

# Regulation of renal proximal tubular epithelial cell hyaluronan generation: Implications for diabetic nephropathy

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## Regulation of renal proximal tubular epithelial cell hyaluronan generation: Implications for diabetic nephropathy.

**Background.** Proximal tubular cells (PTCs) contribute to pathological changes in the renal interstitium by the generation of cytokines and alterations in the composition of the extracellular matrix. Hyaluronan (HA) is a ubiquitous connective tissue polysaccharide that regulates cell function and tissue remodeling. In the current study, we investigated the regulation of HA generation by PTCs.

**Methods.** Primary cultures of human PTCs were grown to confluence and stimulated under serum-free conditions with either interleukin-1 (IL-1) or 25 mmol/L D-glucose. Alterations in HA generation were detected by enzyme-linked immunosorbent assay, and alterations in HA synthase gene expression were examined by reverse transcription-polymerase chain reaction. Subsequently, the mechanisms of IL-1 $\beta$  and glucose-induced alterations in HA were examined utilizing HK-2 cells.

**Results.** Stimulation of human PTCs (HPTCs) with either IL-1 $\beta$  or 25 mmol/L D-glucose led to a significant increase in the HA concentration in the culture supernatant. In contrast, stimulation of HPTCs with transforming growth factor- $\beta$ 1, basic fibroblast growth factor, or platelet-derived growth factor-AB did not stimulate HA production. The addition of IL-1 $\beta$  or 25 mmol/L D-glucose also increased HA generation in HK-2 cells and was associated with the induction of HAS2 mRNA. HAS3 mRNA was constitutively expressed and was not influenced by the addition of either stimulus. HAS1 mRNA expression was not detected in either unstimulated or stimulated cells. Inhibition of gene transcription or protein synthesis abolished HA production in response to either IL-1 $\beta$  or glucose. Inhibition of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation either by sulindac or by the proteasome inhibitor (PSI) abrogated both IL-1 $\beta$  and glucose-mediated alteration in HA synthesis.

**Conclusion.** This study demonstrates, to our knowledge for the first time, that increased HA synthesis in response to either IL-1 $\beta$  or elevated 25 mmol/L D-glucose is associated with NF- $\kappa$ B-activated transcription of HAS2.

Renal failure is a common complication of diabetes mellitus that occurs in approximately 30% of patients with insulin-dependent diabetes mellitus (IDDM) and 10 to 20% of patients with non-insulin-dependent diabetes (NIDDM) [1]. Epidemiological evidence suggests that the complications of diabetes are related to poor glycemic control [2, 3]. Since only 30% of diabetic patients develop nephropathy, hyperglycemia alone is insufficient to initiate pathological changes that are thus likely to be multifactorial in origin. The aim of our work was to identify factors that in combination with hyperglycemia may contribute to both the initiation and progression of diabetic renal disease. As it is now evident that a progressive decline in renal function in diabetes is closely correlated with the degree of renal interstitial fibrosis [4, 5], we have focused on those mechanisms that may induce changes in the interstitium, particularly the mechanisms by which the proximal tubular epithelial cell (PTC) may be involved in their initiation.

It is now clear that cell function can be modified by the composition of the extracellular matrix. Hyaluronan (HA) is a ubiquitous connective tissue polysaccharide that in vivo is present as a high molecular mass component of the extracellular matrix. In addition to its role in providing cellular support, it is now known that under normal circumstances, HA regulates cell-cell adhesion, migration, proliferation, differentiation, and the movement of interstitial fluid and macromolecules [reviewed in 6]. As a result, it is likely to be an important contributor to and a regulator of wound healing and tissue remodeling. A role for HA in the pathogenesis of diabetic nephropathy is suggested by the observations that increased HA production is involved in initiation of glomerular hypercellularity in the streptozotocin model of diabetes [7].

To date, many studies have focused on the role of profibrotic cytokines such as transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (FGF2) in the pathogenesis of both the glomerular and interstitial changes associated with diabetic nephropathy. Recent studies have also im-

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plicated macrophage infiltration in the pathogenesis of diabetic nephropathy. In vivo studies on streptozotocin-induced diabetic rats have demonstrated prominent macrophage infiltration [8, 9]. In addition, studies of renal biopsies taken from patients with NIDDM mellitus have suggested that macrophages and their products are involved in the initiation of the pathological changes of human diabetic nephropathy [10]. Recent studies have shown that stimulation of fibroblasts with both of the macrophage-derived proinflammatory cytokines, interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) increased HA generation. These observations suggest that macrophage-derived proinflammatory cytokines that act on resident cells may be involved in the pathogenesis of renal fibrosis in diabetes.

In our current study, we examined the regulation of PTC HA generation in response to profibrotic and proinflammatory cytokines and elevated glucose concentration. The data demonstrate that both elevated glucose and IL-1 $\beta$  increase HA generation by PTCs. This was associated with an induction of HA synthase (HAS) 2 gene expression and was mediated by activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) by both stimuli. In contrast, the profibrotic cytokines TGF- $\beta$ 1, PDGF, and FGF2 did not increase HA synthesis.

## METHODS

### Cell culture

Initial experiments were performed using primary cultures of human renal proximal tubular cells (HPTCs) isolated and characterized as previously described [11]. Cells were grown to confluence and were serum deprived for 48 hours. Growth arrested cells were subsequently stimulated with recombinant cytokines (TGF- $\beta$ 1, PDGF-AB, FGF2, or IL-1 $\beta$ ) at concentrations ranging from 0 to 100 ng/mL under serum-free conditions. Supernatant samples were collected up to 24 hours after the addition of the cytokines for quantitation of HA. The effect of elevated glucose concentration was determined by stimulation with 25 mmol/L D-glucose and collection of supernatant samples at time points up to 96 hours. Control experiments were performed by the addition of 25 mmol/L L-glucose as the osmolar control or 5 mmol/L D-glucose as a "normoglycemic" control.

Subsequently, the mechanisms involved in HA generation were studied utilizing HK-2 cells [human renal proximal tubular epithelial cells immortalized by transduction with human papilloma virus (HPV) 16 E6/E7 genes] [12]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Biological Industries Ltd., Cumbernauld, UK), glutamine (Life Technologies Ltd.), insulin, transferrin, hydrocortisone, and sodium selenite (Sigma, Poole, UK). Fresh

growth medium was added to cells every three to four days until confluent. Cells were grown to confluence and growth arrested in serum-free medium for 48 hours, and all experiments were subsequently performed under serum-free conditions. Cells were stimulated with the same range of cytokines and glucose concentrations as for HPTC. In addition, the effect of costimulation of HK-2 cells with IL-1 $\beta$  and elevated glucose concentration was examined by the addition of IL-1 $\beta$  (1 ng/mL) together with 25 mmol/L D-glucose and collection of supernatant samples for HA quantitation over the subsequent 96 hours.

### Alteration in HA synthesis

**Determination of HA concentration.** In all experiments, the HA concentration in the cell culture supernatant was determined by an enzyme-linked binding protein assay (HA "Chugai" quantitative test kit; Chugai Diagnostics, TCS Biologicals Ltd., Botolph-Claydon, UK). Interassay precision ranged from 6.2 to 7.0% (CV), and intra-assay precision ranged from 3.6 to 4.7% (CV). The assay is sensitive to 10 ng/mL, with no cross-reactivity with other glycosaminoglycan compounds. To ensure that equal numbers of cells were studied in all experiments, total cell protein was determined using a micro bicinchoninic acid kit (Pierce-Wariner, Chester, UK). There were no differences in cell protein in any experiments. All experiments were performed using the same volume of medium; therefore, the HA results are expressed as an absolute concentration of HA.

