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Differential effect of molecular mass hyaluronan on lipopolysaccharide-induced damage in chondrocytes

Giuseppe M. Campo, Angela Avenoso, Salvatore Campo, Angela D'Ascola, Paola Traina, Carmela A. Rugolo, Alberto Calatroni

Department of Biochemical, Physiological and Nutritional Sciences, Section of Medical Chemistry School of Medicine University of Messina, Policlinico Universitario, Messina, Italy

Hyaluronan is a biological polysaccharide that may exist in different degrees of polymerization. Several investigations reported that low molecular mass hyaluronan may have pro-inflammatory activity, while high molecular mass hyaluronan can exert beneficial effects. Starting from these data, the aim of this study was to investigate the effect of hyaluronan of different molecular mass in mouse articular chondrocyte cultures stimulated with lipopolysaccharide (LPS). Inflammation was induced in chondrocytes by acute treatment with 2.0 μg/ml LPS. High levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, interferon (IFN)-γ and iNOS gene expression and their related proteins were found in chondrocytes 24 h after treatment with LPS. High concentrations of NO, NF-κB activation, IκBα phosphorylation and apoptosis, evaluated by the increase in caspase-3 expression and its related protein amount were also produced by LPS stimulation. In contrast, LPS reduced aggrecan and collagen type II (Col2A) expression and their protein production. The treatment of chondrocytes with hyaluronan of different molecular mass produced the following effects: (i) low molecular mass hyaluronan exerted a slight inflammatory effect in untreated chondrocytes, while in LPS-treated chondrocytes it enhanced cytokine production and decreased aggrecan and Col2A compared with cells treated with LPS alone; (ii) no effect was exerted on LPS-induced apoptosis and NO production; (iii) medium molecular mass hyaluronan did not exert any inflammatory/anti-inflammatory activity in LPS-untreated/treated cells and failed to reduce apoptosis; and (iv) high molecular mass hyaluronan had no inflammatory effect in LPS-untreated cells while it was able to reduce all the detrimental effects stimulated by LPS treatment. These data confirm the multifactorial role played by hyaluronan and suggest, in particular, that hyaluronan may modulate inflammation during pathologies by its different degrees of polymerization.

Keywords: hyaluronan, chondrocytes, LPS, NF-κB, nitric oxide, inflammatory cytokines, apoptosis

Introduction

Arthritis is a degenerative joint disease characterized by the progressive disruption of articular cartilage. This is accompanied by varying degrees of infiltration of the synovial membrane by mononuclear cells.[1] The processes that cause progressive cartilage disruption seem to be related to the production of pro-inflammatory cytokines by chondrocytes, synoviocytes and mononuclear cells.[2–4] Pro-inflammatory cytokines comprise tumour necrosis (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ, IL-6, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS) and proteinases (MMPs). These have an important role not only in the priming, but also in the maintenance, of inflammatory mechanisms.[3] Chemokines directly contribute to the modulation of immunocellular functions. They are able both to recruit mononuclear cells in the damaged areas

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Correspondence to: Giuseppe M. Campo PhD, 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 3330; E-mail: gcampo@unime.it

and to stimulate specific cellular functions, such as proliferation, and production of degradative enzymes and ROS.[3,4] One ROS, nitric oxide (NO), is a highly reactive, cytotoxic molecule that has been strongly implicated in arthritis.[5] Increased levels of NO promoted numerous effects on chondrocytes, including inhibition of matrix synthesis,[6] activation of MMPs[7] and apoptosis.[8]

Lipopolysaccharide (LPS) is the principal component of the outer membrane of Gram-negative bacteria. The cell response to LPS includes the expression of a variety of inflammatory cytokines and cytotoxic mediators such as NO, or a systemic inflammatory response syndrome[9] similar to the one produced during arthritis.[10,11] These processes may also involve caspase activation that, in turn, determines apoptosis and cell death. It is also known that all these events are modulated mainly by nuclear transcription factor NF-κB activation which is significantly involved in the regulation of inflammation as well as in arthritis.[12]

In many tissues, cell–matrix interactions serve to regulate cellular homeostasis.[13] Such interactions are particularly important in tissue such as articular cartilage, as this is a tissue rich in extracellular matrix but with limited vascular access for systemic control of homeostasis. Chondrocytes are thus highly dependent on cell–matrix interactions as a primary means to 'sense' changes in the extracellular environment.[14] However, cell–matrix interactions involving hyaluronan, once thought to be predominantly structural in Nature, are now beginning to attract increasing attention as initiators of cell signalling. Hyaluronan, a high molecular mass polysaccharide produced by type B synovial cells, is one of the main components of synovial fluid.[15] Within the joint cavity, hyaluronan plays a major role in joint lubrication and in maintaining homeostasis.[15] The remarkable rheological properties of synovial fluid are dependent on hyaluronan concentration and its molecular mass.[16] Both hyaluronan concentration and molecular mass usually decline in inflamed joints, such as in arthritis.[16] Previous research suggested that hyaluronan degradation occurs in pathological conditions, probably because of ROS depolymerization of the hyaluronan chains.[17] However, it was also reported that both low and high molecular mass hyaluronan were produced during pathological conditions.[18,19] *In vitro* studies have demonstrated that hyaluronan has different biological functions depending on its molecular mass. For instance, hyaluronan fragments induce the expression of genes involved in the inflammation processes,[20–22] while high molecular mass hyaluronan was reported to exert the opposite effects.[23–25]

Starting from these previous findings, the aim of the present study was to investigate the anti-inflammatory activity of different doses of hyaluronan at low, medium and high molecular mass in an experimental model of LPS-induced inflammation in mouse articular chondrocyte cultures.

Materials and Methods

Materials

Hyaluronan sodium salt at low molecular mass (50 kDa, HYA-50K-1 SelectHA^{TM}50K) and medium molecular mass (1000 kDa, HYA-1000K-1 SelectHA^{TM}1000K) were obtained from NorthStar Bioproducts (East Falmouth, USA), high molecular mass (5000 kDa, HEALON) was purchased from Pharmacia Corporation (Kalamazoo, USA), and LPS from *Salmonella enterica* was obtained from Sigma-Aldrich S.r.l. (Milan, Italy). Mouse TNF-α (cat. IB49688), IL-1β (cat. IB49700), IL-6 (cat. IB49686), and IFN-γ (cat. IB49687) commercial ELISA kits were provided from Immuno-Biological Laboratories Inc. (Minneapolis, MN, USA). Aggrecan and collagen type II (Col2A) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Inducible nitric oxide (iNOS), caspase-3 monoclonal antibodies and horseradish peroxidase-labeled goat anti-rabbit antibodies were obtained from GenWay Biotech, Inc., (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypsin-EDTA solution and phosphate buffered saline (PBS) were obtained from GibcoBRL (Grand Island, NY, USA). All cell culture plastics were obtained from Falcon (Oxnard, CA, USA). Sulphanilamide, naphthyl-ethylenediamine dihydrochloride, phosphoric acid, sodium nitrite, sucrose, ethylenediaminetetraacetic acid (EDTA), potassium phosphate, RNase, proteinase K, protease inhibitor cocktail, sodium dodecylsulphate (SDS), phenylmethylsulfonyl fluoride (PMSF) and all other general laboratory chemicals were obtained from Sigma-Aldrich.

Cell culture

Normal knee mouse chondrocytes (DPK-CACC-M, strain: C57BL/6J; Dominion Pharmakine, Bizkaia, Spain) were cultured in 75-cm^2 plastic flasks containing 15.0 ml of DMEM to which was added 10% FBS, L-glutamine (2.0 mM) and penicillin/streptomycin (100 U/ml, 100 μg/ml), and were incubated at 37°C in humidified air with 5% CO_2. Experiments were performed using chondrocyte cultures between the third and the fifth passage.

Lipopolysaccharide stimulation and hyaluronan treatment

Chondrocytes were cultured in 6-well culture plates at a density of 1.3×10^5 cells/well. Twelve hours after plating (time 0), the culture medium was replaced with 2.0 ml of fresh medium containing LPS at concentration of 2.0 μg/ml. Four hours later, low molecular mass hyaluronan, medium molecular mass hyaluronan and high molecular mass hyaluronan at concentrations of 0.15 and 0.30 mg/ml were added separately to wells. Twenty-four hours later, the cells and medium underwent morphological and biochemical evaluation.

