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IGF-1 stimulates synthesis of undersulfated proteoglycans and of hyaluronic acid by peritubular cells from immature rat testis

Bénédicte Thiébot, Lahcen Bichoualne, Monique Langris *, Pierre-Jacques Bonnamy, Pierre Barbey, Serge Carreau, Jean Bocquet

Laboratoire de Biochimie, EP CNRS 009, IRBA, Université de Caen, Esplanade de la Paix, F-14032 Caen Cedex, France

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Abstract

The exposure of confluent peritubular (PT) cells from immature rat testis to insulin-like growth factor-1 (IGF-1) induced a time and dose-dependent increase of [^{35}S]-sulfate and [^{3}H]-D-glucosamine incorporations in newly synthesized proteoglycans (PG). This increased content of PG was the result of an enhancement of PG synthesis rather than a decreased rate of degradation. IGF-1 had no effect on the molecular weight of synthesized PG nor on the nature and distribution of the constitutive glycosaminoglycan chains, both in medium and in cell layer. The stimulation of PG synthesis by IGF-1 appeared to be due, at least partially, to an increase of glycosylation processes. IGF-1 effect was mediated by the classical tyrosine kinase signalling process, since IGF-1 action on PG synthesis was abolished by genistein and tyrphostin A9, two well known tyrosine kinase inhibitors. The increase of PG synthesis was accompanied with an undersulfation of constitutive glycosaminoglycan (GAG) chains (chondroitin sulfate and heparan sulfate chains) since the [^{35}S]/[^{3}H] ratio was reduced by about 20–25% in presence of IGF-1. Although the mechanism of hyaluronic acid synthesis was completely different from those of other GAG, IGF-1 also dramatically enhanced its production by PT cells. © 1997 Elsevier Science B.V.

Keywords: Peritubular cell; Rat testis; IGF-1; Proteoglycan; Hyaluronic acid; Tyrosine kinase; Tyrosine kinase inhibitor

1. Introduction

In the testis, peritubular cells are a mesenchymal cell type that surround the seminiferous tubule adjacent to the basal surface of the Sertoli cells and these two cells are separated by a basement membrane. One important peritubular cell function is to provide structural support for the seminiferous tubule and help to maintain the proper cytoarchitecture of the epithelium [1]. In this view, both peritubular and Sertoli cells cooperate in the production of components of the extracellular matrix (ECM) and formation of the basement membrane [2]. This cooperation requires an exchange of information between the two cell types which may occur through soluble factors and through proteoglycan participation [3].

Proteoglycans (PG) are important constituents of the extracellular matrix (ECM); they are necessary for stable assembly of this matrix and functional cell–ECM interactions [4]. In addition to the extracel-

* Corresponding author. Fax: +33 (2) 31565320.

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lular matrix, one other major area in which the proteoglycans are widely distributed is the cell membrane. A growing body of evidence suggests that cell-associated proteoglycans and particularly proteoheparans, play an important role in the control of cell–cell and cell–matrix interactions acting upon tissue architecture and morphogenesis, cell adhesion and migration [5].

Soluble factors have been shown to potentially mediate regulatory interactions between peritubular cells and Sertoli cells. The non-mitogenic peptide P-Mod-S, secreted by peritubular cells modulates Sertoli cell functions [6]. Additional soluble factors that have been identified are growth factors. Testicular somatic and germinal cells may be the site of production and the target of several growth factors which are thought to have a local action by autocrine or paracrine mechanisms [7]. For example, rat Sertoli cells [8] were shown to secrete a peptide identical to IGF-1 and to possess specific membrane receptors for IGF-1 [9]. Moreover, Leydig cells and peritubular cells [8] from immature rats also secrete IGF-1. Thus, IGF-1 is ubiquitously produced in the testis.

IGF-1 has been reported to stimulate the PG synthesis in several cell types. IGF-1 effects on articular cartilage have received much attention and are now well documented. For example, the [^{35}S]-sulfate incorporation in aggrecan is stimulated by IGF-1 in chondrocyte cultures and the synthesis of large PG is maintained by IGF-1 [10].

Taking into account the importance of the PG in cell–cell and cell–ECM interactions, we have shown in a previous work that peritubular cells synthesize PG which are membrane-associated and also constituents of the ECM [11]. In the present work, we have examined the effects of IGF-1 on the synthesis of PG by testicular peritubular cells isolated from immature rats. The hydrodynamic size of the different populations of PG, the GAG chain lengths and the nature of these PG have been analyzed in presence of IGF-1.

IGF-1 signal transmission pathways are now well documented and it is known that IGF-1 acts through the tyrosine kinase activity of its receptor [12]. Accordingly, we have studied the putative involvement of this transduction system in the expression of the IGF-1 effects on PG synthesis, by using two tyrosine kinase inhibitors (genistein and tyrphostin).

2. Materials and methods

2.1. Materials

Sprague–Dawley rats were obtained from our own colony. Phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), HAM's F12 medium and trypsin (USP Grade) for cell culture were from Gibco-BRL (Cergy-Pontoise, France). Collagenase-dispase, fetal calf serum (FCS) and IGF-1 were from Boehringer-Mannheim (Meylan, France). Bovine pancreas deoxyribonuclease I (DNAse I), testicular hyaluronidase (type I-S), trypsin inhibitor, Triton X-100, chondroitinase ABC, *para*-nitrophenyl-β-D-xyloside (PNPX), *para*-nitrophenyl-β-D-galactoside (PNPG) and cycloheximide (CHX) were purchased from Sigma (Saint-Quentin-Fallavier, France). [^{35}S]-sulfate (39–59 TBq/mmol) and [^{3}H]-D-glucosamine hydrochloride (1.2 TBq/mmol) were purchased from NEN (Les Ulis, France). Genistein, daidzein and tyrphostins A1 and A9 were from Calbiochem and France-Biochem and *Streptomyces* hyaluronidase was from Calbiochem (Meudon, France). DEAE-Trisacryl was provided by IBF-Biotechnics (Villeneuve-la-Garenne, France). Superose 6 was obtained from Pharmacia (Saint-Quentin- en Yvelynes, France). Cationic nylon (Zeta-Probe) and the microfold dot blot apparatus were from Bio-Rad (Ivry-sur-Seine, France).