**Alteration in HAS mRNA expression.** Recently, three distinct cDNA sequences encoding HASs (HAS1 to HAS3) have been cloned. The effect of various stimuli on the expression of HAS1, HAS2, and HAS3 mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) as previously described [11]. Following stimulation of growth-arrested monolayers of HK-2 cells with either IL-1 $\beta$  or 25 mmol/L D-glucose, total cellular RNA was extracted for RT. Following PCR, 1/10th of the PCR reaction from both test and control ( $\alpha$ -actin) product was mixed and separated by flat bed electrophoresis in 3% wt/vol NuSieve GTG agarose gels (Flowgen Instruments Ltd., Sittingbourne, UK), stained with ethidium bromide (Sigma), and photographed. The negatives were scanned using a densitometer (Model 620 video densitometer; Bio-Rad Laboratories, Ltd., Hercules, CA, USA), and the density of the bands was compared with those of the housekeeping gene  $\alpha$ -actin.

**Oligonucleotide synthesis.** The sequences of the amplification primers are in Table 1.

To confirm that HA generation was dependent on induction of HAS transcription, HK-2 cells were stimulated with IL-1 $\beta$  (1 ng/mL) or 25 mmol/L D-glucose in the presence of increasing doses of either actinomycin-D

**Table 1.** Amplification primer sequences

Gene	Primers	Product size
<i>HAS1</i>	5'-AGCAGGACGCGCCCAAGCCCACTC-3' 5'-TCCTCCGCCTCCACCTCCCGATAG-3'	562 bp
<i>HAS2</i>	5'-GCAGGCGGAAGAAGGGACAAC-3' 5'-TCAGGCGGATGCACAGTAAGGA-3'	313 bp
<i>HAS3</i>	5'-AGTGCAGCTTCGGGGATGA-3' 5'-TGATGGTAGCAATGGCAAAGAT-3'	453 bp
$\alpha$ -Actin	5'-CCTTCTGGGCATGGAGTCCT-3' 5'-GGAGCAATGATCTTGATCTT-3'	204 bp

(0 to 250 ng/mL) to inhibit transcription or cycloheximide (0 to 5  $\mu$ g/mL) to inhibit mRNA translation. The toxicity of actinomycin D and cycloheximide was assessed using cellular adenosine 5'-triphosphate (ATP) measurements as previously described [13], and only doses that did not lead to a reduction in total cellular ATP or to detachment of the cell monolayer were used. We have previously demonstrated the ability of actinomycin D and cycloheximide to inhibit gene transcription or mRNA translation, respectively, over these dose ranges [11].

### Mechanism of altered HA synthesis

**Effect of glucose on IL-1 $\beta$  generation.** To determine whether the glucose-induced alterations in HA generation were mediated by alterations in IL-1 $\beta$  production, supernatant samples from HK-2 cells stimulated with 25 mmol/L D-glucose or 5 mmol/L D-glucose were collected and IL-1 $\beta$  assayed by enzyme-linked immunosorbent assay (ELISA; interleukin-1 $\beta$  [(h)IL-1 $\beta$ ] human ELISA system; Amersham, Pharmacia Biotech U.K. Ltd., Little Chalfont, UK).

**Potential signaling pathways involved in HA generation.** Previous studies examining the potential signaling mechanisms by which glucose may mediate its effects have demonstrated that glucose-induced alterations in cell function may be mediated by numerous pathways, including increased polyol pathway activity as well as activation of kinase pathways such as protein kinase C, extracellular signal-regulated protein kinase/mitogen-activated protein (ERK/MAP) kinase, or p38 MAP kinase. Similarly, IL-1 $\beta$  activation of PKC, p38 MAP kinase, and ERK/MAP kinase cascades have also been demonstrated. The involvement of each of these pathways in either IL-1 $\beta$  or 25 mmol/L D-glucose-mediated alterations in HA were investigated by the use of specific inhibitors.

HK-2 cells were stimulated with IL-1 $\beta$  (1 ng/mL) alone or IL-1 $\beta$  in the presence of the PKC inhibitor RO-31-8220 (0 to 100 nmol/L), MAP kinase inhibitor PD 98059 (0 to 4  $\mu$ mol/L), or the p38 MAP kinase inhibitor SB203580 (0 to 1200 nmol/L). Supernatant samples were collected at 24-hour poststimulation for HA analysis. In

parallel experiments, cells were stimulated with 25 mmol/L D-glucose alone or in the presence of increasing doses of either the polyol pathway inhibitor sorbinil (0 to 100  $\mu$ mol/L; Pfizer Inc., Groton, CT, USA) or the inhibitors of PKC, p38 MAP kinase, or ERK/MAP kinase. Toxicity of each inhibitor was assessed by measurement of cellular ATP. None of the inhibitors were toxic over the dose range used. Supernatant samples were collected at 96-hours poststimulation for HA analysis. All inhibitors were obtained from Calbiochem (Nottingham, UK).

**Involvement of NF- $\kappa$ B.** The dependence of HA generation of NF- $\kappa$ B activation was determined following its inhibition by the use of Sulindac. Growth-arrested HK-2 cells were stimulated with either IL-1 $\beta$  (10 ng/mL) or 25 mmol/L D-glucose for up to 96 hours in the presence of increasing doses of either sulindac (0 to 10 mmol/L) or indomethacin (0 to 5  $\mu$ mol/L; both obtained from Calbiochem). Total cellular RNA was isolated, and *HAS2* mRNA expression was examined by RT-PCR. The concentration of HA in the culture supernatant was determined as described previously in this article.

Phosphorylation of I- $\kappa$ B is followed by proteasome-mediated degradation, which results in the release of active NF- $\kappa$ B [14]. The role of NF- $\kappa$ B was further examined by the use of the proteasome inhibitor (PSI; Calbiochem). Growth-arrested HK-2 cells were stimulated with either IL-1 $\beta$  (10 ng/mL) or 25 mmol/L D-glucose in the presence of an increasing dose of PSI (0 to 60  $\mu$ mol/L). Supernatant samples were collected at time points of up to 96 hours for determination of HA concentration by ELISA.

### Statistical analysis

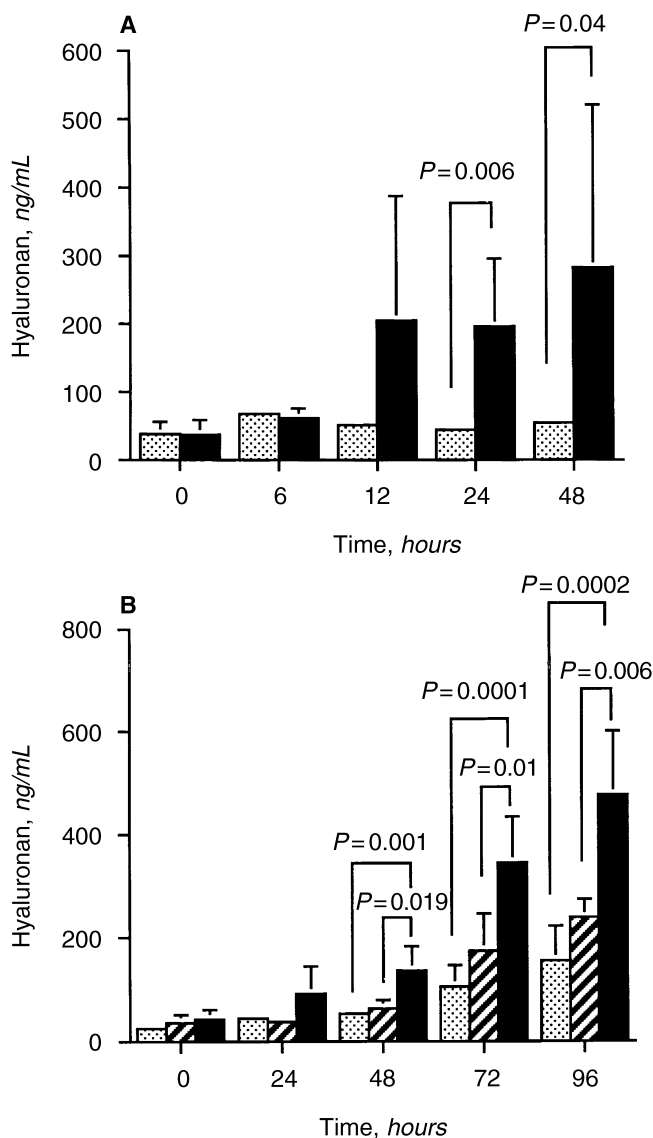
Statistical analysis was performed using the unpaired Student *t* test, with a value of  $P < 0.05$  considered to represent a significant difference. The data are presented as means  $\pm$  SD of *N* experiments. For each individual experiment, the mean of duplicate determinations was calculated.