Isolation of RNA, cDNA synthesis and PCR real-time quantitative amplification

Total RNA was isolated from chondrocytes for reverse-PCR real-time analysis of TNF-α, IL-1β, IL-6, IFN-γ, iNOS, caspase-3, aggrecan and collagen type II (Col2A) (RealTime PCR system, Mod. 7500, Applied Biosystems, USA) using an Omnizol Reagent Kit (Euroclone, West York, UK). The first strand of cDNA was synthesized from 1.0 μg 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 TNF-α, IL-1β, IL-6, IFN-γ, iNOS, caspase-3, aggrecan and Col2A mRNAs. 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 TNF-α, IL-1β, IL-6, IFN-γ, iNOS, caspase-3, aggrecan, Col2A, and β-actin mRNA. The C_T values were plotted against the log input RNA concentration in serially diluted total RNA of chondrocyte samples and used to generate standard curves for all mRNAs analysed. The amounts of specific mRNA in samples were calculated from the standard curve, and normalized with the β-actin mRNA. After normalization, the mean value of normal cartilage cell 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).

Western blot assay of iNOS, caspase-3, aggrecan and Col2A proteins

For SDS-PAGE and Western blotting, chondrocytes were washed twice in ice-cold PBS and subsequently dissolved in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue). β-Actin protein was used as an endogenous control to allow the normalization of iNOS, caspase-3, aggrecan and Col2A proteins. Aliquots of protein extracted from the culture media (10–25 μl/well) in the presence of 1 nM PMSF and protease inhibitor cocktail. Following centrifugation at $3{,}500 \times g$ for 10 min at 4°C, the protein content of the supernatant was measured.[26] Then, after denaturation, the samples were separated on a mini gel (10%). The proteins were blotted onto polyvinylidene difluoride membranes (Amersham Biosciences) using a semi-dry apparatus (Bio-Rad). The blots were flushed with double distilled H_2O, dipped into methanol, and dried for 20 min before proceeding to the next steps. Subsequently, the blots were transferred to a blocking buffer solution (1 × PBS, 0.1% Tween 20, 5% w/v non-fat dried milk) and incubated for 1 h. The membranes were then incubated with the specific diluted (1 : 1) primary antibody in 5% bovine serum albumin, 1 × PBS, and 0.1% Tween 20 and stored in a roller bottle overnight at 4°C. After being washed in three stages in wash buffer (1 × 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% non-fat dried milk. After 45 min of gentle shaking, the blots were washed five times in wash buffer and the proteins were made visible using a UV/visible transilluminator (EuroClone, Milan, Italy) and Kodak BioMax MR films. A densitometric analysis was also run in order to quantify each band.

ELISA assays for TNF-α, IL-1β, IL-6, and IFN-γ

Samples of cell-secreted protein extracted from the culture media in the presence of 1 nM PMSF and protease inhibitor cocktail were centrifuged at $3{,}500 \times g$ for 10 min at 4°C. The analysis of TNF-α, IL-1β, IL-6, IFN-γ 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 were added to each well. After 120 min incubation at 20–22°C, the liquid from the wells was discarded. Then, after washing wells three times, 100 μl of streptavidin-horseradish peroxidase were added. After a further incubation for 60 min and a new washing of the wells, 100 μl of a substrate chromogen solution were added. After incubation for 10 min, and the addition of 100 μl of stop solution, the absorbance of each well was read spectrophotometrically at $\lambda = 450$ nm. Tumour-necrosis factor-α, IL-1β, IL-6, IFN-γ values were expressed as pg/ml.

Nuclear factor-κB p50/65 transcription factor assay

Nuclear factor-κB p50/65 DNA binding activity in nuclear extracts of chondrocytes was evaluated in

order to measure the degree of NF-κB activation. The analysis was performed according to the manufacturer's protocol for a commercial kit (NF-κB p50/65 Transcription Factor Assay Colorimetric, cat. SGT510; Chemicon International, USA). In brief, cytosolic and nuclear extraction was performed by lysing the cell membrane with an apposite hypotonic lysis buffer containing protease inhibitor cocktail and tributylphosphine (TBP) as reducing agent. After centrifugation at 8000 *g*, the supernatant containing the cytosolic fraction was stored at −70°C, while the pellet containing the nuclear portion was re-suspended in the apposite extraction buffer and the nuclei disrupted by a series of drawing and ejecting actions. The nuclei suspension was then centrifuged at 16,000 *g*. The supernatant fraction was the nuclear extract. After the 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-κB 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 $\lambda = 450$ nm. Values are expressed as relative optical density (OD) per mg protein.

Identification of IκBα

The IκBα loss was quantified in chondrocytes in order to confirm NF-κB activation. The test is based on a solid-phase sandwich ELISA assay. The cytosolic fraction, which was obtained during the nuclei extraction procedure for NF-κB assay, was used for IκBα evaluation. The assay was carried out using a commercial kit (IκBα, Total Human BioAssay ELISA Kit, cat. 12500-05T; US Biological, USA). Briefly, 100 µl of solution of standards, samples and controls were added to each well of the coated microplate and 100 µl of anti-IκBα antibodies was then added to each well. After 1-h incubation at 20–22°C, the liquid was removed from the wells and 100 µl of anti-rabbit IgG-horseradish peroxidase was added. After incubation for a further 30 min, 100 µl of stabilized chromogen was added. Absorbance was measured using a spectrophotometric microplate reader set at $\lambda = 450$ nm. Values are expressed as relative OD/mg protein.

Nitric oxide release assay

At the end of the experiments, culture media were removed and assayed for nitrite production (a stable metabolic by-product of NO generation) using the Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% phosphoric acid). In brief, 0.1 ml medium or sodium nitrite standards were transferred to a 96-well plate, followed by the addition of 0.1 ml of Griess reagent. The absorbance values were read at $\lambda = 540$ nm using an automated microtiter plate reader (DAS srl, Rome, Italy). Nitric oxide levels were calculated with reference to the standard curve of sodium nitrite generated by known concentrations. Nitric oxide levels are expressed as nmol/mg protein.

Protein determination

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.[26]

Statistical analysis

Data are expressed as the mean ± SD values of at least seven experiments for each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. The statistical significance of differences was set at $P < 0.05$.

RESULTS

Tumour-necrosis factor-α, IL-1β, IL-6, IFN-γ, iNOS, caspase-3, aggrecan and Col2A mRNA expression, ELISA and Western blot analysis

Tumour-necrosis factor-α (Fig. 1), IL-1β (Fig. 2), IL-6 (Fig. 3), IFN-γ (Fig. 4), iNOS (Fig. 5), caspase-3 (Fig. 6), aggrecan (Fig. 7) and Col2A (Fig. 8) mRNA evaluation (panel A of Figs 1–6), ELISA assay (panel B of Figs 1–4) and Western blot analysis with densitometric evaluation (panels B and C of Figs 5–8) showed a marked increase in the expression and protein synthesis of all inflammatory cytokines, the iNOS and the apoptotic initiator after stimulation of chondrocytes with LPS, while aggrecan and Col2A were significantly reduced. Treatment with hyaluronan exerted the following effects: (i) low molecular mass hyaluronan slightly increased TNF-α, IL-1β, IL-6, IFN-γ and iNOS in both unstimulated and in LPS-stimulated cells; no effect was exerted in caspase-3 activation, while aggrecan and Col2A in both unstimulated and in LPS-stimulated cells were significantly reduced with the highest dose; (ii) medium molecular mass hyaluronan exerted no significant effect on inflammatory cytokines and apoptosis activation, both in unstimulated or LPS-stimulated chondrocytes; and (iii) high molecular mass hyaluronan at both doses

