression levels.

The effect exerted by HA on the parameters considered could be due to the ability of HA to bind protein structures, such as PKC,

thereby exerting a block that reduced NF- κ B activation. A similar effect exerted directly on the CD44 receptor should be excluded, since the high-molecular mass HA interaction with CD44 seems to be just one step in a pathway of intermediary metabolism involving the catabolism of native HA into small fragments [55]. The rationale for the use of high-molecular mass HA in place of a specific antibody targeted at CD44 may be explained by the fact that a block of the CD44 receptor leads to the abrogation of several fundamental CD44 functions, such as cell adhesion, cell-substrate interaction, and lymphocyte recruitment [39].

Blocking PKC isoforms using specific agents was also previously attempted, but the effects are not very encouraging because of the high toxicity of these agents [56]. The choice of HA as a modulating agent was derived essentially from its natural endogenous origin. The number of active charged HA sites (carboxylic groups) is responsible for HA action. In fact, high molecular weight HA contains many charged groups that may completely wrap protein structures such as PKC, thereby preventing its activation. The mechanism could be similar to a well known lock-and-key model which applies to all the better-known effector-receptors where the effector is chemically specific and a similar molecule with several charged groups may block the receptor. In this work, the larger size of HA molecule was able to mask the PKC active site completely. The resultant inhibition of PKC thereby produced a reduction in NF- κ B activation that in turn limited the transcription of the detrimental inflammatory intermediates with

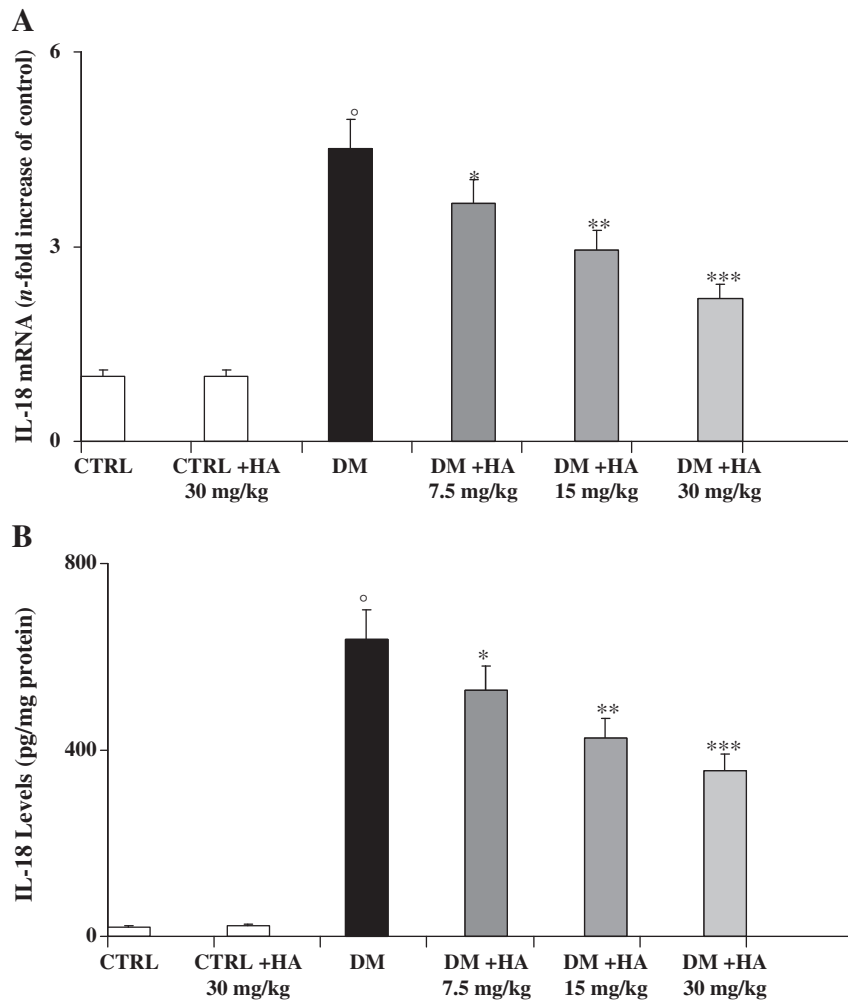


Fig. 8. Effect of HA treatment on IL-18 mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as picograms per milligram of protein (B) for IL-18 protein levels. $^{\circ}p < 0.001$ vs control; $*p < 0.01$, $**p < 0.005$, and $***p < 0.001$ vs diabetic.

a consequent reduction in tissue injury, in particular in the limitation of diabetic nephropathy as the results of this study reported.