## RESULTS

### Alteration in HA production

Stimulation of HPTCs with IL-1 $\beta$  (1 ng/mL) led to a time-dependent increase in the HA concentration in the culture supernatant. This became significant 24 hours after the addition of IL-1 $\beta$ , at which time it represented a fivefold increase over control values ( $P = 0.006$ ; Fig. 1A).

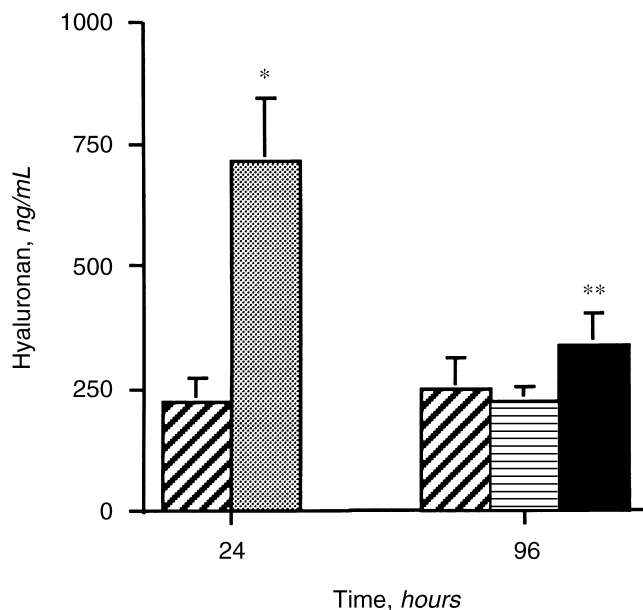
Stimulation of HPTCs with 25 mmol/L D-glucose also led to an increase in the concentration of HA in the culture supernatant (Fig. 1B). In contrast to IL-1 $\beta$ , the effect of elevated glucose was significant 48 hours after its addition and represented only a 2.5-fold increase in the amount of HA over the control ( $P = 0.0016$ ). This increase was unrelated to alterations in osmolarity since the addition of 25 mmol/L L-glucose did not affect the production of HA.



**Fig. 1. Stimulation of hyaluronan (HA) production in human proximal tubular cells (HPTCs).** The effect of interleukin-1 $\beta$  (IL-1 $\beta$ ) on HA generation (A) was determined by the addition of IL-1 $\beta$  (1 ng/mL) to confluent growth-arrested HPTCs under serum-free conditions (■). In control experiments, serum-free 5 mmol/L D-glucose in the absence of IL-1 $\beta$  was added (▨). Time-dependent effects of elevated glucose concentration (B) on HA generation were determined by the addition of either 25 mmol/L D-glucose (■), 25 mmol/L L-glucose (▩), or 5 mmol/L D-glucose (▧) to growth-arrested HPTCs under serum-free conditions. Supernatant samples were collected at time points up to 48 hours for quantitation of HA by ELISA. Data represent mean  $\pm$  SD of six individual experiments.

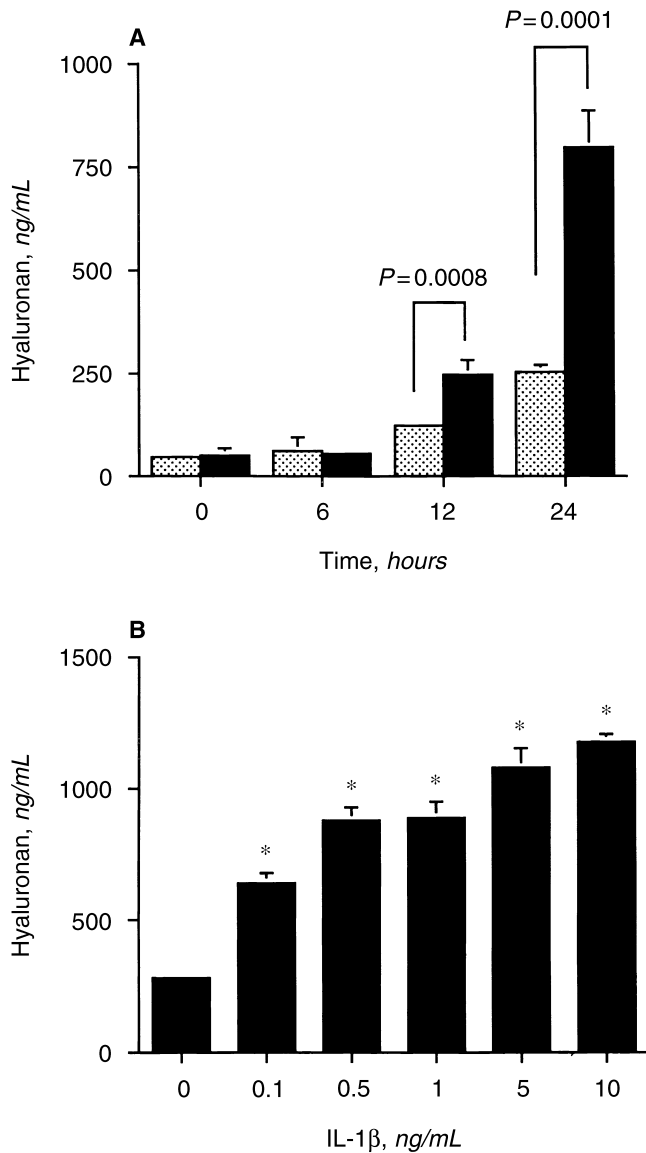
In contrast, stimulation of HPTC with either TGF- $\beta$ 1 (0 to 100 ng/mL), FGF2 (0 to 100 ng/mL), or PDGF-AB (0 to 100 ng/mL) had no effect on HA production, as assessed by ELISA (data not shown).

Experiments using HK-2 cells confirmed that their HA production was influenced in the same way as HPTCs. The addition of either IL-1 $\beta$  or 25 mmol/L D-glucose



**Fig. 2. Stimulation of HA in HK-2 cells.** IL-1 $\beta$  (1 ng/mL; ■), 25 mol/L D-glucose (■), 25 mmol/L L-glucose (▩), or 5 mmol/L D-glucose (▨) were added to confluent HK-2 cells under serum-free conditions. Supernatant samples were collected at time points up to 96 hours for quantitation of HA by ELISA. Data represents mean  $\pm$  SD of 20 individual experiments. \* $P$  < 0.005 IL-1 $\beta$  vs. control; \*\* $P$  < 0.005 25 mmol/L D-glucose vs. 5 mmol/L D-glucose and 25 mmol/L L-glucose.