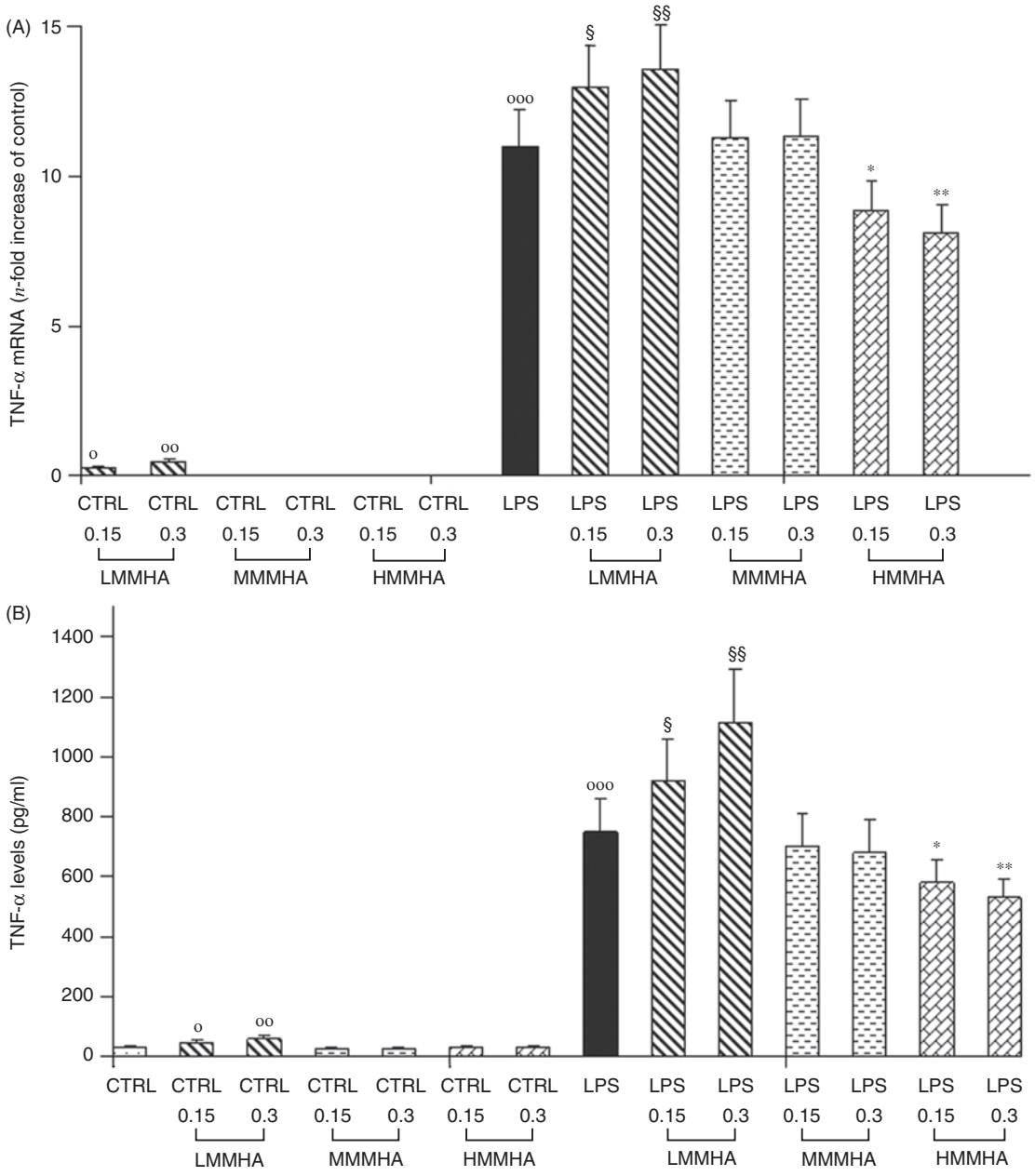

Fig. 1. Effect of hyaluronan treatment at different molecular mass on chondrocyte TNF-α mRNA expression (A) and related protein production (B) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as pg/ml (B) for the TNF-α protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$, and $^{\circ\circ\circ}P<0.001$ versus control; $^{*}P<0.01$, $^{**}P<0.005$ versus LPS; $^{\S}P<0.05$ and $^{\S\S}P<0.01$ versus LPS.

significantly reduced the increment in inflammatory cytokines and apoptosis induced by LPS and partially restored aggrecan and Col2A but no effect was exerted in unstimulated cells.

Nitric oxide production

Figure 9 shows the changes in NO levels of chondrocyte cultures after LPS stimulation and treatment with different molecular masses of hyaluronan. A significant increase in NO release was seen in cells stimulated with LPS alone. Hyaluronan treatment exerted the following effects: (i) low molecular mass hyaluronan did not exert any significant effect in either unstimulated or LPS stimulated cells; (ii) medium molecular mass hyaluronan slightly reduced NO levels in LPS-stimulated cells, while it had no effect in unstimulated cells; (iii) high molecular mass hyaluronan significantly reduced NO levels in LPS-stimulated cells, and had no effect in

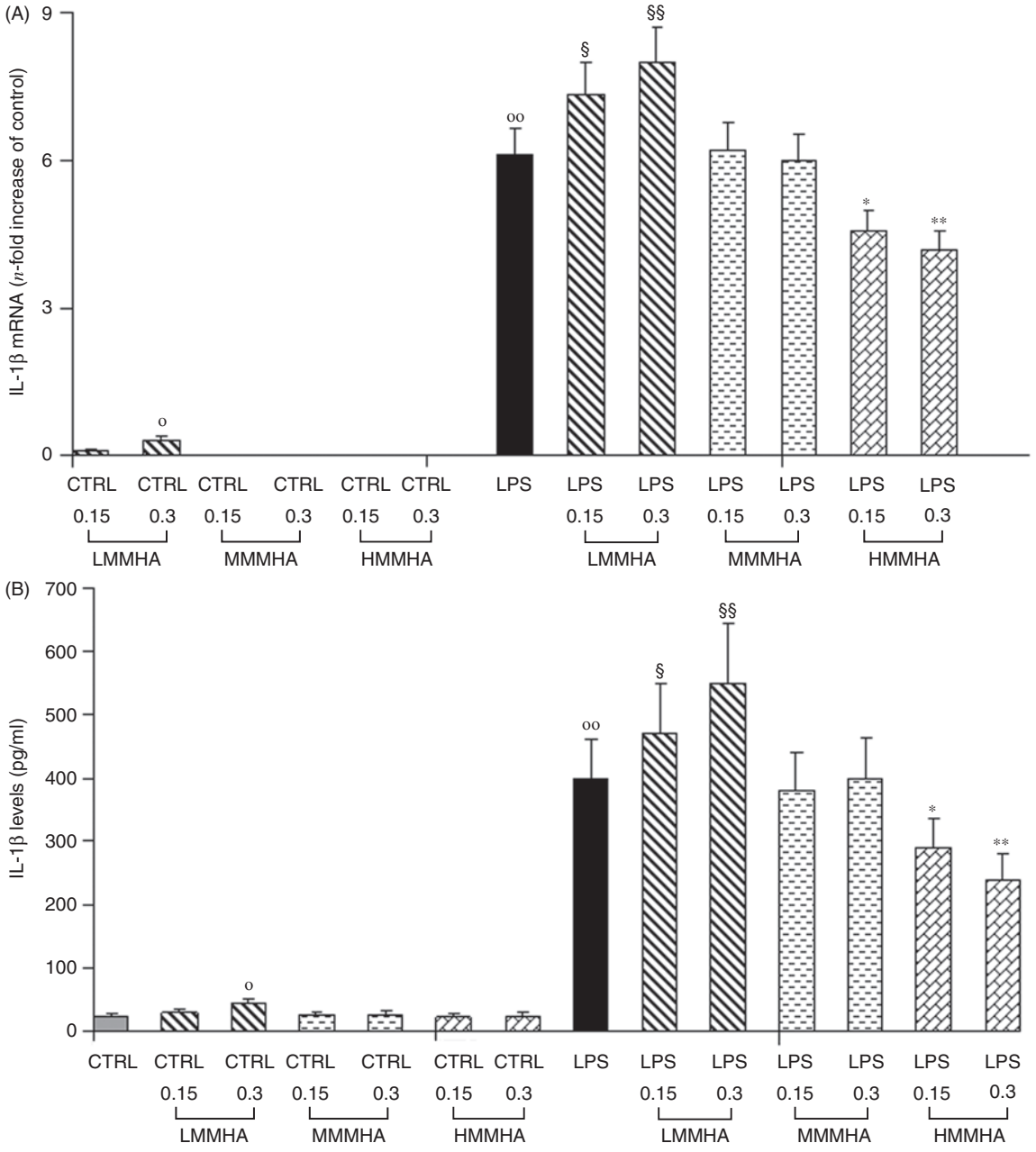

Fig. 2. Effect of hyaluronan treatment at different molecular mass on chondrocyte IL-1β mRNA expression (A) and related protein production (B) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as pg/ml (B) for the IL-1β protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.05$, and $^{\circ\circ}P<0.001$ versus control; $^{*}P<0.005$, and $^{**}P<0.001$ versus LPS; $^{\S}P<0.05$ and $^{\S\S}P<0.01$ versus LPS.

unstimulated cells; and (iv) a more significant reduction was exerted by high molecular mass hyaluronan than by medium molecular mass hyaluronan.