2.2. Cell cultures

Peritubular cells were isolated from the testes of 18–20 day-old rats by sequential enzymatic digestions at 32°C as previously described [13]. Briefly, decapsulated testes were treated with trypsin (1 mg/ml) and DNAse (0.02 mg/ml) in HAM F12/DMEM (1:1 v/v; H/D) containing bicarbonate (2.2 g/l) and antibiotics (50 IU/ml penicillin, 50 mg/ml streptomycin, 50 mg/ml kanamycine, 0.25 mg/ml fungizone) for 25 min at 32°C. After addition of soybean trypsin inhibitor (0.1 mg/ml), interstitial cells were discarded and seminiferous tubules were washed three times with H/D, then submitted to digestion by a mixture of collagenase–dispase (1 mg/ml) and testicular hyaluronidase (2 mg/ml) for 25 min at 32°C. After unit-gravity sedimentation (5 min), peritubular cells in the supernatant were recov-

ered by centrifugation ($800 \times g$ for 10 min) washed twice with H/D and resuspended in H/D containing 10% FCS; the cells were then plated onto 75 cm^2 plastic flasks at a density of 30 000 cells/cm^2. The cells were maintained in a humidified atmosphere containing 5% CO_2, at 32°C. Culture medium was changed every 2 days and peritubular cells were grown to confluence. Confluent cultures were trypsinized (0.25% trypsin in PBS plus 0.02% EDTA) and replated at a density of 30 000 cells/cm^2 in either 24 wells or 75 cm^2 plastic flasks depending upon the experiments. At confluence, cells were incubated in H/D without FCS, 24 h prior to radiolabeling.

2.3. Determination of PG and GAG synthesis

Confluent monolayers were double labeled in serum free H/D containing carrier free [^{35}S]-sulfate (0.37 MBq/ml or 10 μCi/ml) and [^{3}H]-D-glucosamine (0.07 MBq/ml or 2 μCi/ml) for 24 h, in presence or in absence of agents mentioned below. After labeling, the culture medium was collected, the cell layer was washed three times with PBS. The medium and PBS were centrifuged ($800 \times g$ for 30 min). To the supernatant an equal volume of Tris–HCl buffer (100 mM Tris, pH 7.2, containing 8 M urea, 1% Triton X-100 (v:v) and a mixture of protease inhibitors: 20 mM EDTA, 200 mM 6-aminohexanoic acid, 10 mM benzamidine-HCl, 2 mg/ml pepstatin, 20 mM N-ethyl-maleimide, 0.4 mM phenylmethylsulfonide fluoride) (TUT buffer at double concentration) was added. The cell layer was solubilized with the same buffer, under gentle shaking for 24 h at 4°C, boiled for 3 min and cleared by centrifugation ($800 \times g$, 30 min).

The quantification of PG from the medium and cell layer extract was determined by slightly modified solid phase assay using cationic nylon membrane [14]. Samples prepared as indicated above were spotted on a membrane previously soaked for 30 min in 50 mM Tris–HCl, 0.15 M NaCl, pH 7.0 (TBS) and sandwiched in a 96-well dot blot apparatus. The membrane was successively washed for 1 h with TBS containing 0.15 M NaCl then with TBS composed of NaCl 0.9 M, to remove free GAG, then rinsed with distilled water and ethanol. Spots were cut and their radioactivity was determined by scintillation counting. The radioactivity in the 0.15 M NaCl-treated dot represented the radioactivity incorporated in PG-associated or free GAG chains/fragments whereas radioactivity measured in the 0.9 M NaCl-treated dot represented only the radioactivity incorporated in PG-associated GAG chains. For each culture well, radioactivity incorporated in free GAG chains was determined by subtracting the radioactivity of the 0.15 M treated dot from that of the 0.9 M treated dot.

To assess the effects of IGF-1 on GAG synthesis, cell cultures were treated with 1 mM *para*-nitrophenyl-β-D-xyloside (PNPX) for 24 h, in the presence or absence of IGF-1. PNPX-GAG and residual PG were radiolabeled with [^{3}H]-glucosamine and their synthesis was determined as described above.

2.4. Pulse–chase experiment

The effect of IGF-1 on proteoglycan degradation in peritubular cells was investigated by pulse–chase experiments. Confluent peritubular cells in 24-well culture dishes were radiolabeled with 10 μCi/ml [^{35}S]-sulfate and 2 μCi/ml [^{3}H]-D-glucosamine for 24 h at 32°C. The medium was then removed and the cell layer was extensively washed with H/D to remove free labeled precursors. Separated wells were then 'chased' for 6.5 to 24 h, either in the presence or absence of IGF-1 at a 50 ng/ml concentration. After each 'chase' period (6.5, 12 and 24 h), the proteoglycans in the medium and extracted from the cell layer were quantified on the Zeta-probe membrane.

2.5. Analysis of proteoglycans and hyaluronic acid

Double labeled proteoglycans were first purified by anion-exchange chromatography. The medium or the cell extracts were dialysed against TUT buffer. The samples were applied to a DEAE-Trisacryl column (1×5 cm) previously equilibrated with TUT buffer. Elution was performed with 10 ml of TUT buffer, then with a linear gradient of NaCl (0–1.2 M) in the same buffer. A flow rate of 15 ml/h was used and 1 ml fractions were collected. The fractions corresponding to the peak of PG and GAG (material eluted between 0.4–0.5 M NaCl) were pooled and precipitated with ethanol. The pellet was redissolved in distilled water, aliquoted, lyophilized and samples were reconstituted with different buffers depending upon the subsequent treatments.

An aliquot of PG was dissolved in TUT buffer, cleared by centrifugation (10 000 × *g* for 10 min) and then submitted to gel filtration on a Superose 6 column (1 × 30 cm). The elution was carried out with TUT buffer at a flow rate of 12 ml/h and 0.4 ml fractions were collected.

Lyophilized PG were then submitted to β-elimination in the presence of 0.05 M NaOH and 1 M $NaBH_4$ [15]. After 48 h incubation at 45°C, the mixture was neutralized with glacial acetic acid and precipitated with ethanol. The pellet was dissolved in TUT buffer and centrifuged (10 000 × *g* for 10 min) prior to chromatography on a Superose 6 column as indicated above.