However, further studies are needed to fully confirm these hypotheses.

Acknowledgements

This study was supported by a grant PRA (Research Athenaeum Project 2007–2008) from the University of Messina, Italy.

References

- [1] T. Zelmanovitz, F. Gerchman, A.P. Balthazar, F.C. Thomazelli, J.D. Matos, L.H. Canani, Diabetic nephropathy, *Diabetol. Metab. Syndr.* 1 (2009) 1–17.
- [2] T.W. Tervaert, A.L. Mooyaart, K. Amann, A.H. Cohen, H.T. Cook, C.B. Drachenberg, F. Ferrario, A.B. Fogo, M. Haas, E.D. Heer, K. Joh, L.H. Noel, J. Radhakrishnan, S.V. Seshan, I.M. Bajema, J.A. Bruijn, Pathological classification of diabetic nephropathy, *J Am Soc Nephrol* 21 (2010) 556–563.
- [3] J.F. Navarro-Gonzales, C. Mora-Fernandez, The role of inflammatory cytokines in diabetic nephropathy, *J Am Soc Nephrol* 19 (2008) 433–442.
- [4] C.C. Wu, J.S. Chen, K.C. Lu, C.C. Chen, S.H. Lin, P. Chu, H.K. Sytwu, Y.F. Lin, Aberrant cytokines/chemokines production correlate with proteinuria in patients with overt diabetic nephropathy, *Clin Chim Acta* 411 (2010) 700–704.
- [5] F.A. Hakim, A. Pflueger, Role of oxidative stress in diabetic kidney disease, *Med Sci Monit* 16 (2010) RA37–48.
- [6] J.F. Navarro-Gonzales, A. Jarque, M. Muros, C. Mora, J. Garcia, Tumor necrosis factor- α as a therapeutic target for diabetic nephropathy, *Cytokine Growth Factor Rev* 20 (2009) 165–173.
- [7] R.B. Goldberg, Cytokine and cytokine-like inflammation markers, endothelial dysfunction, and imbalanced coagulation in development of diabetes and its complications, *J Clin Endocrinol Metab* 94 (2009) 3171–3182.
- [8] K. Miyauchi, Y. Takiyama, J. Honjyo, M. Tateno, M. Haneda, Upregulated IL-18 expression in type 2 diabetic subjects with nephropathy: TGF- β 1 enhanced IL-18 expression in human renal proximal tubular epithelial cells, *Diabetes Res. Clin. Pract.* 83 (2009) 190–199.
- [9] H. Xiao, Y. Li, J. Qi, H. Wang, K. Liu, Peroxynitrite plays a key role in glomerular lesions in diabetic rats, *J Nephrol* 22 (2009) 800–808.
- [10] K.M. Thrallkill, R.C. Bunn, J.L. Fowlkes, Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy, *Endocr* 35 (2009) 1–10.
- [11] S. Okada, M. Saito, E. Kazuyama, T. Hanada, Y. Kawaba, A. Hayashi, K. Satoh, S. Kanzaki, Effects of *N*-hexacosanol on nitric oxide synthase system in diabetic rat nephropathy, *Mol Cell Biochem* 315 (2008) 169–177.
- [12] G. Gruden, P.C. Perin, G. Camussi, Insight on the pathogenesis of diabetic nephropathy from the study of podocyte and mesangial cell biology, *Curr. Diabetes Rev.* 1 (2005) 27–40.
- [13] G. Nagy, A. Koncz, D. Fernandez, A. Perl, Nitric oxide, mitochondrial hyperpolarization, and T cell activation, *Free Radic Biol Med* 42 (2007) 1625–1631.
- [14] K.H. Han, S.Y. Han, H.S. Kim, Y.S. Kang, D.R. Cha, Prolonged administration enhanced the renoprotective effect of pentoxifylline via anti-inflammatory activity in streptozotocin-induced diabetic nephropathy, *Inflammation* 33 (2010) 137–143.
- [15] J.J. Li, S.H. Lee, D.K. Kim, R. Jin, D.S. Jung, S.J. Kwak, S.H. Kim, S.H. Han, J.E. Lee, S.J. Moon, D.R. Ryu, T.H. Yoo, D.S. Han, S.W. Kang, Colchicine attenuates inflammatory cell infiltration and extracellular matrix accumulation in diabetic nephropathy, *Am. J. Physiol. Renal Physiol.* 297 (2009) F200–F209.
- [16] J.R. Fraser, T.C. Laurent, U.B. Laurent, Hyaluronan: its nature, distribution, function and turnover, *J Intern Med* 242 (1997) 27–33.
- [17] P.W. Noble, Hyaluronan and its catabolic products in tissue injury and repair, *Matrix Biol* 21 (2002) 25–29.
- [18] D. Jiang, J. Liang, P.W. Noble, Hyaluronan in tissue injury and repair, *Annu Rev Cell Dev Biol* 23 (2007) 435–461.