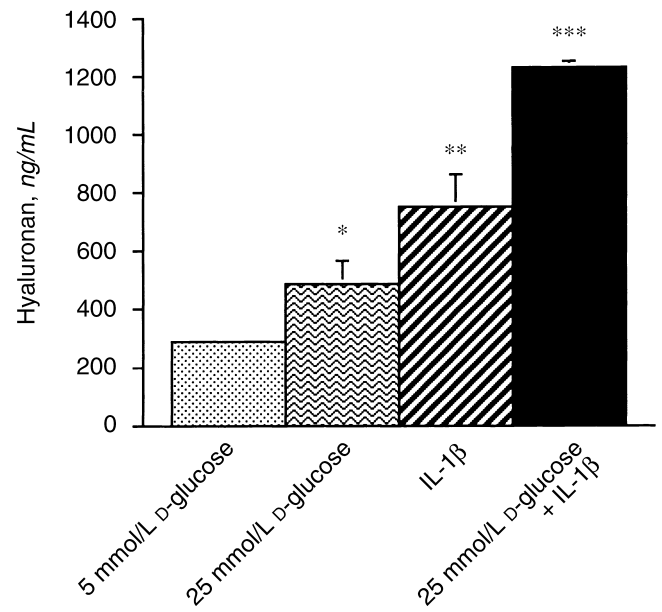
led to a time-dependent increasing HA generation (Fig. 2). The effect of IL-1 $\beta$  was significant at 12 hours (Fig. 3A) and was dose dependent (Fig. 3B). The effect of 25 mmol/L D-glucose was only apparent 96 hours following its addition and was much smaller in its magnitude as compared with that seen following the addition of IL-1 $\beta$  (Fig. 2). Costimulation of cells with IL-1 $\beta$  and 25 mmol/L D-glucose led to an additive rather than an augmented increase in HA generation (Fig. 4). HAS mRNA expression in HK-2 cells was examined by RT-PCR. A small time-dependent increase in HAS2 mRNA expression was seen in unstimulated cells under serum-free conditions. IL-1 $\beta$  stimulation led to induction of HAS2 mRNA above that seen in the control cells (Fig. 5A). HAS3 mRNA was constitutively expressed by HK-2 cells and was not influenced by the addition of IL-1 $\beta$  (Fig. 5B). In contrast, HAS1 mRNA expression was not detected in either unstimulated or stimulated cells. HAS1 mRNA has previously been demonstrated to be expressed in lymphoid tissue; splenic homogenate was used as a positive control to demonstrate the efficacy of the oligonucleotide primers. RT-PCR of RNA extracted from splenic tissue demonstrated constitutive expression of a HAS1 mRNA transcript of the expected size. An increase in HA following addition of IL-1 $\beta$  was dependent of both de novo HAS gene transcription and protein synthesis, as inhibition of de novo gene transcription by actinomycin-D (Fig. 6A) or protein synthesis/



**Fig. 3. Time- and dose-dependent effects of IL-1 $\beta$ .** Time-dependent effects of IL-1 $\beta$  were examined (A) by the addition of 1 ng/mL of IL-1 $\beta$  (■) or 5 mmol/L D-glucose (▨) to serum-deprived HK-2 cells under serum-free conditions. Supernatant samples were collected at time points up to 24 hours for HA determination. Dose-dependent effects (B) were determined by the addition of 0 to 10 ng/mL of IL-1 $\beta$  to growth-arrested HK-2 cells for 24 hours. The supernatant HA concentration was determined by ELISA. Data presented are the means  $\pm$  SD of four individual experiments. \* $P < 0.05$ .

mRNA translation by the addition of cycloheximide (Fig. 6B) completely abrogated HA generation in response to the addition of IL-1 $\beta$ .

The addition of 25 mmol/L D-glucose had similar effects on the expression of HAS, with an induction of HAS 2 mRNA expression, and no effect on constitutive HAS3 mRNA (Fig. 7). The induction of HAS2 mRNA was apparent 48 hours after the addition of 25 mmol/L D-glucose. The addition of 25 mmol/L D-glucose did not



**Fig. 4. Costimulation with IL-1 $\beta$  and 25 mmol/L D-glucose leads to an additive increase in HA generation.** Five mmol/L D-glucose, 25 mmol/L D-glucose, IL-1 $\beta$  (1 ng/mL), or both IL-1 $\beta$  (1 ng/mL) and 25 mmol/L D-glucose were added to confluent monolayers of HK-2 cells for 96 hours. Supernatant samples were subsequently collected and HA quantitated by ELISA. Data are the means  $\pm$  SD of eight individual experiments. \* $P = 0.005$  vs. 5 mmol/L D-glucose; \*\* $P = 0.001$  vs. 5 mmol/L D-glucose; \*\*\* $P = 0.0001$  vs. both 25 mmol/L D-glucose and IL-1 $\beta$ .

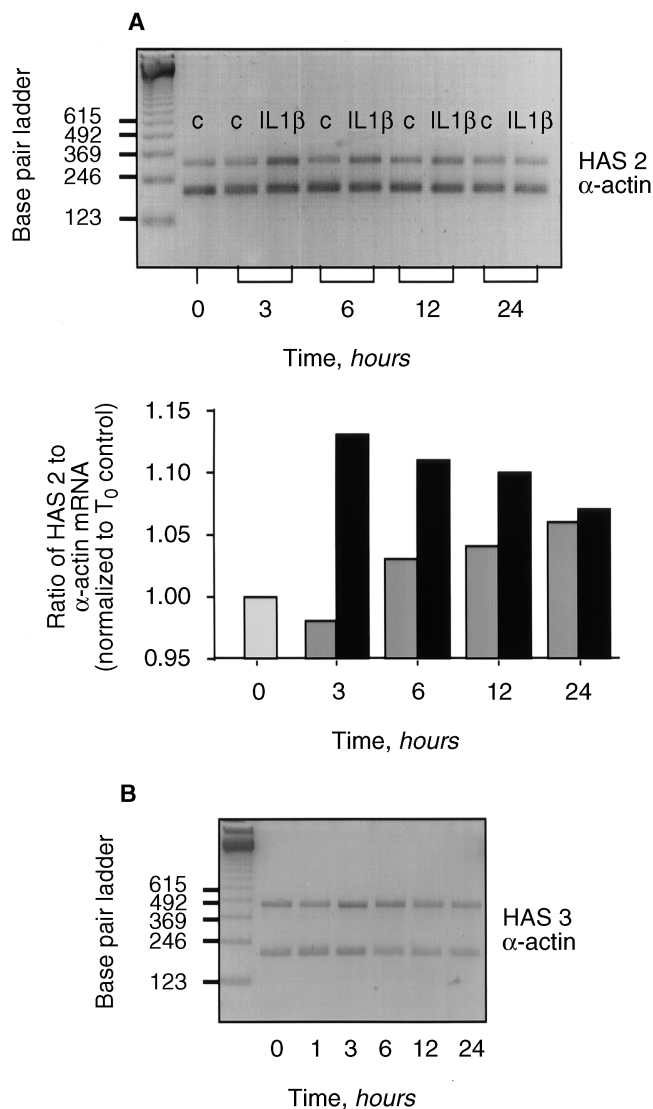
induce the expression of HAS1 mRNA, which again remained undetectable by RT-PCR in RNA extracted from HK-2 cells. The stimulation of HA synthesis following the addition of 25 mmol/L D-glucose was abrogated by the addition of either actinomycin D (Fig. 8A) or cycloheximide (Fig. 8B), demonstrating it to be dependent on both stimulation of gene transcription and protein synthesis.

#### Mechanism of HA stimulation

The temporal differences in HA generation following the addition of either IL-1 $\beta$  or 25 mmol/L D-glucose raised the possibility that the effects of glucose may be the result of 25 mmol/L D-glucose stimulation of IL-1 $\beta$ . The addition of 25 mmol/L D-glucose to HK-2 cells, however, did not increase IL-1 $\beta$  production at time points of up to 96 hours, as assessed by ELISA of supernatant samples collected from 25 mmol/L D-glucose stimulated HPTC or HK-2 cells (data not shown).