Heparan side chains were degraded by deaminative cleavage using the pH 1.5/HNO_2 method [16]. Chondroitin sulfate chains were digested with chondroitinase ABC (1 IU/ml) in 50 mM Tris–HCl

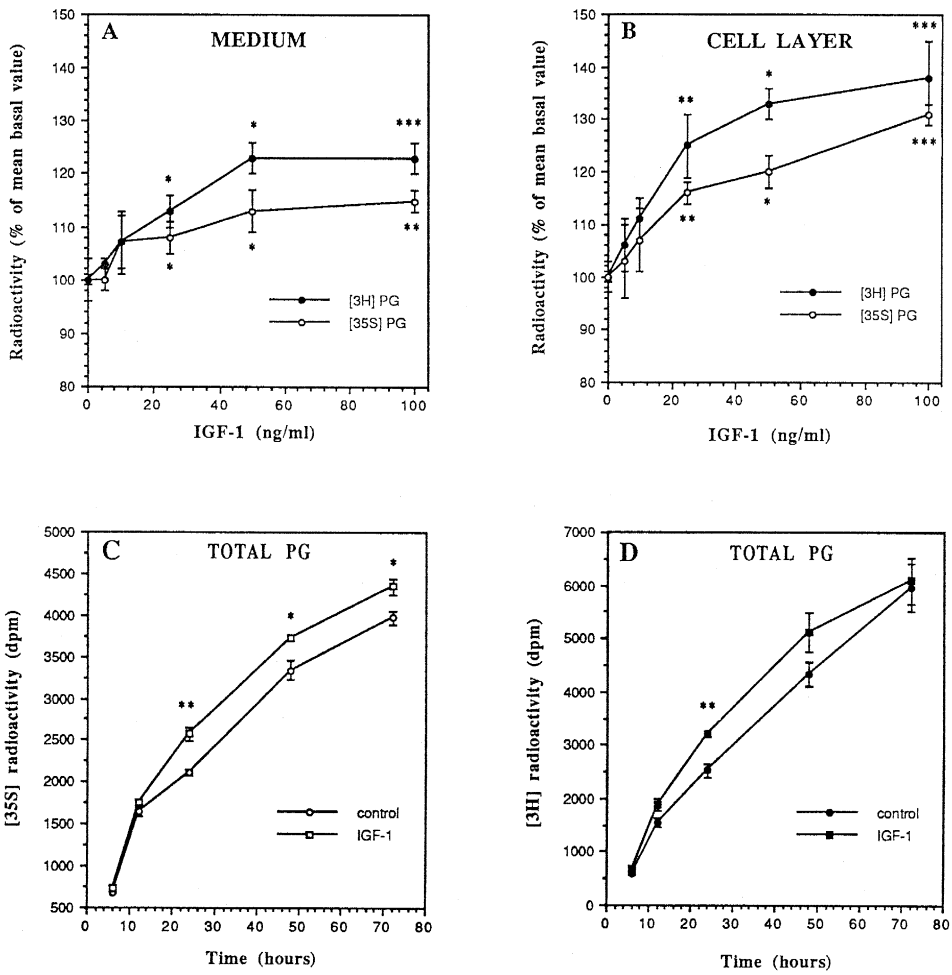

Fig. 1. Dose related and time course effects of IGF-1 on proteoglycan biosynthesis. Peritubular cells were cultured in serum-free medium 24 h prior to the experiments, then incubated in the same medium containing [^{35}S]-sulfate and [^{3}H]-glucosamine, in the presence or absence of IGF-1. Double labeled secreted and cell layer associated PG were quantified as described in Section 2. (A and B) Dose related effect of IGF-1. [^{35}S]-sulfate (-○-○-) and [^{3}H]-glucosamine (-●-●-) incorporations were performed for 24 h, in the presence of increasing concentrations of IGF-1 (0–100 ng/ml). Radioactivities of PG from medium (A) and cell layer (B) were expressed as percentage of their respective mean basal value. (C and D) Time course effect of IGF-1. Cells were cultured during indicated time-periods, in the absence or presence (50 ng/ ml) of IGF-1, labeled with [^{35}S]-sulfate ((C) -○-○-, control; -□-□-, IGF-1) and [^{3}H]-glucosamine ((D) -●-●-, control; -■-■-, IGF-1). Total PG (medium + cell layer) were quantified. Values are means ± SEM from 4 wells in 3 separated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to their respective control.

buffer (pH 8.0) containing 60 mM sodium acetate, 50 mM NaCl, 1 mM NaF, 0.1 mg/ml bovine serum albumin [17].

Hyaluronic acid was degraded by *Streptomyces* hyaluronidase (2 UI/ml) in 0.02 M acetate buffer, pH 5, for 24 h at 37°C [18].

In all cases, the degradation extent of polysaccharides was determined by Superose 6 chromatography.

2.6. General techniques

The viability of peritubular cells was assessed by the trypan blue dye exclusion test and was never below 98%. The purity of peritubular cell cultures was determined using histochemical markers for peritubular cells (alkaline phosphatase) [19], Leydig cells (3β-hydroxysteroid dehydrogenase) [20] or Sertoli cells (staining neutral lipid droplets with Oil Red O) [21]. The Leydig cell marker was not detected in any of the cultures and Sertoli cells represented less than 5% of the total cell population. The purity of the peritubular cell cultures thus reached at least 95%.

DNA content of cells was determined fluorimetrically using Hoechst 33258 reagent according to West and al. [22].

2.7. Statistical analysis

All experimental data were presented as the mean $\pm$ SEM of four independent incubations and each experiment was repeated at least three times.

Statistical significance between groups were determined by the Student's *t*-test using the Statworks package on a Macintosh computer (Apple Computer, Cupertino, CA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Dose related and time course effects of IGF-1 on [^{35}S]-sulfate and [^{3}H]-D-glucosamine incorporations in proteoglycans

IGF-1 had an optimal stimulatory effect on PG synthesis for a 50 ng/ml concentration and no additional effect was observed when IGF-1 was used at 100 ng/ml. In the presence of 50 ng/ml IGF-1, the incorporations of [^{35}S]-sulfate and of [^{3}H]-D-glucosamine were significantly increased by 13 and 23% into secreted PG (Fig. 1A) and by 20 and 30% in cell-associated PG (Fig. 1B), respectively. Although peritubular cells were confluent, we examined the possibility that IGF-1 induced stimulation of the PG synthesis may be due to an increase of cell proliferation. However, the DNA content was not modified by IGF-1 (50 ng/ml) in our experimental conditions (control: 582 ± 21 ng DNA/well, IGF-1 treatment: 576 ± 20 ng DNA/well).