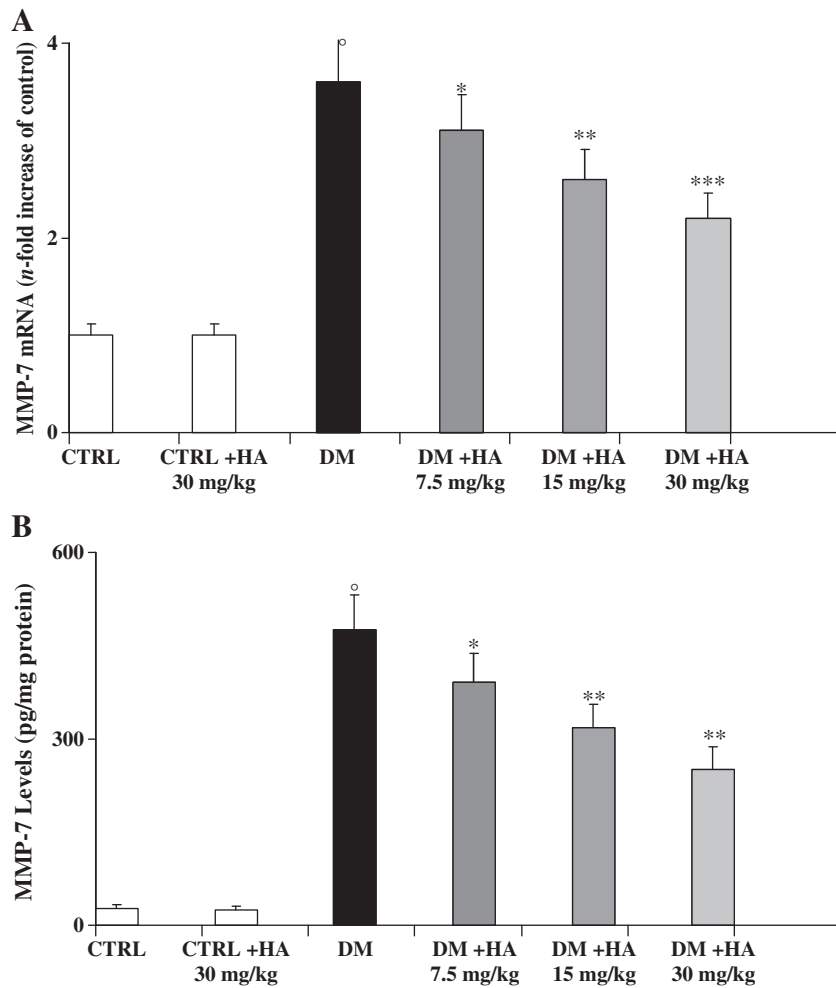


Fig. 9. Effect of HA treatment on MMP-7 mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as picograms per milligram of protein (B) for MMP-7 protein levels. $^{\circ}p < 0.001$ vs control; $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$ vs diabetic.