Nuclear factor-κB activation and IκBα degradation

Figure 10A shows the changes in NF-κB p50/p65 heterodimer translocation over the course of the experiment. Lipopolysaccharide stimulation induced massive NF-κB translocation into the nucleus. Treatment with hyaluronan at different molecular masses showed the following effects: (i) low molecular mass hyaluronan activated NF-κB at both doses used and the increase was significant in both unstimulated and in LPS-stimulated chondrocytes; (ii) medium molecular mass hyaluronan did not exert any significant effect in NF-κB activation, in either unstimulated or

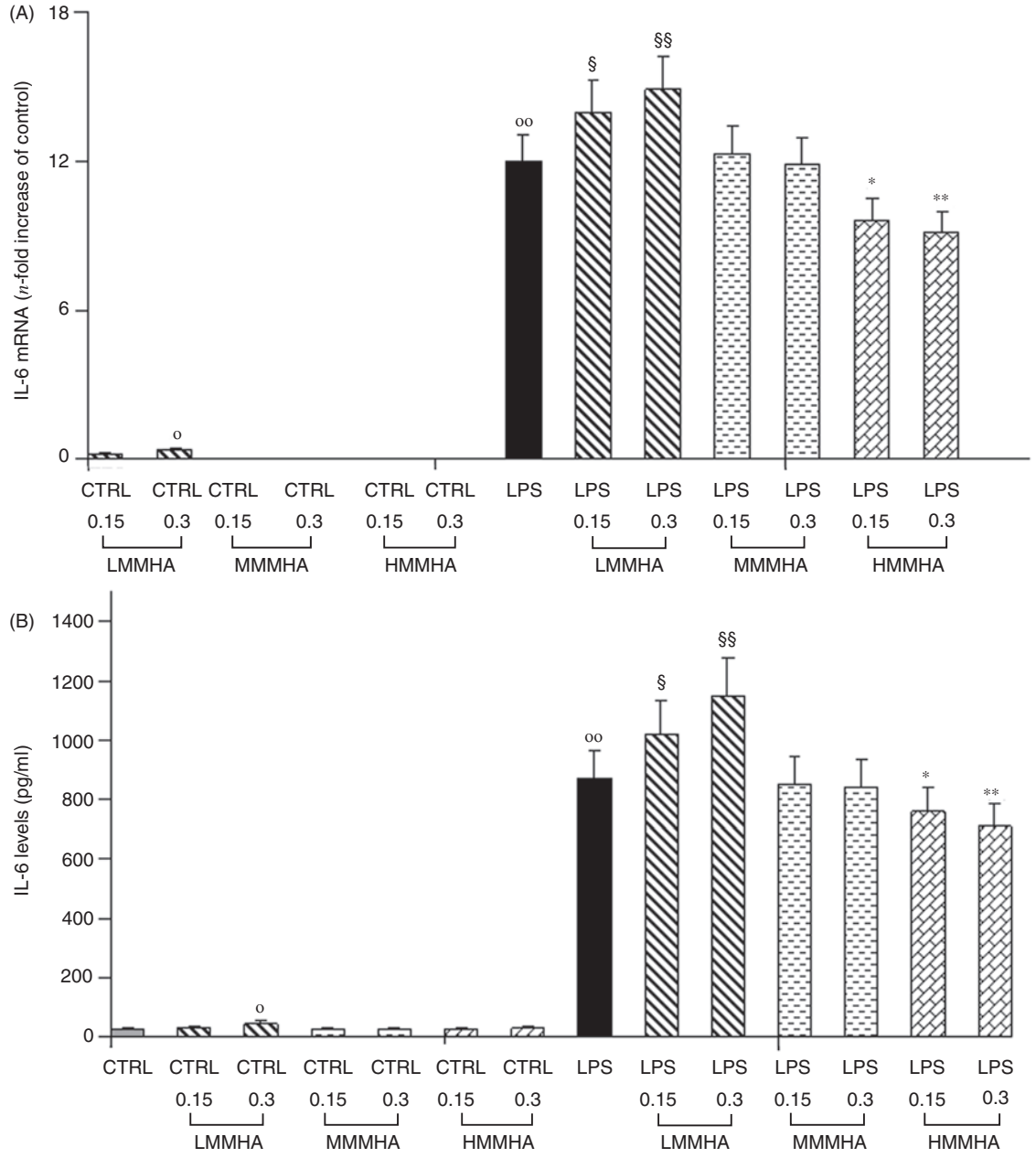

Fig. 3. Effect of hyaluronan treatment at different molecular mass on chondrocyte IL-6 mRNA expression (A) and related protein production (B) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as pg/ml (B) for the IL-6 protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.05$, and $^{\circ\circ}P<0.001$ versus control; $^{*}P<0.05$, and $^{**}P<0.005$ versus LPS; $^{\S}P<0.05$ and $^{\S\S}P<0.01$ versus LPS.

LPS-stimulated cells; and (iii) high molecular mass hyaluronan at both doses significantly inhibited NF-κB activation in LPS-stimulated cells while there was no effect in unstimulated cells.

As IκBα protein is normally associated with NF-κB transcription factor, this assay was performed in order to evaluate the degree of NF-κB activation. Figure 10B shows that LPS stimulation induced a marked loss in IκBα protein due to its phosphorylation. The changes in IκBα degradation exerted by hyaluronan confirmed the data obtained for NF-κB activation.

DISCUSSION

Hyaluronan confers to synovial fluid its unique rheological properties, which together with lubricin provides exceptionally efficient biomechanical protection of