Treatment of the peritubular cells with IGF-1 (50

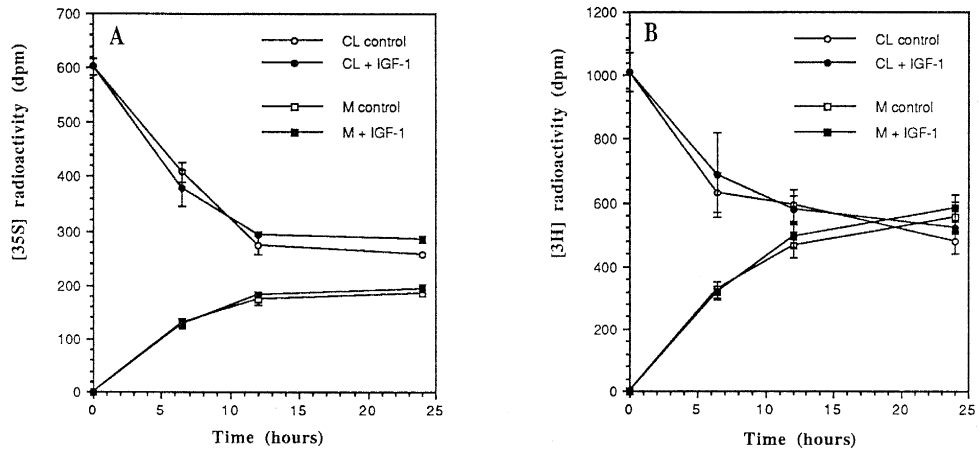

Fig. 2. Pulse–chase experiments. Cell cultures were labeled with [^{35}S]-sulfate (A) and [^{3}H]-glucosamine (B) for 24 h. The medium was discarded, the cell layer was washed and cell culture was 'chased' for various times, either in the absence (control) or presence of IGF-1 (+IGF-1, 50 ng/ml). For each time, PG associated to the cell layer (CL) and released into the culture medium (M) were quantified. Values are means $\pm$ SEM from 3 separated experiments.

ng/ml) generated time-dependent increases in the total proteoglycan biosynthesis. The stimulations were maximal after 24 h of incubation (+20% and +30% for [^{35}S]-sulfate (Fig. 1C) and [^{3}H]-D-glucosamine (Fig. 1D) incorporations, respectively). Beyond a 24 h period of incubation, the stimulatory effects of IGF-1 were less pronounced, since after 48 h treatment, [^{35}S] and [^{3}H] radioactivities were only increased by 11 and 18%, respectively.

3.2. Pulse–chase studies

Treatment of pulse-labeled cell cultures with IGF-1 during the chase period did not affect significantly the rate of PG disappearance from the cell layer. The rate of PG loss corresponded to an apparent $t_{1/2}$ of 11 and 24 h when calculated after prelabeling with [^{35}S]-sulfate (Fig. 2A) and [^{3}H]-D-glucosamine (Fig. 2B), respectively. [^{3}H] and [^{35}S] PG radioactivity was also determined in the chase medium. The results showed that [^{3}H]-PG radioactivity released from the cell layer was completely recovered in the culture medium (Fig. 2B). On the contrary, after 24 h of chase, the [^{35}S] PG radioactivity determined in the medium was less than that released from the cell layer (Fig. 2A). This difference suggested that [^{35}S]-PG, during their transfer from cell to medium, may be partially desulfated. IGF-1 had no additional influence on this process.

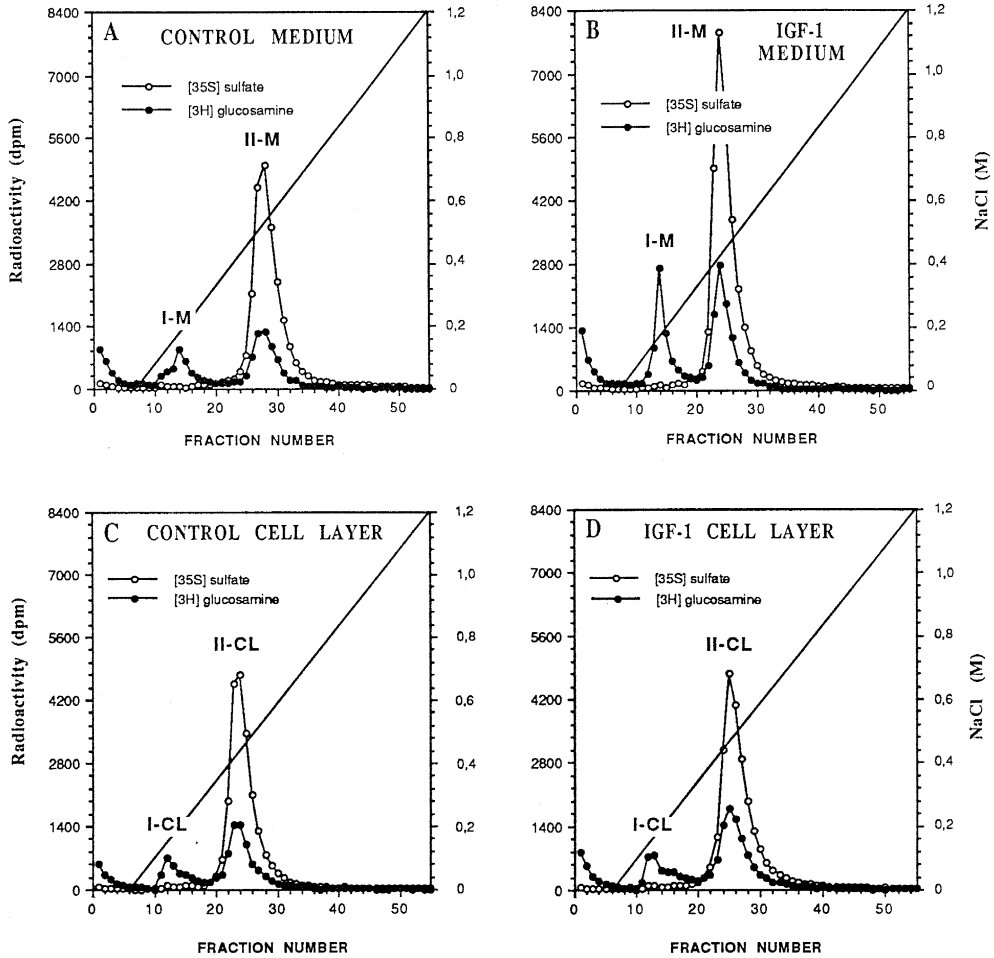

Fig. 3. DEAE-Trisacryl chromatography of culture medium (A and B) and cell layer extract (C and D). Cell cultures were incubated without (A and C) or with IGF-1 (50 ng/ml) (B and D) and simultaneously labeled for 24 h with [^{35}S]-sulfate (-○-○-) and [^{3}H]-glucosamine (-●-●-). Medium and cell layer extract were applied to a DEAE-Trisacryl column and eluted with a 0–1.2 M NaCl gradient (solid line).