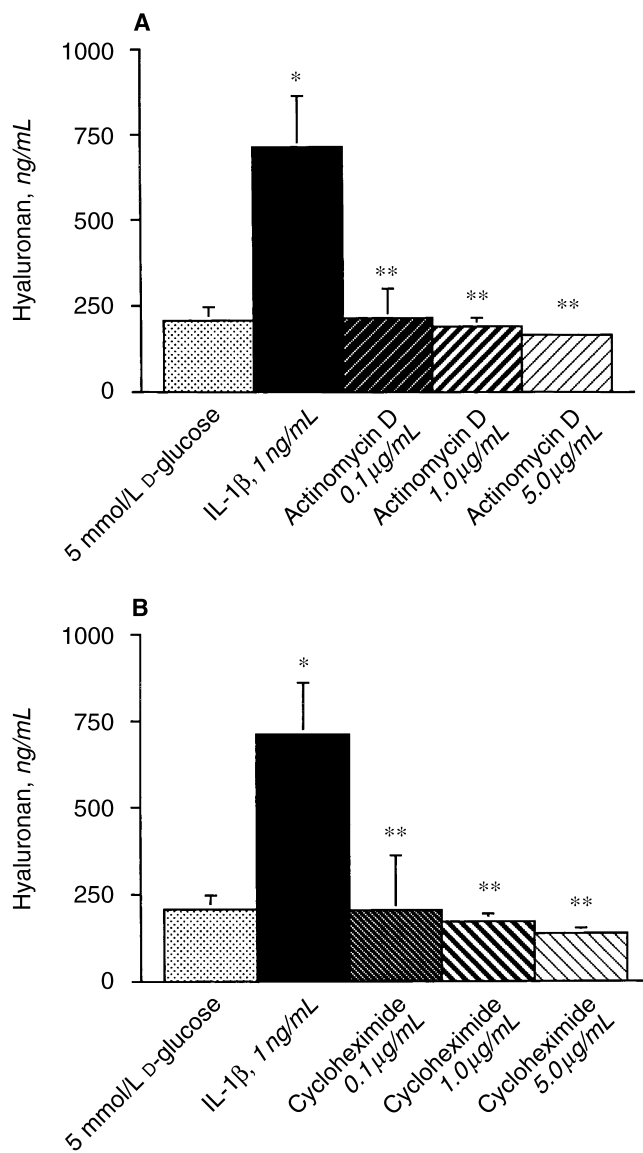
#### Signaling

Stimulation of HA by IL-1 $\beta$  was unaffected by the inhibition of PKC, p38 MAP kinase, or ERK/MAP kinase (data not shown). Similarly, inhibition of PKC, p38 MAP kinase, or ERK/MAP kinase did not influence glucose-mediated alterations in HA synthesis (data not



**Fig. 5. Hyaluronan synthase (HAS) expression: Effect of IL-1 $\beta$  stimulation on HAS2 mRNA.** Following the addition of 1 ng/mL IL-1 $\beta$  (■) to confluent monolayers of HK-2 cells under serum-free conditions, total mRNA was extracted, and HAS2 mRNA (A) expression was examined by RT-PCR at time points of up to 24 hours. In control experiments, 5 mmol/L D-glucose was added in the absence of IL-1 $\beta$  (□). PCR products were separated by electrophoresis on a 3% agarose gel. Densitometric ratios of HAS2 to  $\alpha$ -actin mRNA are shown with the results normalized to the ratio for the unstimulated control cells at time 0 (■). (B) Similarly, following the addition of 1 ng/mL IL-1 $\beta$  to confluent monolayers of HK-2 cells under serum-free conditions and extraction of total cellular RNA, HAS3 mRNA expression was examined by RT-PCR. Scanning densitometry confirmed the lack of induction of HAS3 following the addition of IL-1 $\beta$ . One representative experiment of four individual experiments is shown.

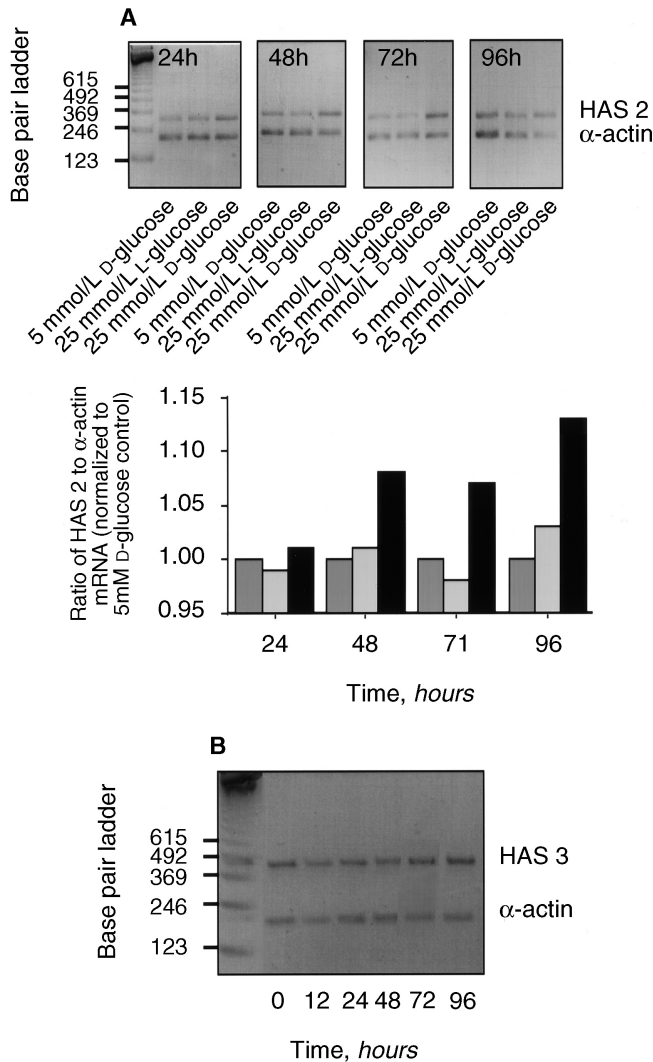
shown). Furthermore, the induction of HA by 25 mmol/L D-glucose in HK-2 cells was not affected by the use of the polyol pathway inhibitor sorbinil (data not shown) over a dose range that we have previously demonstrated to be effective in inhibiting sorbitol generation following the addition of 25 mmol/L D-glucose to PTCs [15].



**Fig. 6. IL-1 $\beta$ -stimulated HA production is inhibited by inhibition of de novo gene transcription (A) and by inhibition of protein synthesis (B).** Actinomycin-D (0 to 5  $\mu$ g/mL; A) or cycloheximide (0 to 5  $\mu$ g/mL; B) were added to growth-arrested HK-2 cells. Following incubation for one hour, the cells were washed with phosphate-buffered saline (PBS), pH 7.3, and subsequently stimulated with 1 ng/mL IL-1 $\beta$ . Supernatant samples were collected after 24 hours for determination of HA. Data presented are the means  $\pm$  SD of eight individual experiments. \* $P$  < 0.005 IL-1 $\beta$  vs. control; \*\* $P$  < 0.05 actinomycin-D vs. IL-1 $\beta$ .