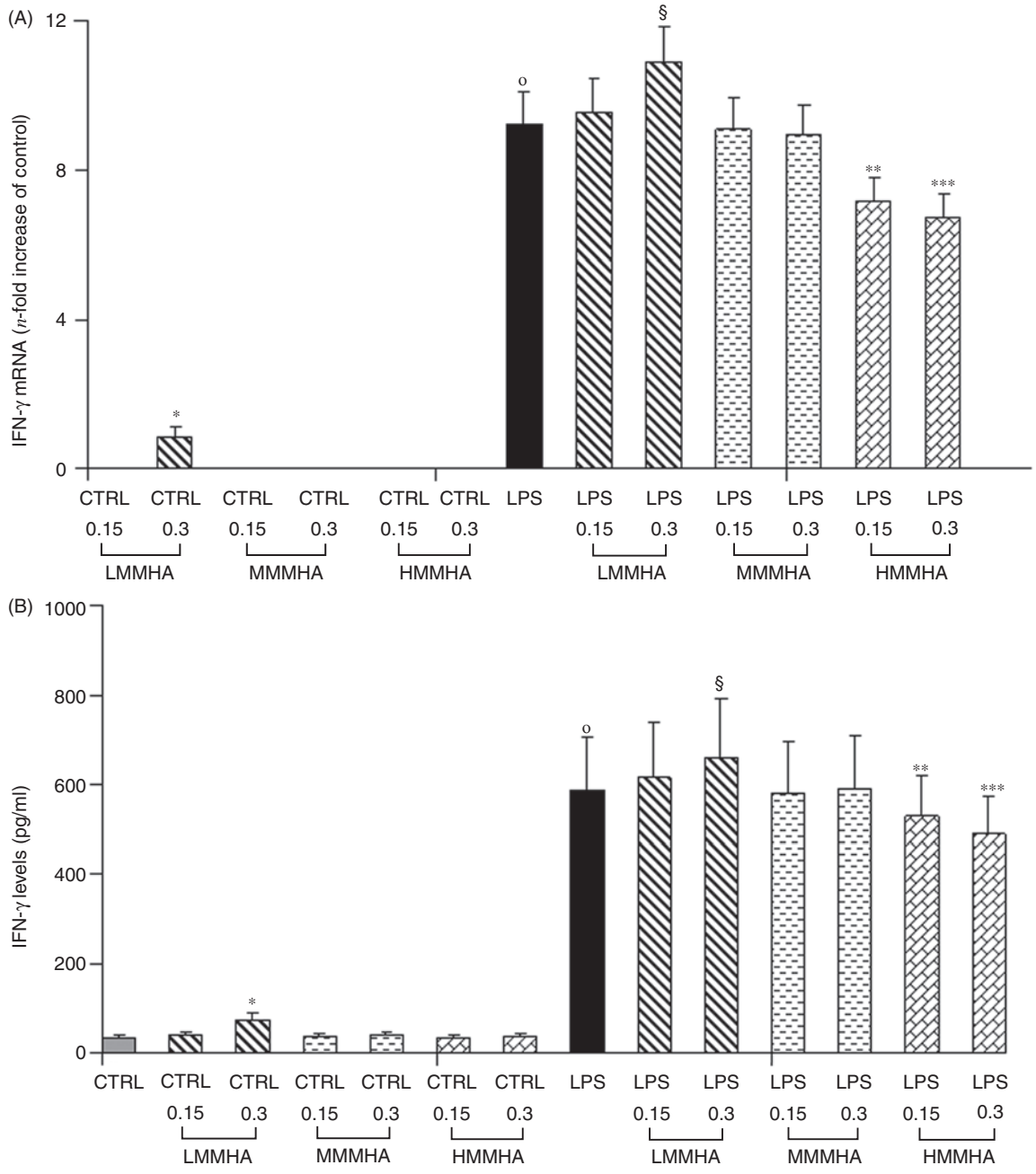

Fig. 4. Effect of hyaluronan treatment at different molecular mass on chondrocyte IFN-γ mRNA expression (A) and related protein production (B) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as pg/ml (B) for the IFN-γ protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.001$ versus control; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ versus LPS; $^{\S}P<0.05$ versus LPS.

articular cartilage and peri-articular tissues.[15,16] In arthritic joints, synovial fluid hyaluronan concentration and molecular mass are decreased,[27] compromising its visco-elasticity and lubricating ability. Various preparations of hyaluronan with a range of molecular mass differentially stimulated synthesis of high molecular mass hyaluronan by synovial cells in culture.[28] However, a large number of clinical trials, consisting to an intra-articular supplementation of hyaluronan at high molecular mass for different periods in patients with osteoarthritis, have shown a marked reduction in cartilage erosion, joint inflammation, pain and swelling, and a general amelioration of the disease symptoms.[29–31]

Pro-inflammatory cytokines (NO and other detrimental intermediates, such as MMPs) produced by stimulated cells play a critical role in inflammatory diseases such as sepsis and arthritis.[2–6] Lipopolysaccharide-stimulated chondrocytes show an inflammatory response by

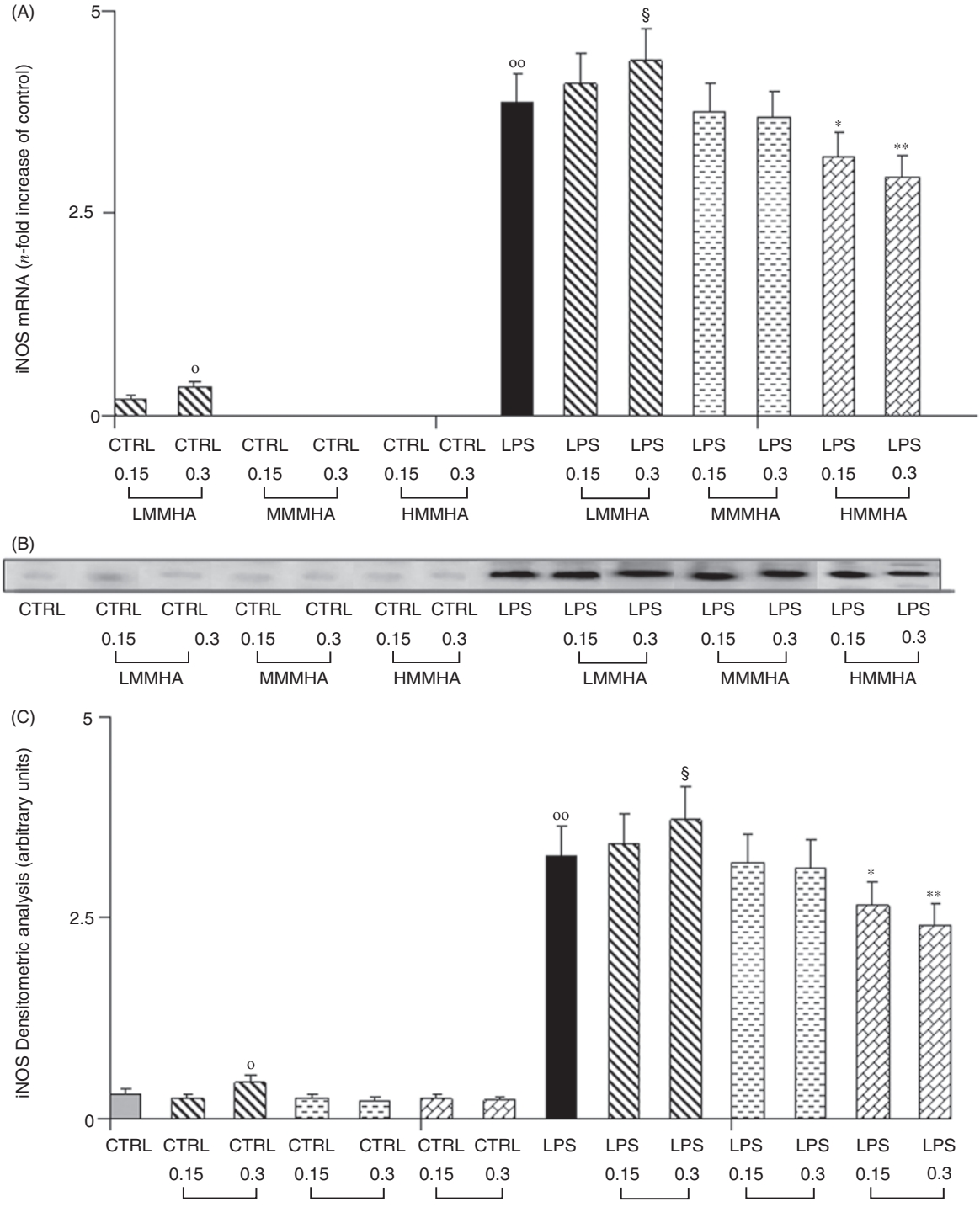

Fig. 5. Effect of hyaluronan treatment at different molecular mass on chondrocyte iNOS mRNA expression (A) and related protein production (B,C) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for the iNOS protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.05$ and $^{\circ\circ}P<0.001$ versus control; $^{*}P<0.01$, and $^{**}P<0.005$ versus LPS; $^{\S}P<0.05$ versus LPS.

increasing TNF-α production, which in turn activates NF-κB translocation into the nucleus. The activation of NF-κB involves the phosphorylation of IκB species via the IκB kinase (IKK) signalosome complex. The resulting free NF-κB is then translocated to the nucleus, where it binds to κB binding sites in the promoter regions of target genes, and induces the transcription of pro-inflammatory mediators such as TNF-α, IL-1β, IFN-γ,