- [19] G.M. Campo, A. Avenoso, S. Campo, A. D'Ascola, P. Traina, A. Calatroni, Differential effect of molecular size HA in mouse chondrocytes stimulated with PMA, *Biochim Biophys Acta* 1790 (2009) 1353–1367.
- [20] G.M. Campo, A. Avenoso, S. Campo, A. D'Ascola, G. Nastasi, A. Calatroni, Molecular size hyaluronan differently modulates toll-like receptor-4 in LPS-induced inflammation in mouse chondrocytes, *Biochimie* 92 (2010) 204–215.
- [21] A. Aruffo, I. Stamenkovic, M. Melnick, C.B. Underhill, B. Seed, CD44 is the principal cell surface receptor for hyaluronate, *Cell* 61 (1990) 1303–1313.
- [22] P. Heldin, E. Karousou, B. Bernert, H. Porsch, K. Nishitsuka, S.S. Skandalis, Importance of hyaluronan–CD44 interactions in inflammation and tumorigenesis, *Connect Tissue Res* 49 (2008) 215–218.
- [23] L. Weiss, S. Slavina, S. Reich, P. Cohen, S. Shuster, R. Stern, E. Kaganovsky, E. Okon, A.M. Rubinstein, D. Naor, Induction of resistance to diabetes in non-obese diabetic mice by targeting CD44 with a specific monoclonal antibody, *Proc Natl Acad Sci USA* 97 (2000) 285–290.
- [24] L. Weiss, A.M. Botero-Anug, C. Hand, S. Slavina, D. Naor, CD44 gene vaccination for insulin-dependent diabetes mellitus in non-obese diabetic mice, *Isr Med Assoc J* 10 (2008) 20–25.
- [25] M. Zoller, P. Gupta, R. Marhaba, M. Vitacolonna, P. Freyschmidt-Paul, Anti-CD44-mediated blockade of leukocyte migration in skin-associated immune diseases, *J Leukoc Biol* 82 (2007) 57–71.
- [26] G. Baier, J. Wagner, PKC inhibitors: potential in T cell-dependent immune diseases, *Curr Opin Cell Biol* 21 (2009) 262–267.
- [27] R.F. Thorne, J.W. Legg, C.M. Isacke, The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events, *J Cell Sci* 117 (2004) 373–380.
- [28] C. Larsson, Protein kinase C and the regulation of the actin cytoskeleton, *Cell Signal* 18 (2006) 276–284.
- [29] I. Okamoto, Y. Kawano, M. Matsumoto, M. Suga, K. Kaibuchi, M. Ando, H. Saya, Regulated CD44 cleavage under the control of protein kinase C, calcium influx, and the Rho family of small G proteins, *J Biol Chem* 274 (1999) 25525–25534.
- [30] D. Liu, T. Liu, R. Li, M.S. Sy, Mechanisms regulating the binding activity of CD44 to hyaluronan, *Front Biosci* 3 (1998) d631–d636.
- [31] K.A. Fitzgerald, A.G. Bowie, B.S. Skeffington, L.A.J. O'Neill, Ras, protein Kinase C and I κ B kinase 1 and 2 are downstream effectors of CD44 during the activation of NF- κ B by hyaluronan fragments in T-24 carcinoma cells, *J Immunol* 164 (2000) 2053–2063.
- [32] A. Kumar, Y. Takada, A.M. Boriek, B.B. Aggarwal, Nuclear factor- κ B: its role in health and disease, *J Mol Med* 82 (2004) 434–448.
- [33] J. Menne, M. Meier, J.K. Park, M. Boehne, T. Kirsch, C. Lindschau, R. Ociepka, M. Leitges, J. Rinta-Valkama, H. Holthofer, H. Haller, Nephron loss in experimental diabetic nephropathy is prevented by deletion of protein kinase C α signalling in-vivo, *Kidney Int* 70 (2006) 1456–1462.
- [34] D. Wu, F. Peng, B. Zhang, A.J. Ingram, D.J. Kelly, R.E. Gilbert, B. Gao, J.C. Krepinsky, PKC- β 1 mediates glucose-induced Akt activation and TGF- β 1 upregulation in mesangial cells, *J Am Soc Nephrol* 20 (2009) 554–566.
- [35] P.R. Nagareddy, H. Soliman, G. Lin, P.S. Rajput, U. Kumar, J.H. McNeill, K.M. MacLeod, Selective inhibition of protein kinase C β (2) attenuates inducible nitric oxide synthase-mediated cardiovascular abnormalities in streptozotocin-induced diabetic rats, *Diabetes* 58 (2009) 2355–2364.
- [36] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, *Anal Biochem* 72 (1976) 248–254.
- [37] D.R. Garris, B.L. Garris, Estrogenic restoration of functional pancreatic islet cytoarchitecture in diabetes (*db/db*) mutant C57BL/KsJ mice: relationship to estradiol localization, systemic glycemia, and persistent hyperinsulinemia, *Cell Tissue Res* 319 (2005) 231–242.
- [38] L. Lebel, J.R. Fraser, W.S. Kimpton, J. Gabrielsson, B. Gerdin, T.C. Laurent, A pharmacokinetic model of intravenously administered hyaluronan in sheep, *Pharm Res* 6 (1989) 677–682.
- [39] D. Naor, S. Nedvetzki, M. Walmsley, A. Yayon, E.A. Turley, I. Golan, D. Caspi, L.E. Sebban, Y. Zick, T. Garin, D. Karussis, N. Assayag-Asherie, I. Raz, L. Weiss, S. Slavina, I. Golan, CD44 involvement in autoimmune inflammations: the lesson to be learned from CD44-targeting by antibody or from knockout mice, *Ann. N.Y. Acad. Sci.* 1110 (2007) 233–247.
- [40] J. Melin, O. Hellberg, K. Funa, R. Hallgren, E. Larsson, B.C. Fellstrom, Ischemia-induced renal expression of hyaluronan and CD44 in diabetic rats, *Nephron Exp Nephrol* 103 (2006) e86–e94.