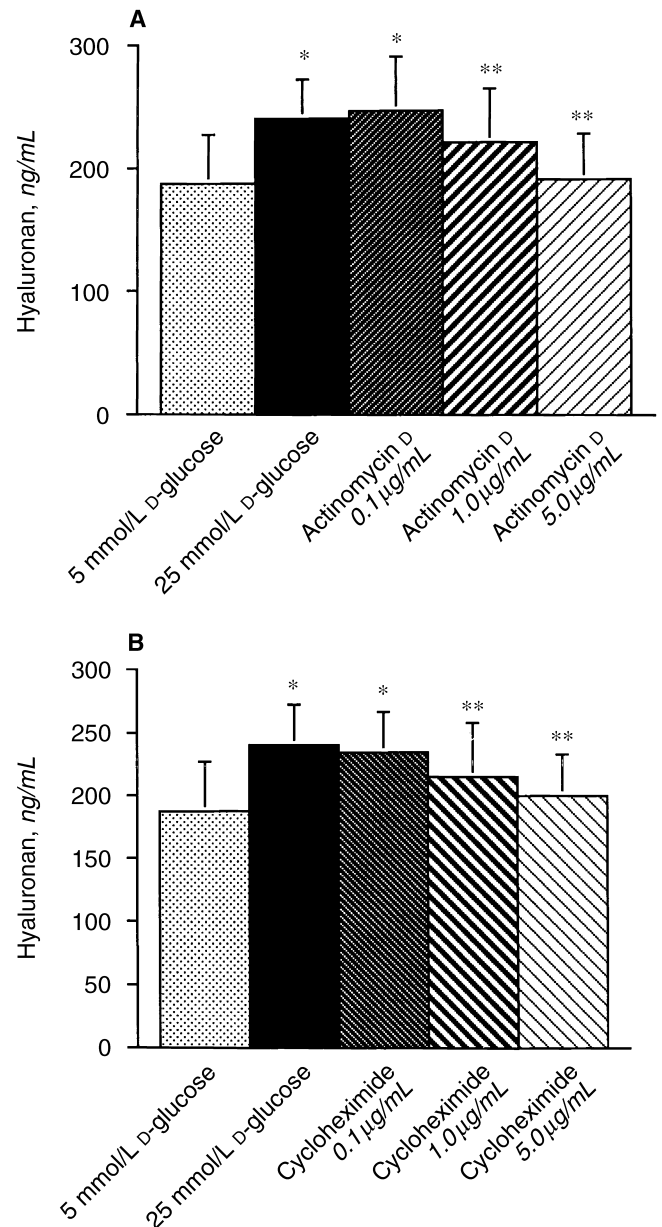
### Involvement of NF- $\kappa$ B

Both IL-1 $\beta$  and 25 mmol/L D-glucose-induced alterations in HA generation by HK-2 cells were abrogated by the addition of sulindac in a dose-dependent manner (Fig. 9). In addition, stimulation of cells with either IL-1 $\beta$  or 25 mmol/L D-glucose in the presence of sulindac abrogated the induction of HAS2 mRNA seen following the addition of either stimulus in the absence of sulindac



**Fig. 7. HAS expression: Effect of addition of 25 mmol/L D-glucose.** HAS2 mRNA expression following the addition of 25 mmol/L D-glucose (■) was examined in confluent monolayers of HK-2 cells under serum-free conditions (A). Total cellular RNA was isolated at time points up to 96 hours for HAS analysis by RT-PCR. In control experiments, either 5 mmol/L D-glucose (□) or 25 mmol/L L-glucose (□) were added to the cells. PCR products were separated by electrophoresis on a 3% agarose gel. Densitometric ratios of HAS2 to  $\alpha$ -actin mRNA are shown with the results normalized to the ratio for the unstimulated control cells to which 5 mmol/L D-glucose was added. Similarly, following the addition of 25 mmol/L D-glucose to confluent monolayers of HK-2 cells under serum-free conditions, and extraction of total cellular RNA, HAS3 mRNA expression was examined by RT-PCR (B). Scanning densitometry confirmed the lack of induction of HAS3 following the addition of 25 mmol/L D-glucose. One representative experiment of four individual experiments is shown.

(Fig. 10). In contrast, indomethacin had no effect on the generation of HA following the addition of either stimulus (Fig. 11). Furthermore, the stimulation of cells with either IL-1 $\beta$  or 25 mmol/L D-glucose in the presence of indomethacin did not influence HAS2 mRNA expression (Fig. 10). The inhibition of NF- $\kappa$ B activation by the addition of the proteasome inhibitor PSI also abrogated

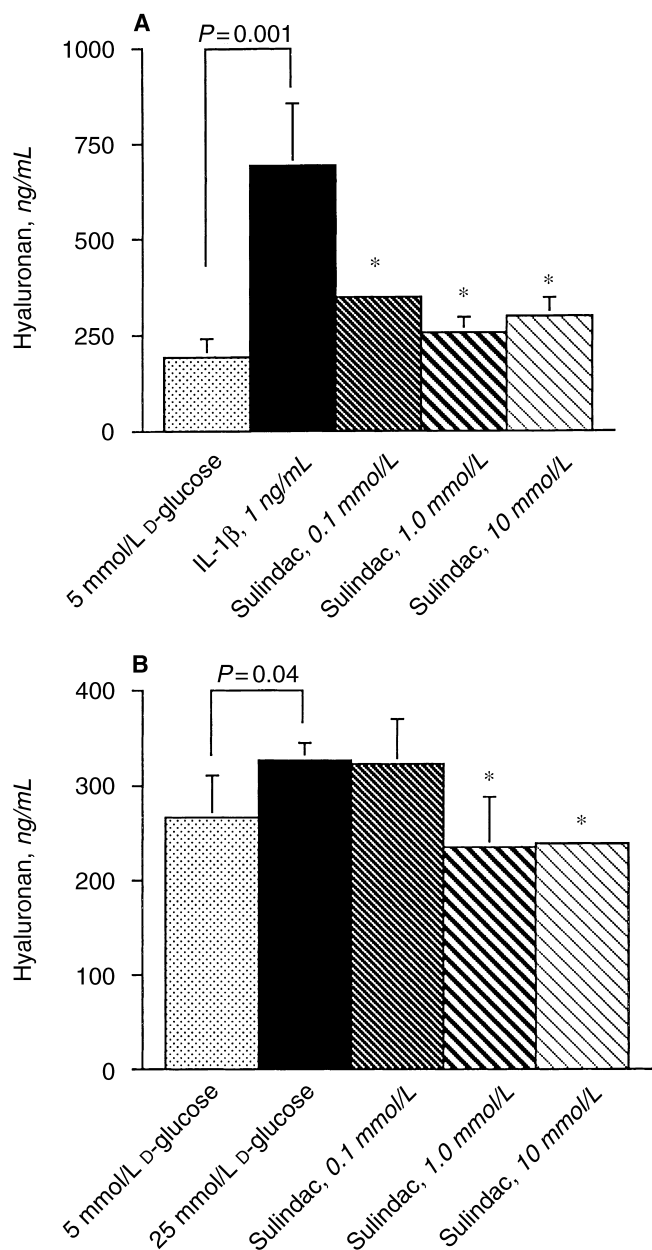


**Fig. 8. Twenty-five mmol/L D-glucose-stimulated HA production is inhibited by inhibition of de novo gene transcription (A) and by inhibition of protein synthesis (B).** Actinomycin-D (0 to 5  $\mu$ g/mL; A) or cycloheximide (0 to 5  $\mu$ g/mL; B) were added to growth-arrested HK-2 cells. Following incubation for one hour, the cells were washed with PBS, pH 7.3, and subsequently stimulated with 25 mmol/L D-glucose. Supernatant samples were collected after 96 hours for determination of HA. Data presented are the means  $\pm$  SD of eight individual experiments, \* $P$  < 0.005 25 mmol/L D-glucose vs. control; \*\* $P$  < 0.05 actinomycin-D vs. 25 mmol/L D-glucose.

both the IL-1 $\beta$  and 25 mmol/L D-glucose-stimulated increase in HA (Fig. 12).

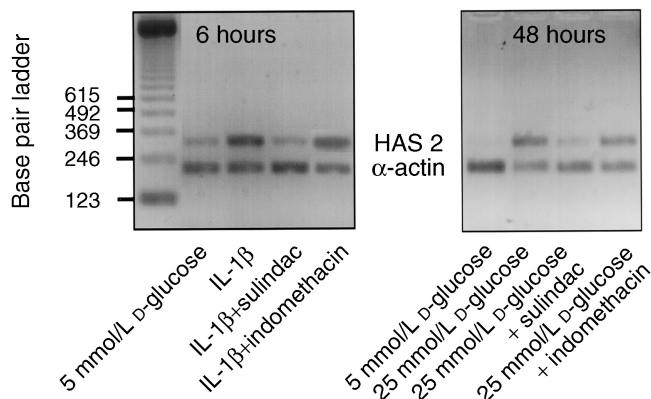
**DISCUSSION**

Hyaluronan is a water-soluble glycosaminoglycan that is a key constituent of the pericellular matrix and has



**Fig. 9. Sulindac inhibits IL-1 $\beta$  (A) and 25 mmol/L D-glucose (B) induced HA production.** Confluent monolayers of HK-2 cells were stimulated with either 1 ng/mL of IL-1 $\beta$  (A) or 25 mmol/L D-glucose in the presence of increasing doses of sulindac. Supernatant samples were collected 24 hours after the addition of IL-1 $\beta$  and 96 hours following the addition of 25 mmol/L D-glucose for determination of HA concentration. Data represent mean  $\pm$  SD of four individual experiments. \* $P < 0.005$  inhibitor vs. IL-1 $\beta$  (A) or 25 mmol/L D-glucose (B).

important structural functions in the extracellular matrix of all tissues. It has only recently been recognized that it can perform more subtle functions than just serving as a structural scaffold. HA may function as a cellular signaling molecule, following either binding to its cell surface receptors (CD44 and RHAMM) or following internalization via CD-44-mediated endocytosis [16].