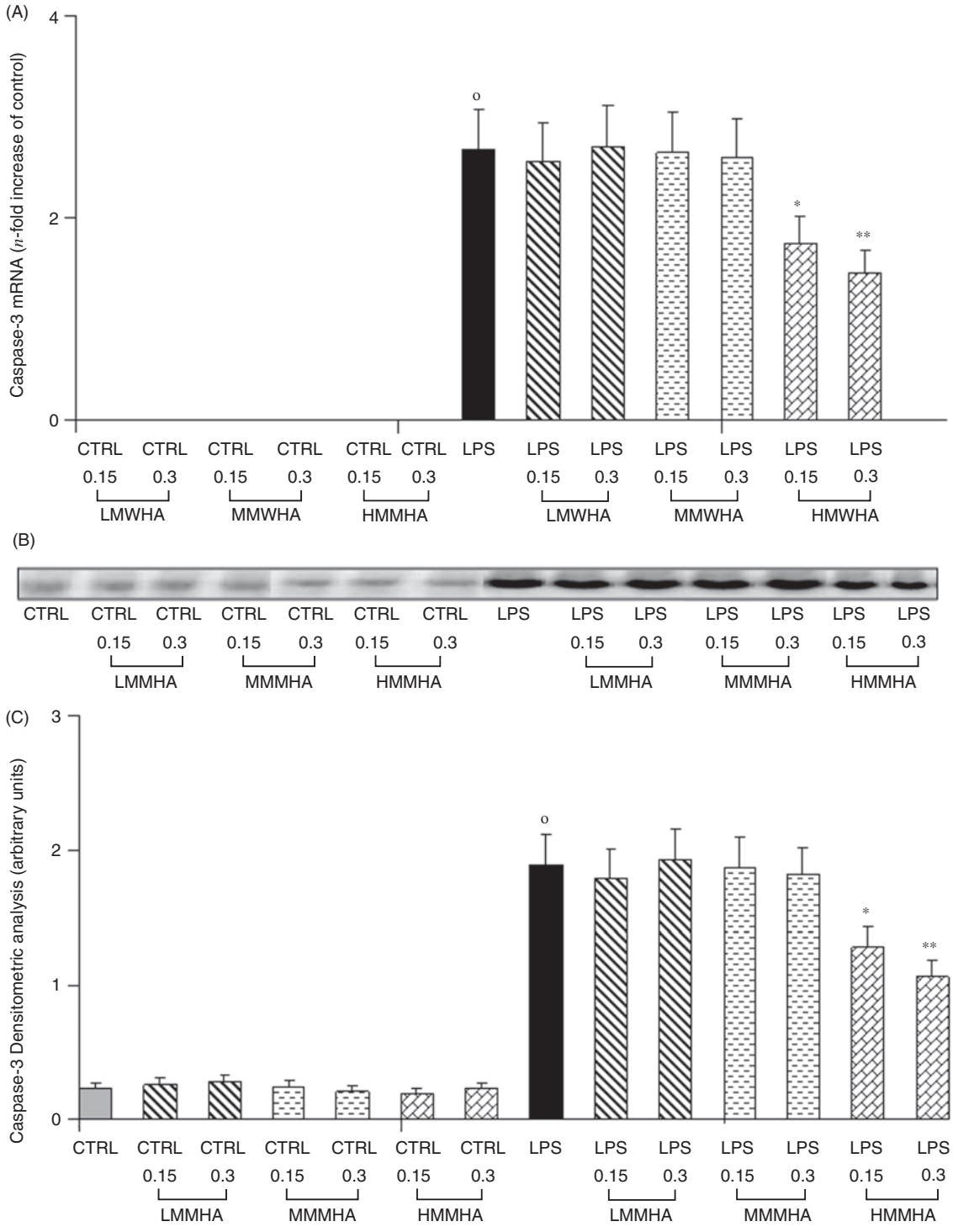

Fig. 6. Effect of hyaluronan treatment at different molecular mass on chondrocyte caspase-3mRNA expression (A) and related protein production (B,C) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for the caspase-3 protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P < 0.001$ versus control; $^{*}P < 0.005$, and $^{**}P < 0.001$ versus LPS.

iNOS, MMPs and caspases.[32] The activation of all these factors contributes to cell death and tissue disruption.

The two main pathways for apoptosis are known as the extrinsic and the intrinsic or mitochondria-dependent pathway; both pathways are important. The intrinsic apoptotic pathway follows a prescribed sequence of events that centre on the mitochondria. Consequently, mitochondria play a central role in the regulation

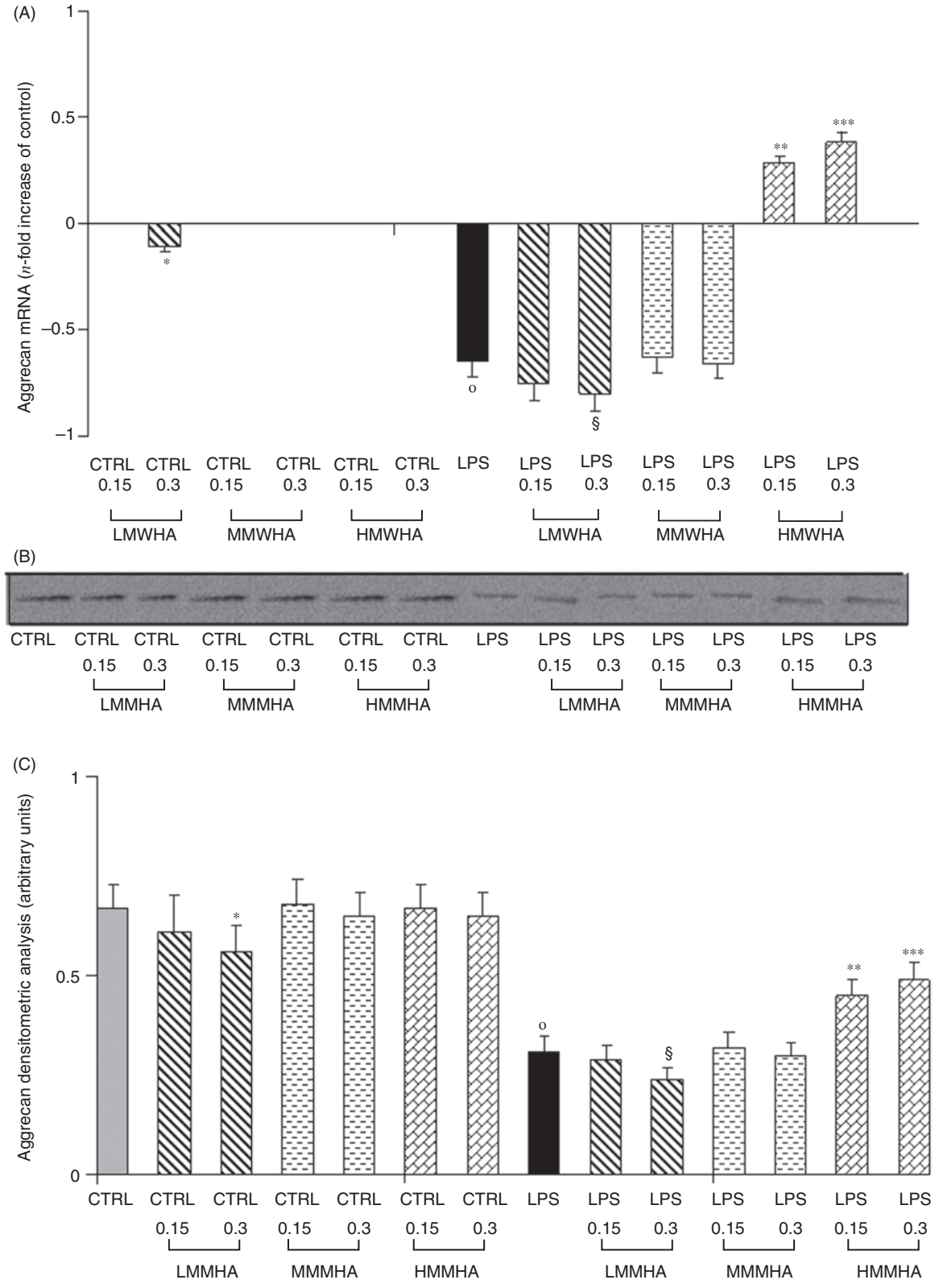

Fig. 7. Effect of hyaluronan treatment at different molecular mass on chondrocyte aggrecan mRNA expression (A) and related protein production (B,C) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for the aggrecan protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.001$ versus control; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ versus LPS; $^{\S}P<0.05$ versus LPS.