3.3. Characterization of proteoglycans and hyaluronic acid synthesized in presence of IGF-1

After labeling for 24 h, secreted material was purified by ion-exchange chromatography (Fig. 3). The elution profile of the control (Fig. 3A) and IGF-1 treated media (Fig. 3B) showed two peaks. The first peak (peak I-M), eluted in the gradient at 0.2 M NaCl, was only labeled with [^{3}H]-glucosamine (Fig. 3A). When submitted to Superose 6 chromatography, peak I-M emerged in the excluded volume and this peak was entirely susceptible to digestion by *Streptomyces* hyaluronidase (Fig. 4A). Thus, [^{3}H]-labeled molecules from peak I-M corresponded to large molecular-weight hyaluronic acid. Addition of IGF-1 to peritubular cell cultures induced about a two fold increase of hyaluronic acid synthesis (Fig. 3B and Fig. 4B).

A second peak (peak II-M), [^{3}H]- and [^{35}S]-double labeled, emerged from the DEAE- Trisacryl column at 0.55 M NaCl concentration for control medium (Fig. 3A) and at 0.44 M NaCl for IGF-1 treated medium (Fig. 3B). Peak II-M contained PG (see below). PG synthesized in the presence of IGF-1 had a lower charge density than those originating from control cultures. This observation was confirmed by the values of the [^{35}S]/[^{3}H] ratio that were 3.65 and 3 for control and IGF-1 treated cultures, respectively. Thus, IGF-1 induced a 18% decrease in the relative [^{35}S]-sulfate incorporation. The fractions of peak II-M were pooled, concentrated and submitted to gel filtration on a Superose 6 column (Fig. 5A and B). PG from control medium were eluted at $K_{av} = 0.12$ in a homogenous peak (Fig. 5A). The elution profile of PG secreted in the presence of IGF-1 was similar (Fig. 5B). After digestion of peak II-M with chon-

Table 1
Distribution and sulfation level of proteochondroitin sulfate (CSPG) and proteoheparan sulfate (HSPG) in control and IGF-1 treated peritubular cell cultures

		CSPG	HSPG
Distribution CSPG/HSPG (%) [a]			
Medium	control	85 ± 4.4	15 ± 4.0
	IGF-1	83 ± 8.3	17 ± 5.2
Cell layer	control	23 ± 3.7	77 ± 5.5
	IGF-1	20 ± 4.8	80 ± 7.3
Sulfation level [b]			
Medium	control	5.5 ± 0.4	3.1 ± 0.2
	IGF-1	3.6 ± 0.5	2.2 ± 0.3
Cell layer	control	4.5 ± 0.2	3.7 ± 0.2
	IGF-1	3.7 ± 0.3	2.8 ± 0.1

[a] Values are expressed as a percentage of total PG (CSPG + HSPG) present in the medium or in the cell layer. The distribution percentages were calculated from results obtained after Superose 6 chromatography of [^{3}H]-labeled PG from peak II-M (medium) or peak II-CL-a (cell layer) submitted to digestion by chondroitinase ABC or to degradation by nitrous acid (see Fig. 5C–F for peak II-M, Fig. 7A–D for peak II-CL-1).
[b] The sulfation level was evaluated by the [^{35}S]/[^{3}H] ratio calculated from each species of PG which was distinguished as indicated in [a].
Each value represents mean ± SEM for 3 determinations.

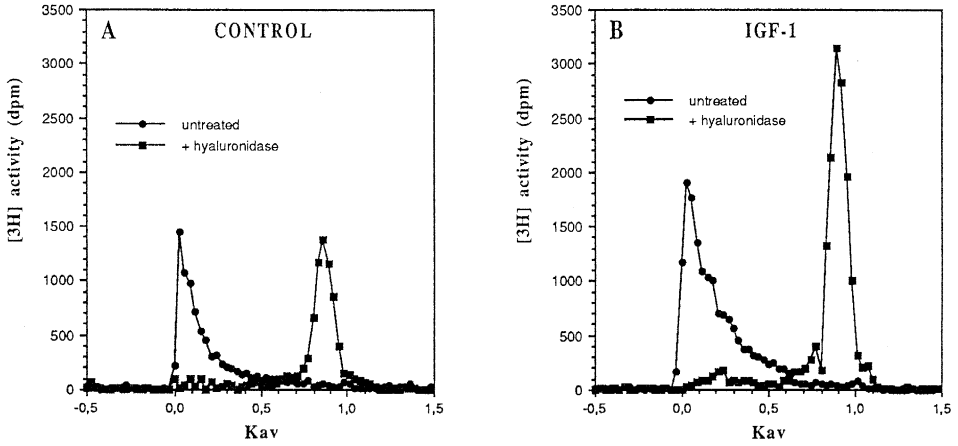

Fig. 4. Gel filtration chromatography of DEAE-Trisacryl purified peak I-M on Superose-6. Peak I-M from control (A) or IGF-1 (50 ng/ml) conditioned medium (B) was chromatographed before (-●-●-) or after *Streptomyces* hyaluronidase digestion (-■-■-).