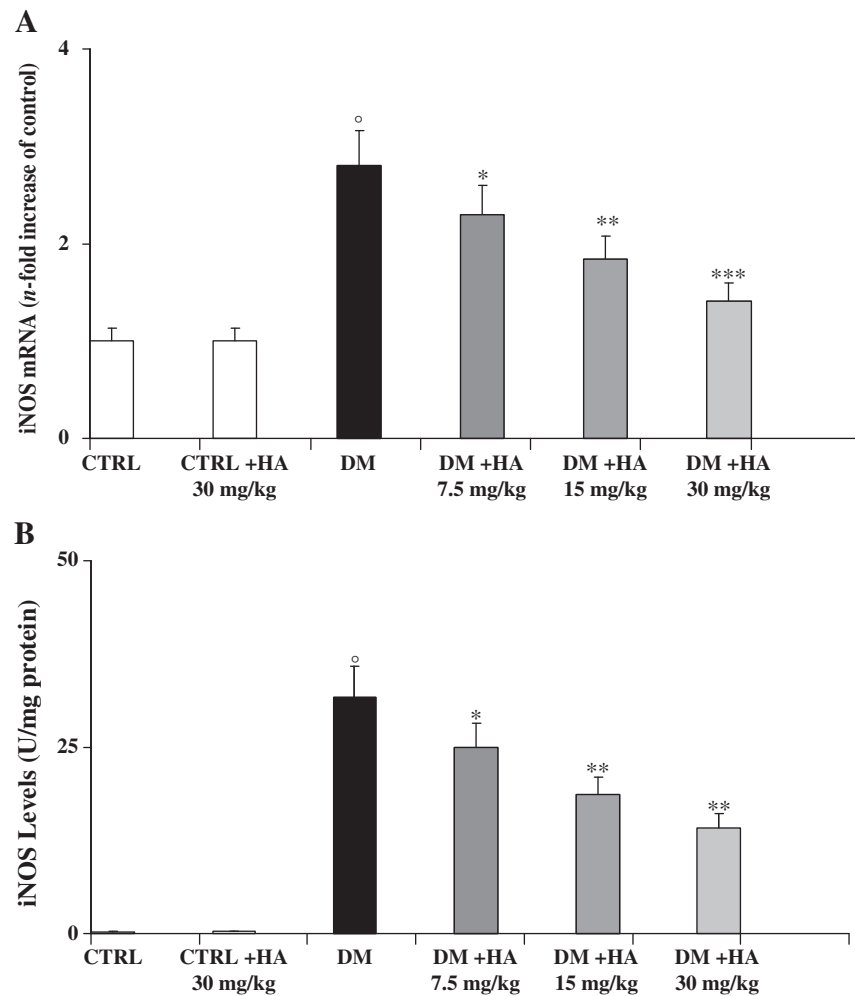


Fig. 10. Effect of HA treatment on iNOS mRNA expression (A) and related protein production (B) in kidney tissue of diabetic mice. Values are the mean \pm SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as picograms per milligram of protein (B) for iNOS protein levels. $^{\circ}p < 0.001$ vs control; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.005$ vs diabetic.