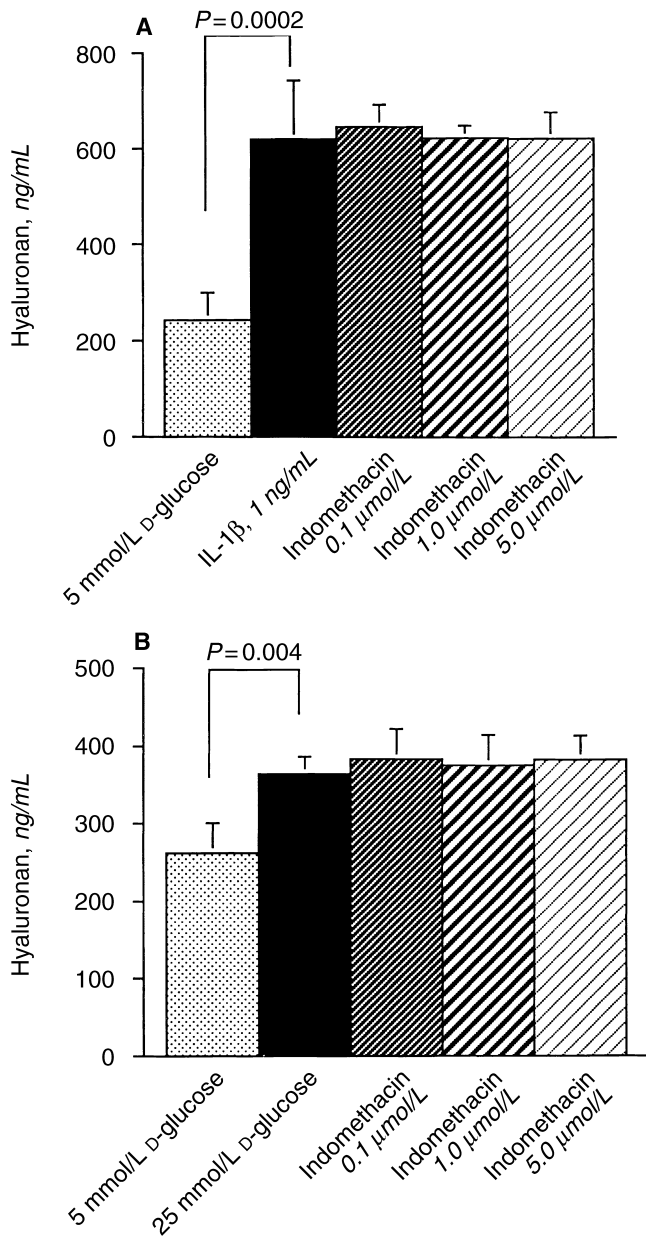


**Fig. 10. Sulindac abrogates HAS2 mRNA induction following the addition of either IL-1 or 25 mmol/L D-glucose.** Confluent growth-arrested HK-2 cells were stimulated with 1 ng/mL of IL-1 $\beta$  or 25 mmol/L D-glucose in the presence or absence of either Sulindac (10 mmol/L) or Indomethacin (5  $\mu$ mol/L). Total cellular RNA was extracted six hours after IL-1 $\beta$  stimulation or 48 hours after the addition of 25 mmol/L D-glucose. HAS2 mRNA expression was subsequently examined by RT-PCR. PCR products were separated by electrophoresis on a 3% agarose gel. One representative experiment of four is shown.

HA has therefore been implicated in a number of biological processes, including embryonic development, tumor growth, chronic inflammation, and wound healing [6]. The data presented in the current manuscript demonstrate that HA generation by proximal tubular epithelial cells was stimulated by both 25 mmol/L D-glucose and the proinflammatory cytokine IL-1 $\beta$ . In addition, the data demonstrate that stimulation of HA synthesis is dependent on the transcriptional activation of HAS2. The different “kinetics” of HA stimulation following the addition of either stimulus, however, suggest that the mechanisms by which they mediate these effects may differ. In contrast, the addition of the profibrotic cytokines TGF- $\beta$ 1, FGF2, or PDGF did not affect HA synthesis. We have also demonstrated the constitutive expression of HAS3 mRNA and inducible expression of HAS2 mRNA in PTCs. HAS2 has been shown to be an inducible transcript in other cell types. Specifically, stimulation of mesothelial cells with PDGF was associated with increased HA synthesis and induction of HAS2 mRNA [17]. Together with the lack of stimulation of HA following the addition of PDGF in our study, this suggests that it is likely that the regulation of HAS genes is cell-type specific.

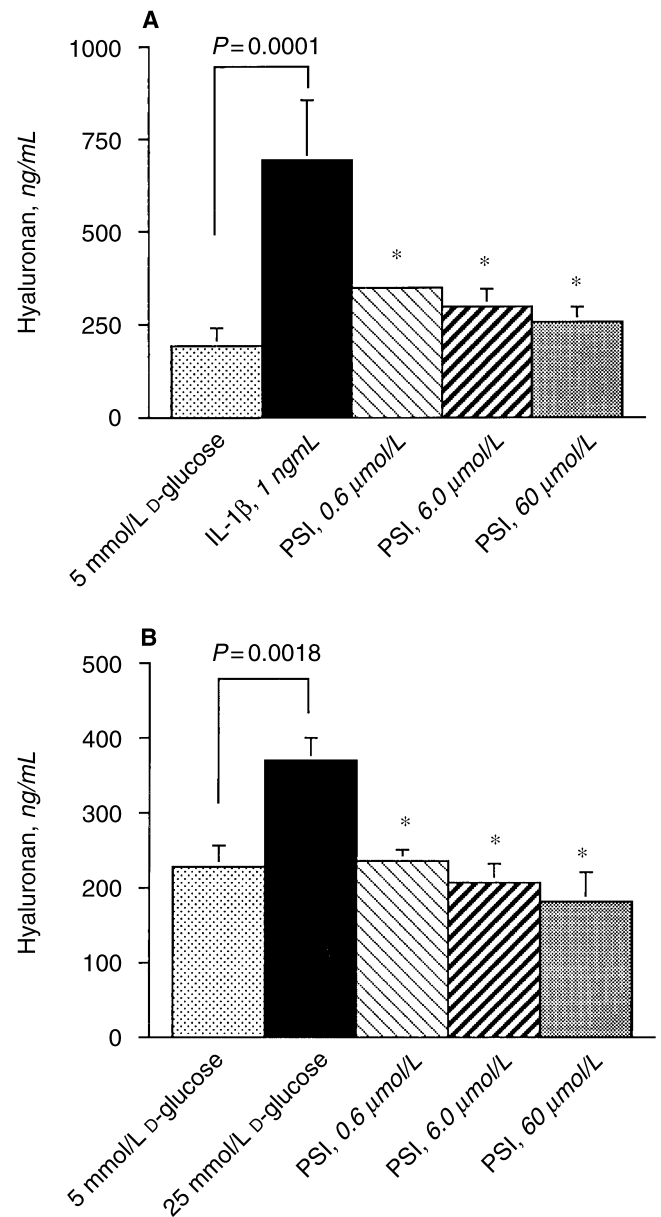
Previous studies have implicated alterations in HA synthesis in the pathogenesis of the glomerular abnormalities associated with diabetic nephropathy [7]. In these studies, alterations in HA were related to an alteration in prostaglandin turnover. Although the effect of both IL-1 $\beta$  and 25 mmol/L D-glucose was inhibited by sulindac, it is unlikely that this is related to an alteration in prostaglandin turnover, as neither IL-1 $\beta$  nor 25 mmol/L D-glucose-induced alterations in HA synthesis were inhibited by