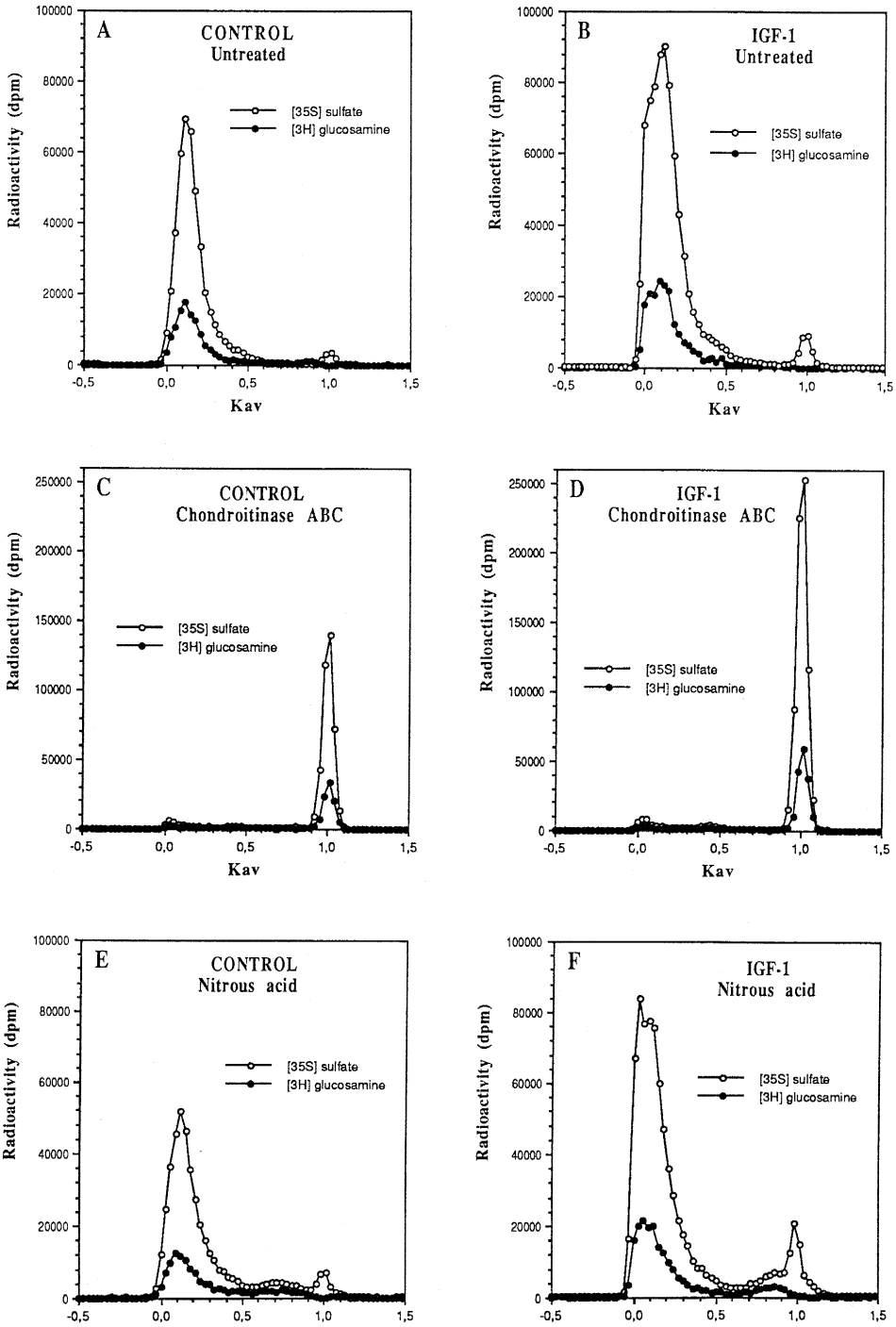

Fig. 5. Gel filtration chromatography of DEAE-Trisacryl purified peak II-M (Fig. 3A and B) on Superose-6. Peak II-M from control (A, C and E) or IGF-1 (50 ng/ml) conditioned medium (B, D and F) was chromatographed without prior treatment (A and B), after chondroitinase ABC digestion (C and D) or after nitrous acid degradation (E and F). (-○-○-): [^{35}S] radioactivity. (-●-●-): [^{3}H] radioactivity.

droitinase ABC, about 85% of [^{35}S]-labeled PG present in the control (Fig. 5C) and in IGF-1 treated (Fig. 5D) cell culture media eluted in the total volume. In both cases, the chondroitinase ABC-resistant material was susceptible to nitrous acid degradation (Fig. 5E and F). These results indicated that PG in the medium were predominantly chondroitin sulfate/dermatan sulfate proteoglycans, containing a minor fraction of heparan sulfate (15%) and IGF-1 had no effect on the nature and distribution of GAG chains (Table 1). The level of undersulfation induced by IGF-1 was evaluated by the [^{35}S]/[^{3}H] ratio calculated for CSPG (proteochondroitin sulfate) and HSPG (proteoheparan sulfate) from the medium (Table 1).

The cell layer extracts (see Section 2) submitted to ion exchange chromatography on DEAE Trisacryl (Fig. 3C and D), gave similar results to those obtained for medium.

Peak I-CL, only labeled with tritium and eluted with 0.2 M NaCl (Fig. 3C), was entirely susceptible to digestion by *Streptomyces* hyaluronidase (data not shown) and consisted of hyaluronic acid. IGF-1 had very little effect on cell-associated hyaluronic acid (Fig. 3D) since this polysaccharide was translocated out into the medium immediately after being synthesized [23]. Peak II-CL, [^{35}S]/[^{3}H] double labeled was eluted from the column with 0.57 and 0.53 M NaCl for the control (Fig. 3C) and IGF-1 treated (Fig. 3D) cells. DEAE Trisacryl purified peak II-CL was chromatographed on a Superose 6 column (Fig. 6A and B). The elution profile revealed two peaks, one at