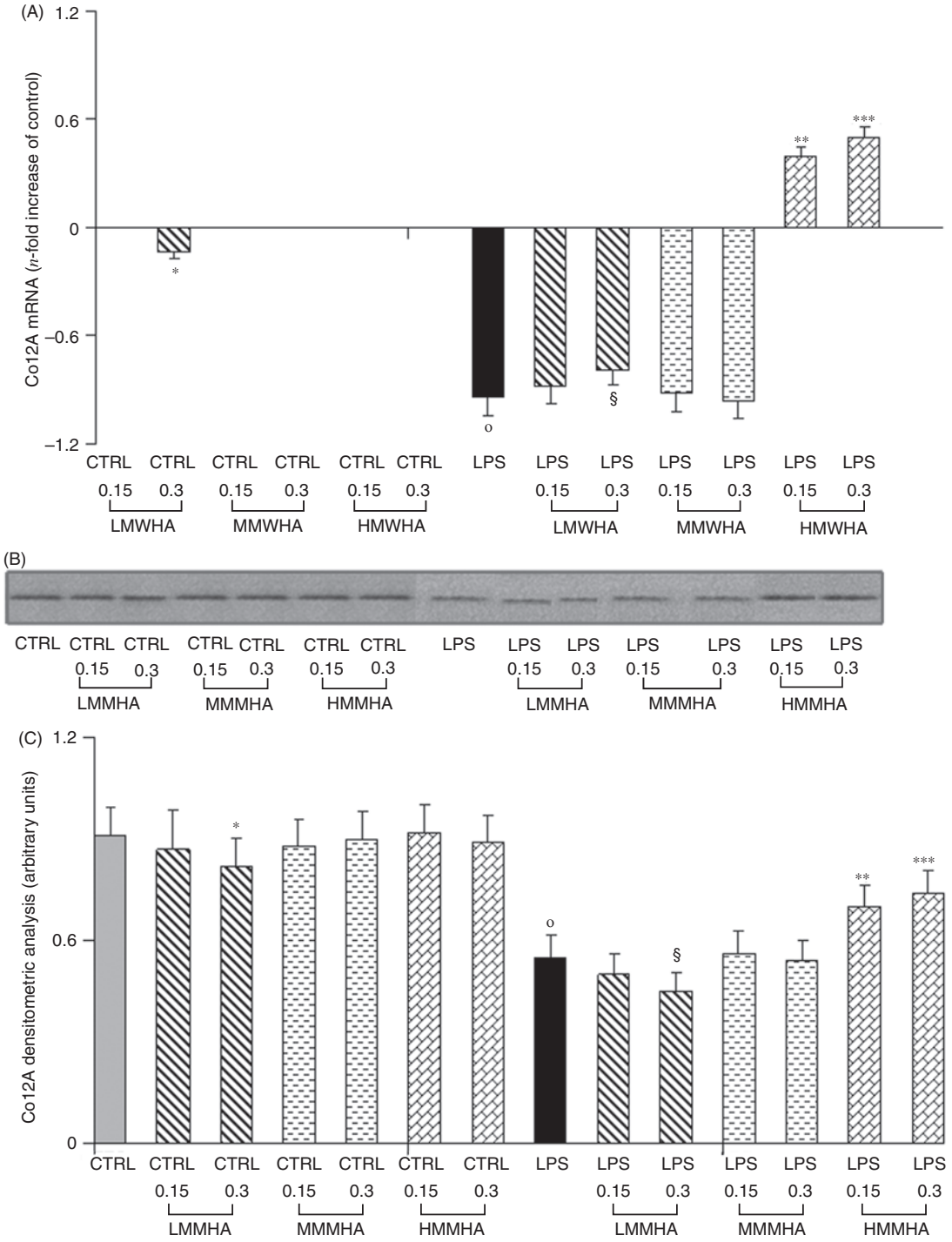

Fig. 8. Effect of hyaluronan treatment at different molecular mass on chondrocyte Col2A mRNA expression (A) and related protein production (B,C) after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as the *n*-fold increase with respect to the control (A) and as both densitometric analysis (C) and Western blot analysis (B) for the Col2A protein levels. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.001$ versus control; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ versus LPS; $^{\S}P<0.05$ versus LPS.

of apoptosis.[33] Mitochondrial pro-apoptotic proteins, such as cytochrome c, second mitochondria-derived activator of caspases/direct IAP-binding protein with low PI (Smac/DIABLO), and apoptosis inducing factor (AIF) are confined to the inter-membrane space and are liberated into the cytosol upon multiple apoptotic stimuli.[34] Cytochrome c collaborates with adaptor protein Apaf-1 to activate initiator caspase-9 in the apoptosome.

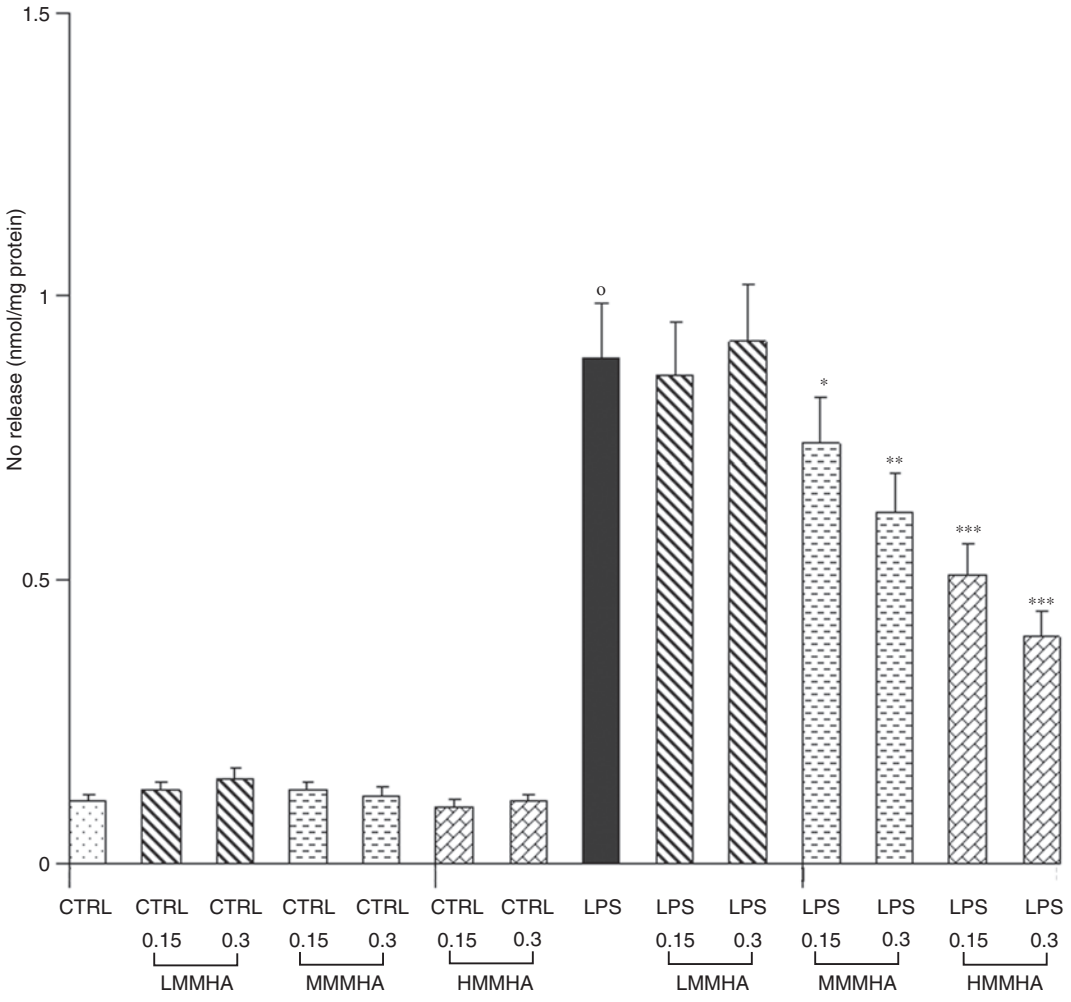

Fig. 9. Effect of hyaluronan treatment at different molecular mass on chondrocyte NO release in normal cells or after LPS stimulation. Values are the mean ± SD of seven experiments and are expressed as nmol/mg protein. LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.001$ versus control; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus LPS.