**Fig. 11. Indomethacin does not influence HA generation in response to the addition of either IL-1 $\beta$  (A) or 25 mmol/L D-glucose (B).** Confluent monolayers of HK-2 cells were stimulated with either 1 ng/mL of IL-1 $\beta$  (A) or 25 mmol/L D-glucose in the presence of increasing doses of indomethacin. Supernatant samples were collected 24 hours after the addition of IL-1 $\beta$  and 96 hours following addition of 25 mmol/L D-glucose for determination of HA concentration. Data represent mean  $\pm$  SD of four individual experiments.

indomethacin. Sulindac is a nonsteroidal anti-inflammatory agent that is related to indomethacin. In addition to its anti-inflammatory properties, it is known to have growth-inhibitory effects. Its anti-inflammatory and growth inhibitory effects are related to its ability to inhibit prostaglandin synthesis by inhibition of cyclooxygenases. Recent studies have also demonstrate that part of its



**Fig. 12. Inhibition of HA generation in response to addition of either IL-1 $\beta$  (A) or 25 mmol/L D-glucose by the proteasome inhibitor (PSI).** Confluent monolayers of HK-2 cells were stimulated with either IL-1 $\beta$  (A) or 25 mmol/L D-glucose in the presence of increasing doses of PSI (0 to 60  $\mu$ mol/L). Control experiments were performed by stimulation with 5 mmol/L D-glucose in the absence of PSI. Supernatant samples were collected 24 hours after the addition of IL-1 $\beta$  and 96 hours following the addition of 25 mmol/L D-glucose for determination of the HA concentration. Data represent mean  $\pm$  SD of four individual experiments. \* $P$  < 0.005 for PSI vs. either IL-1 $\beta$  (A) or 25 mmol/L D-glucose (B) stimulated.

effects are mediated by inhibition of an I- $\kappa$ B kinase (IKK $\beta$ ), which is required to activate NF- $\kappa$ B [18]. Indomethacin does not inhibit IKK $\beta$  and, therefore, does not influence NF- $\kappa$ B activation. That the effect of sulindac in our system was unrelated to inhibition of cyclooxygenase was therefore confirmed by the use of indomethacin.

These findings together with the inhibition HA generation, in response to either IL-1 or 25 mmol/L D-glucose by the use of the proteasome inhibitor PSI, suggest that the induction of HA in our system is associated with activation of NF- $\kappa$ B.

The NF- $\kappa$ B pathway regulates the cellular response to a variety of stimuli, including cytokines, bacterial and viral infection, and activation of cellular stress pathways. To our knowledge, this is the first demonstration that the activation of NF- $\kappa$ B is associated with the synthesis of HA by transcriptional activation of HAS2, although transcriptional activation of HAS1 by TNF- $\alpha$  has recently been demonstrated in a myofibroblast cell line [19]. Activation of NF- $\kappa$ B by elevated concentration of glucose has recently been demonstrated in vascular smooth muscle cells [20]. To our knowledge, however, our data are the first to demonstrate the activation of NF- $\kappa$ B following the addition of 25 mmol/L D-glucose in the kidney.

Although it is known that increased synthesis of HA may be a feature of diabetic glomerulosclerosis, to date little is known regarding alterations in HA in the renal interstitium. It is interesting to speculate on the functional significance of increased HA production. Recent studies suggest that high molecular weight HA and low molecular weight HA oligosaccharides present different signals to cells. In general, high molecular weight HA represents the normal homeostatic state, whereas the generation of low molecular weight HA fragments signals a disruption of the normal homeostatic environment. Several workers have reported that HA oligosaccharides may stimulate gene expression and protein synthesis of chemokines [21] and interstitial collagens [22]. In contrast, high molecular weight HA oligosaccharides inhibit the "bioactivity" of TGF- $\beta$  and stimulate the secretion of tissue inhibitors of metalloproteinases [23, 24]. The key to understanding the implication of the increased HA that we have described therefore may be the analysis of the molecular weight of the HA generated.

Macrophage influx previously has been implicated in the pathogenesis of diabetic nephropathy, both in animal models [8, 9] and in human disease [10]. This suggests that the generation of macrophage-derived cytokines such as IL-1 $\beta$ , in combination with the effect of elevated glucose concentrations, may act synergistically to influence the pathogenesis of diabetic nephropathy. We have previously demonstrated that the combined effects of glucose and IL-1 $\beta$  may modulate the profibrotic potential of the PTCs in diabetic nephropathy, by an increase in the production of the profibrotic cytokine TGF- $\beta$ 1 [25]. More recently, augmented proinflammatory cytokine-induced NF- $\kappa$ B activation by elevated glucose concentrations has been demonstrated in vascular smooth muscle cells [20]. In the current study, we demonstrate that stimulation with the combination of IL-1 $\beta$  and 25 mmol/L D-

glucose had an additive effect on the production of HA, which further supports the concept that the interaction of hyperglycemia and macrophage-derived proinflammatory cytokines may augment renal injury in diabetes.

Gene induction by cellular stresses such as elevated glucose concentration may involve the interplay of multiple signaling pathways, including MAP kinases [MAP kinase, protein kinase C (PKC)] [26] implicated in the pathological complications of diabetes [27, 28], and the p38 pathway [29]. Hypertonicity is also known to induce at least three MAP kinase cascades: the ERK pathway, the JNK pathway, and the p38 pathway [29–32]; however, as the effects of glucose in our study were not mimicked by the addition of 25 mmol/L L-glucose, it is unlikely that activation of NF- $\kappa$ B and subsequent induction of HAS2 relates to hyperosmolarity. Similarly, activation of PKC [33], ERK/MAP kinase [34], and p38 MAP kinase [35–38] have all been implicated as mediators of the effects of macrophage derived cytokines. The data presented in here do not identify the signaling pathways by which either elevated glucose or IL-1 $\beta$  mediate the activation of NF- $\kappa$ B and subsequent stimulation of HA synthesis in PTCs. However, we demonstrate that this process does not involve signaling pathways identified previously as mediators of the biological effects of either glucose or IL-1 $\beta$ .

In summary, we demonstrate, to our knowledge for the first time, that increased HA synthesis in response to either IL-1 $\beta$  or elevated 25 mmol/L D-glucose is associated with NF- $\kappa$ B activated transcription of HAS2. This may have implications for the pathogenesis of renal interstitial changes associated with diabetes mellitus. We also demonstrate that HAS3 is constitutively expressed and not is induced in these cells, although the role of constitutive HA synthesis in these cells remains to be determined. Numerous signaling pathways that might be expected to link these stimuli to activation of NF- $\kappa$ B have been excluded; therefore, identification of the signaling pathways that mediate these effects represent an important area for future research.

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## APPENDIX

Abbreviations used in this article are: ATP, adenosine 5'-triphosphate; ERK, extracellular signal-regulated protein kinase; FGF-2, basic fibroblast growth factor; HA, hyaluronan; HAS, hyaluronan synthases; HPTC, human proximal tubular cells; HPV, human papilloma virus;

IDDM, insulin dependent diabetes mellitus; IL-1 $\beta$ , interleukin-1 $\beta$ ; IKK $\beta$ , I- $\kappa$ B kinase; MAP, mitogen activated protein; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NIDDM, non-insulin dependent diabetes; PDGF, platelet-derived growth factor; PSI, proteasome inhibitor; PTC, proximal tubular cells; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

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