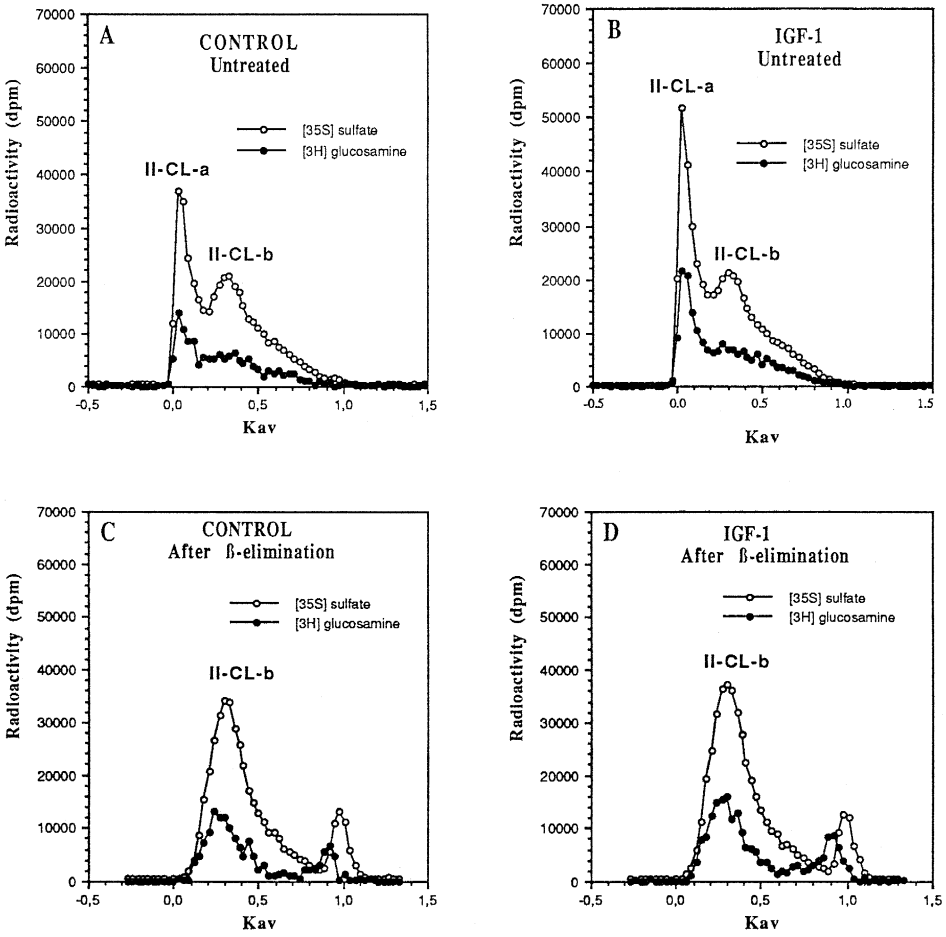

Fig. 6. Gel filtration chromatography of DEAE-Trisacryl purified peak II-CL (Fig. 3C and D) on Superose-6. Peak II-CL from control (A and C) or IGF-1 (50 ng/ml) treated cell layer extract (B and D) was chromatographed without prior treatment (A and B) or after β-elimination (C and D). (-○-○-): [^{35}S] radioactivity. (-●-●-): [^{3}H] radioactivity.

$K_{av} = 0$ (II-CL-a) and the other one at $K_{av} = 0.33$ (II-CL-b) for both the control (Fig. 6A) and IGF-1-treated cell layers (Fig. 6B). Peak II-CL was submitted to β-elimination to release single glycosaminoglycan chains and rechromatographed on Superose 6 (Fig. 6C and D). Peak II-CL-a shifted to $K_{av} = 0.33$ indicating that it was sensitive to β-elimination and proving that it was PG in nature. On the contrary, after alkaline treatment, peak II-CL-b eluted at the same position as untreated peak and thus corresponded to free GAG chains. Identical qualitative results were obtained for control (Fig. 6C) and IGF-1 treated cultures (Fig. 6D). In control cultures, the ratio [^{35}S]-PG (peak II-CL-a)/[^{35}S]-GAG (peak II-CL-b) was approximately 0.65 (Fig. 6C). Under IGF-1 conditions, this ratio increased (0.80) (Fig. 6D) since IGF-1 stimulated production of PG but did not modify those of GAG which presumably represented intracellular degradation products of internalized PG.

Comparison of the [^{35}S]/[^{3}H] ratio calculated in control and IGF-1 treated cultures indicated that IGF-1 induced a 20 and 25% decrease in the relative [^{35}S] incorporation in peak II-CL-a (PG) and peak II-CL-b (GAG), respectively (Fig. 6A and B). As observed for medium PG, IGF-1 altered the sulfation of GAG chains associated to cell layer.

Approximately 20% of PG from peak II-CL-a were sensitive to chondroitinase ABC, representing chondroitin sulfate/dermatan sulfate proteoglycans, while 80% were degraded by nitrous acid and corresponded to heparan sulfate proteoglycans, both in control (Fig. 7A and C) and in IGF-1 treated cells

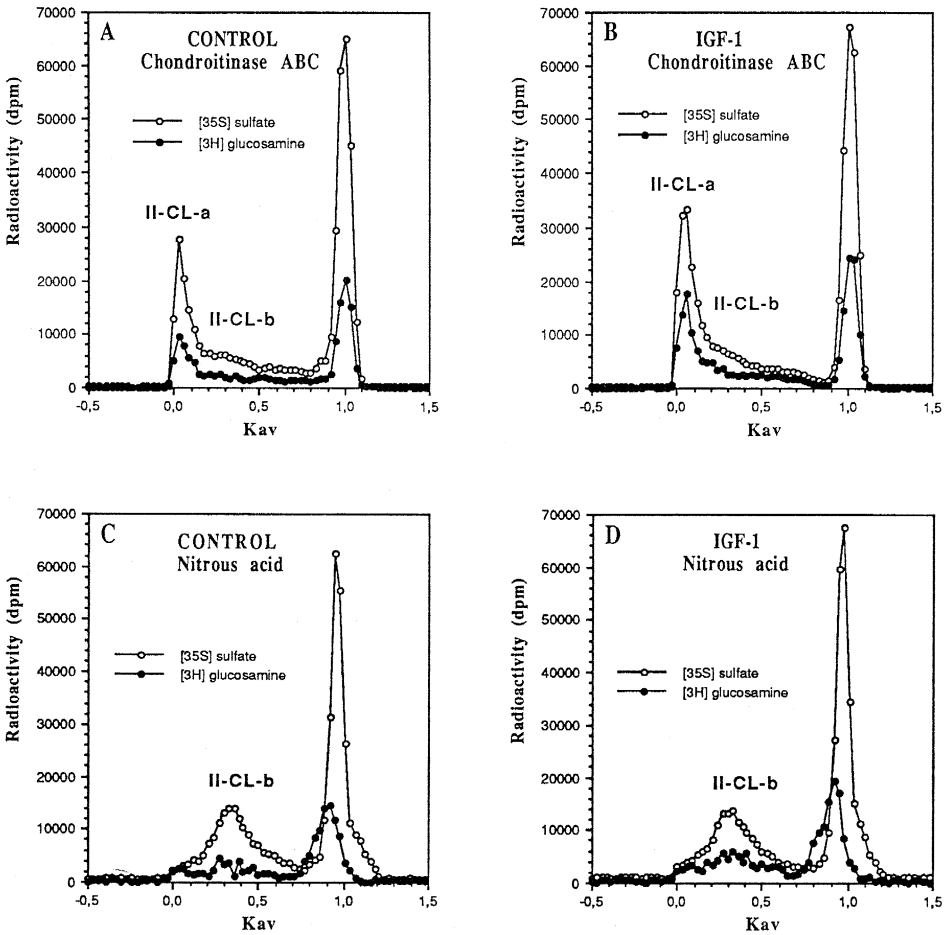

Fig. 7. Gel filtration chromatography of DEAE-Trisacryl purified peak II-CL (Fig. 3C and D) on Superose-6. Peak II-CL from control (A and C) or IGF-1 (50 ng/ml) treated cell layer extract (B and D) was chromatographed after chondroitinase ABC digestion (A and B) or after nitrous acid degradation (C and D). (-○-○-): [^{35}S] radioactivity. (-●-●-): [^{3}H] radioactivity.