Smac/DIABLO facilitates caspase activity by binding to and antagonizing inhibitor of apoptosis (IAP) proteins. Apoptosis inducing factor translocates to the nucleus where it induces caspase-independent DNA fragmentation and chromatin condensation with consequent cell death.[35]

The interaction of cells with the surrounding extracellular matrix is fundamental in many physiological and pathological mechanisms. Proteoglycans may influence cell behaviour through binding events mediated by their glycosaminoglycan (GAG) chains. The specificity of protein–GAG interactions is governed by the ionic attractions of sulphate and carboxylate groups of GAGs with the basic amino acid residues on the protein as well as the optimal structural fit of the GAG chain into the protein binding site.[36] The binding affinity of the interaction depends on the ability of the oligosaccharide sequence to provide optimal charge and surface with the protein.[36]

We previously reported that hyaluronan produced by fibroblasts after TGF-1β stimulation was able to reduce free radical damage in fibroblast cultures exposed to $FeSO_4$ plus ascorbate.[19] The action of hyaluronan, especially at high molecular mass, was also able to inhibit NF-κB and executioner caspase activation.[19] We hypothesized that the inhibition of NF-κB DNA binding to the nucleus may be the consequence of hyaluronan reduced ROS production in the fibroblasts. As ROS are able to activate these pathways,[37] it is likely that hyaluronan treatment reduced the activation of NF-κB by preventing the oxidative burst. We also hypothesized that the same line of reasoning could be extended to apoptosis activation; direct inhibition of NF-κB cannot be excluded, however. In order to study this effect, we employed an LPS-induced inflammation in chondrocyte cultures in which there was no immediate and direct production of ROS, such as $HO^{\bullet}$, H_2O_2 or $O_2^{-\bullet}$.[38] However, LPS produced a marked increase in NO

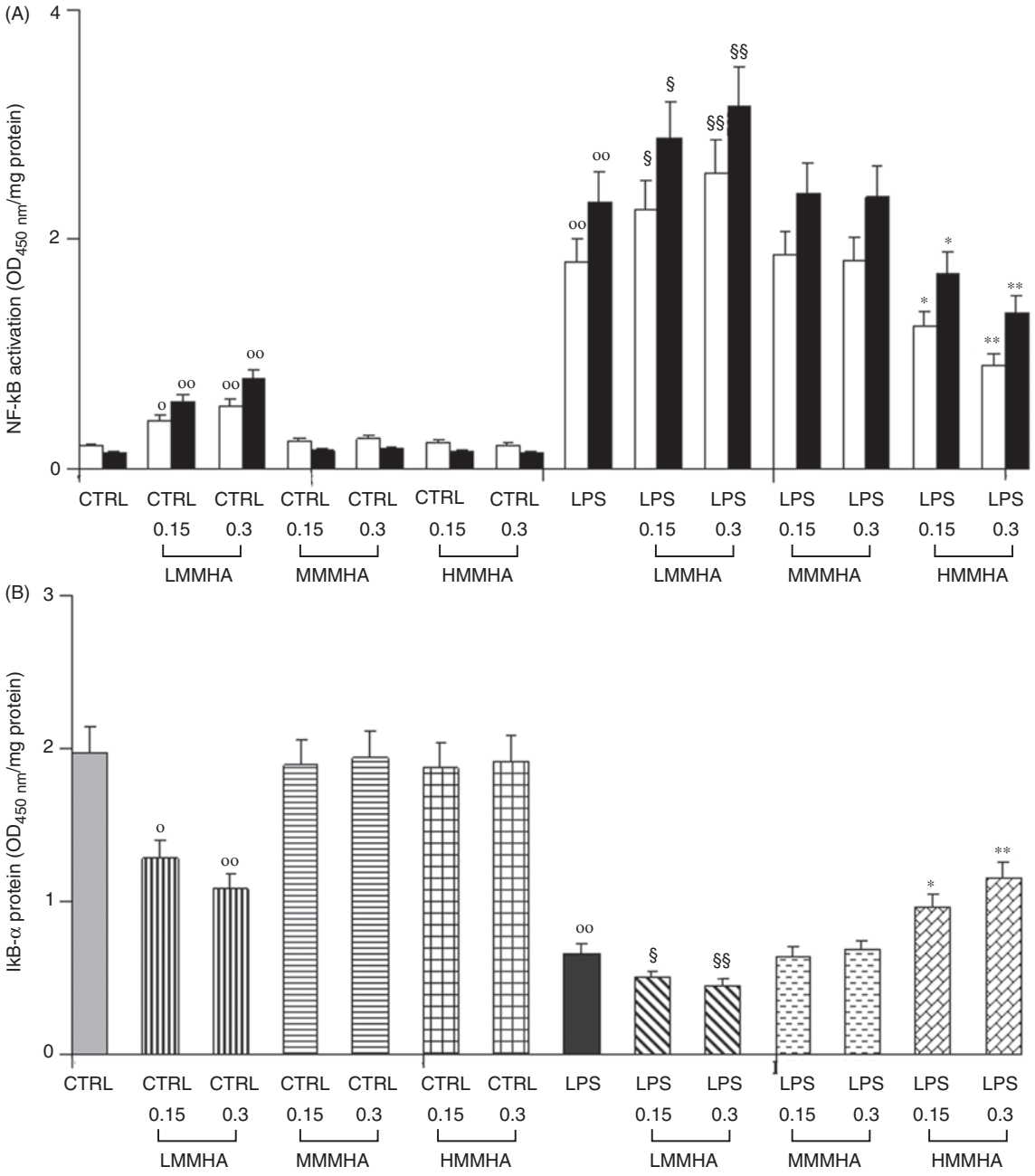

Fig. 10. Effect of hyaluronan treatment at different molecular mass on chondrocyte NF-κB p50/65 transcription factor DNA binding activity (A) and IκBα protein degradation (B) after LPS stimulation In (A), white bars represent the p50 subunit, black bars represent the p65 subunit. Values are the mean ± SD of seven experiments and are expressed as optical density at λ 450 nm/mg protein of nuclear extract (A) and as optical density measured at λ 450 nm/mg protein (B). LMMHA, hyaluronan at low molecular mass; MMMHA, hyaluronan at medium molecular mass; HMMHA, hyaluronan at high molecular mass; $^{\circ}P<0.005$ and $^{\circ\circ}P<0.001$ versus control; $^{*}P<0.01$, and $^{**}P<0.001$ versus LPS; $^{\S}P<0.05$ and $^{\S\S}P<0.01$ versus LPS.

levels through iNOS activation and this, in turn, may also stimulate ROS production.[39]

This study suggests that hyaluronan may have different effects in relation to its molecular mass. In fact, the data obtained show that the size of this polymer was able to modulate inflammation and apoptosis differently in unstimulated or LPS-stimulated normal murine chondrocytes. The medium molecular mass hyaluronan had no effect in all considered parameters except a slight reduction in NO levels. In particular, medium molecular mass hyaluronan reduced NO levels without any effect on iNOS mRNA expression and protein production. This paradox may be justified by the fact that medium molecular mass hyaluronan possesses a free radical scavenger activity and, consequently, may also have directly bound NO with a consequent reduction in

NO concentrations. These findings suggest that hyaluronan via its carboxylic group interactions may interfere with the mechanism of NF-κB activation that, in turn, primes inflammatory cytokines and NO production. When all these mechanisms were heavily stimulated, apoptosis occurred. This could explain the lack of effect on caspase activation by low molecular mass hyaluronan in LPS-unstimulated chondrocytes. Previous studies reported that certain chemokines, including pro-inflammatory cytokines, require interactions with GAGs for their *in vivo* functioning.[40,41] This interaction is thought to play a role in chemokine sequestration and subsequent presentation to the receptor expressed on the leukocyte cell surface.[40,41] It has been suggested that the inhibition of the interaction between pro-inflammatory cytokines such as IFN-γ and membrane-associated GAGs may provide a mechanism for inducing clinically useful immunosuppression.[42,43] In this context, it is conceivable that since NF-κB is able to induce pro-inflammatory cytokine production, and may itself be induced by pro-inflammatory cytokines,[44] high molecular mass hyaluronan could inhibit pro-inflammatory cytokines that, in turn, inhibit NF-κB activation, whilst the interaction of low molecular mass hyaluronan with pro-inflammatory cytokines produced exactly the opposite effect. Otherwise, hyaluronan interaction may directly involve either pro-inflammatory cytokines or NF-κB or both. Therefore, the positive modulatory effect exerted by high molecular mass hyaluronan on all the parameters considered may be due to its efficiency to bind protein structures thereby exerting inhibitory activity, in contrast with low molecular mass hyaluronan which instead had a stimulatory effect by acting as a bridge junction between these proteins and the cell surface. The low molecular mass hyaluronan-induced inflammatory effects could also be due to the activation of a different pathway that finally converge to activate NF-κB.[20] Medium molecular mass hyaluronan, since its structure was unable to mask completely these proteins and to act as bridge between proteins and the cell surface, is not able to inhibit or to stimulate inflammation. We suggest that the number of interaction sites available in the hyaluronan structures may play the key role in the hyaluronan modulatory activity during inflammation.

Conclusions

These data confirm the multifactorial role played by hyaluronan and suggest, in particular, that hyaluronan may differentially modulate inflammation during different pathologies, depending on its degree of polymerization.

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