- [41] H.Y. Lan, Role of macrophage migration inhibition factor in kidney disease, *Nephron Exp Nephrol* 109 (2008) e79–e83.
- [42] M. Ascon, D.B. Ascon, M. Liu, C. Cheadle, C. Sarkar, L. Racusen, H.T. Hassoun, H. Rabb, Renal ischemia–reperfusion leads to long term infiltration of activated and effector–memory T lymphocytes, *Kidney Int* 75 (2009) 526–535.
- [43] A.C. Newton, Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm, *Biochem J* 370 (2003) 361–371 part2).
- [44] A.M. Martelli, C. Evangelisti, M. Nyakern, F.A. Manzoli, Nuclear protein kinase C, *Biochim Biophys Acta* 1761 (2006) 542–551.
- [45] A.C. Newton, Lipid activation of protein kinases, *J. Lipid Res. Suppl* 50 (2009) S266–S271.
- [46] D. Koya, M. Haneda, H. Nakagawa, K. Isshiki, H. Sato, S. Maeda, T. Sugimoto, H. Yasuda, A. Kashiwagi, D.K. Wags, G.L. King, R. Kikkawa, Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC β inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes, *FASEB J* 14 (2000) 439–447.
- [47] Y. Kim, Y.S. Lee, J. Choe, H. Lee, Y.M. Kim, D. Joung, CD44–epidermal growth factor receptor interaction mediates hyaluronic acid–promoted cell motility by activating protein kinase C signalling involving Akt, Rac1, Phox, reactive oxygen species, focal adhesion kinase, and MMP–2, *J Biol Chem* 283 (2008) 22513–22528.
- [48] P.V. Jensen, L.I. Larsson, Actin microdomains on endothelial cells: association with CD44, ERM proteins, and signalling molecules during quiescence and wound healing, *Histochem Cell Biol* 121 (2004) 361–369.
- [49] D.L. Clarke, A. Sutcliffe, K. Deacon, D. Bradbury, L. Corbett, A.J. Knox, PKC β augments NF–kappaB–dependent transcription at the CCL11 promoter via p300/CBP–Associated factor recruitment and histone H4 acetylation, *J Immunol* 181 (2008) 3503–3514.
- [50] R.P. Thylur, Y.D. Kim, M.S. Kwon, H.M. Oh, H.K. Kwon, S.H. Kim, S.H. Im, J.S. Chun, Z.Y. Park, C.D. Jun, Swiprosin-1 is expressed in mast cells and up-regulated through the protein kinase C beta 1/eta pathway, *J Cell Biochem* 108 (2009) 705–715.
- [51] M.W. Greene, M.S. Ruhoff, C.M. Burrington, R.S. Garofalo, S.J. Orena, TNFalpha activation of PKCdelta, mediated by NFkappaB and ER stress, cross-talks with the insulin signalling cascade, *Cell Signal* 22 (2010) 274–284.
- [52] E.A. Yates, C.J. Terry, C. Rees, T.R. Rudd, L. Duchesne, M.A. Skidmore, R. L evy, N.T. Thanh, R.J. Nichols, D.T. Clarke, D.G. Fernig, Protein–GAG interactions: new surface-based techniques, spectroscopies and nanotechnology probes, *Biochem Soc Trans* 34 (2006) 427–430.
- [53] D.A. Steeber, G.M. Venturi, T.F. Tedder, A new twist to the leukocyte adhesion cascade: intimate cooperation is key, *Trends Immunol* 26 (2005) 9–12.
- [54] M. Eberlein, K.A. Scheibner, K.E. Black, S.L. Collins, Y. Chan-Li, J.D. Powell, M.R. Horton, Anti-oxidant inhibition of hyaluronan fragment-induced inflammatory gene expression, *J Inflamm* 5 (2008) 5–20.
- [55] R. Stern, Hyaluronan catabolism: a new metabolic pathway, *Eur J Cell Biol* 83 (2004) 317–325.
- [56] J.A. Beckman, A.B. Goldfine, M.B. Gordon, L. A. Garrett, M.A. Creager, Inhibition of protein kinase C β prevents impaired endothelium-dependent vasodilation caused by hyperglycemia in humans, *Circ Res* 90 (2002) 107–111.

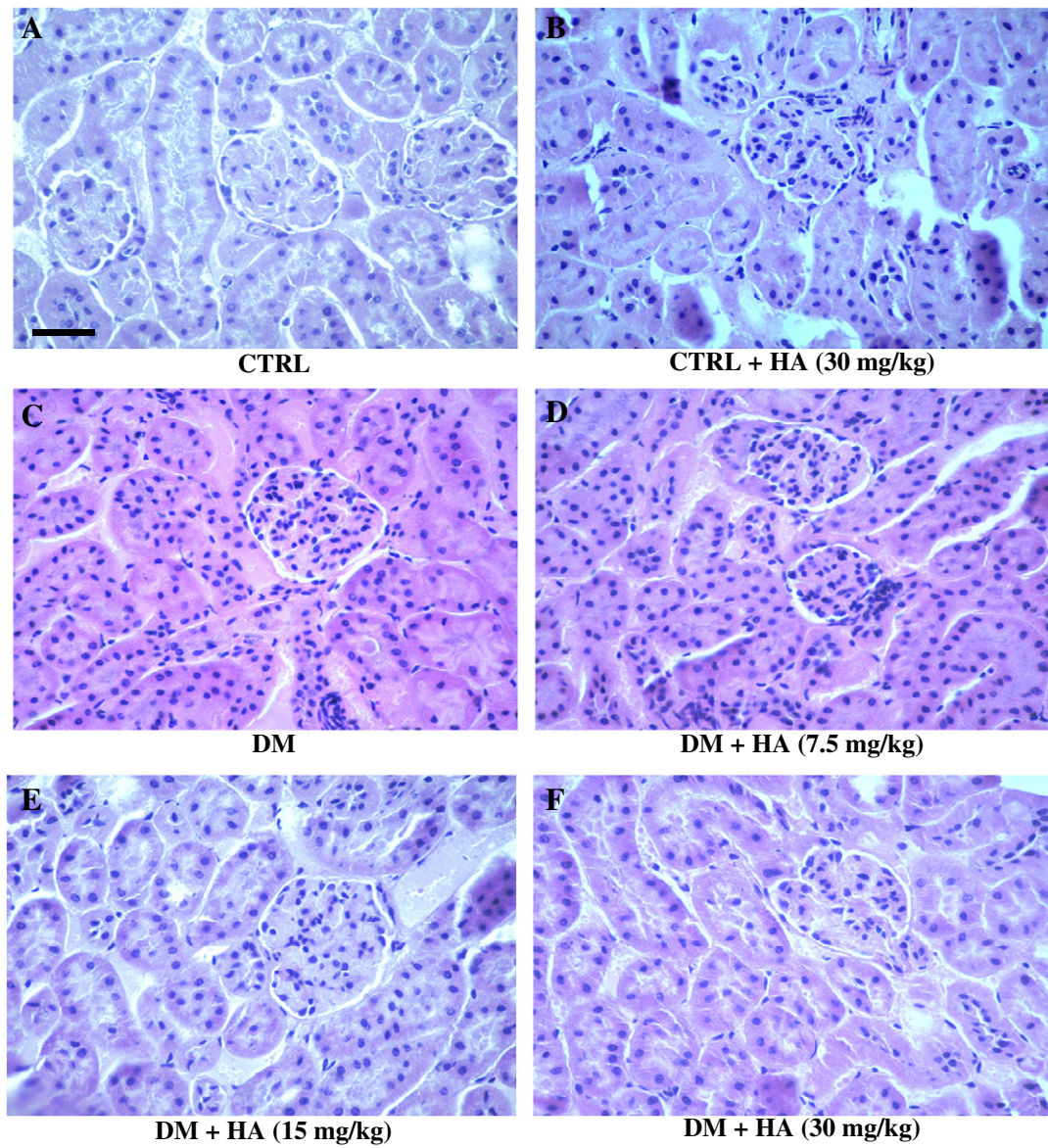


Fig. 11. Effect of HA on the prevention of glomerular hyperplasia and mesangial matrix expansion in congenitally diabetic mice. Hematoxylin–eosin staining. Bar = 50 μm (magnification $\times 2080$).