(Fig. 7B and D and Table 1). The level of undersulfation induced by IGF-1 was evaluated by the [^{35}S]/[^{3}H] ratio calculated for CSPG and HSPG from cell layer extract (Table 1).

In peak II-CL-b, 65% of GAG were chondroitin sulfate/dermatan sulfate in nature, the remaining 35% being heparan sulfate (Fig. 7A and C). These percentages were not modified when IGF-1 was present in peritubular cell cultures (Fig. 7B and D).

3.4. Effects of IGF-1 on PG and GAG synthesis under para-nitrophenyl-β-D-xyloside (PNPX) conditions

In preliminary experiments, we observed that PNPX decreased the total PG synthesis in peritubular cells and dramatically stimulated PNPX-primed GAG production, in a time and dose-dependent manner. The maximum effect of PNPX was reached 24 h after addition of this exogeneous acceptor used at 1 mM (data not shown). 1 mM PNPX reduced by 60% the [^{3}H]-glucosamine incorporation in PG (Fig. 8A) and at the same time, increased by 150% the [^{3}H]-labeling in PNPX-GAG (Fig. 8B).

In order to test the specificity of PNPX upon peritubular cell PG and GAG synthesis, additional experiments were conducted with *para*-nitro-β-D-galactoside (PNPG), a structural analog of PNPX, described as ineffective on PG synthesis [24]. The addition of increasing concentrations of PNPG (0.01–1 mM) to peritubular cell cultures remained unchanged both PG and GAG basal synthesis (data not shown).

Cells were simultaneously incubated with IGF-1 and PNPX, then PG and GAG production was assessed. As presentated above and confirmed in Fig. 8A, in the absence of PNPX, IGF-1 increased PG synthesis. When PNPX and IGF-1 were added in association, the [^{3}H]-glucosamine incorporation in PG appeared significantly enhanced (Fig. 8A). This result indicated that IGF-1 favored the glycosylation of xylosylated core proteins which accumulated in the cell because of the presence of PNPX. The basal level of cellular GAG was not affected by IGF-1 treatment (Fig. 8B) but this factor increased by 30% the synthesis of GAG formed on xyloside primer (Fig. 8B). If IGF-1 enhanced the fixation of glycanic chains both on available core proteins and on PNPX,

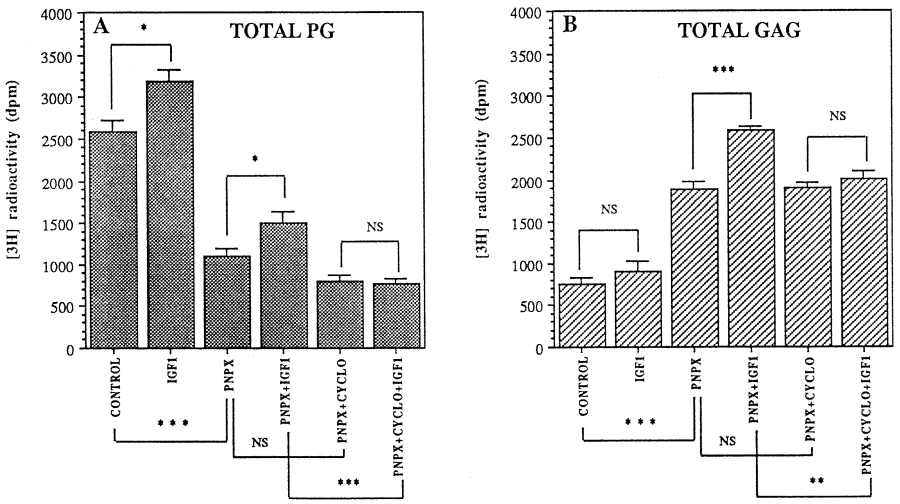

Fig. 8. Effect of IGF-1 on the total synthesis of PG (■, left) and GAG (diagonally striped area, right) under *para*-nitrophenyl-xyloside (PNPX, 1 mM) and cycloheximide (CHX, 20 μM) conditions. Confluent peritubular cells were incubated in culture medium, supplemented or not with IGF-1 (50 ng/ml), in the absence or presence of PNPX and CHX used alone or in combination and double labeled with [^{35}S]-sulfate and [^{3}H]-glucosamine. After 24 h of labeling, the synthesis of total PG and total GAG (medium + cell layer extract) was determined as indicated in Section 2. Only results concerning [^{3}H] incorporation were shown. Similar results were obtained with [^{35}S] labeling but with a lesser extent due to the IGF-1 induced undersulfation of GAG chains (see Table 1). Values are means ± SEM from 4 wells in 3 separated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to their respective control. NS: not significant.

this effect may result from an increase of glycosyltransferase activities and/or from an increase of *de novo* synthesis of these enzymes. To test these possibilities, cycloheximide, an inhibitor of protein synthesis, was used under PNPX conditions. When cycloheximide was introduced conjointly with PNPX, the stimulatory effects of IGF-1 on PG (Fig. 8A) and on PNPX-GAG (Fig. 8B) were suppressed. These results suggested that IGF-1 exerted its action through the synthesis of proteins involved, directly or indirectly, in the glycosylation process.

3.5. Effects of tyrosine kinase inhibitors on IGF-1 stimulated PG synthesis

The action of two specific protein-tyrosine kinase inhibitors, genistein [25] and tyrphostin A9 [26], on IGF-1-stimulated PG synthesis was examined. From 8 to 80 μM, genistein did not significantly modify basal synthesis of total [^{3}H]-labeled proteoglycans (medium + cell layer) but counteracted the stimulating effect of IGF-1 (Fig. 9A). Similarly, tyrphostin A9 used at concentrations ranging from 0.025 to 0.4 μM did not change the basal synthesis of proteoglycans and at 0.2 μM abolished IGF-1 stimulation (Fig. 9B). To rule out a non-specific activity of these inhibitors, daidzein, an inactive analog of genistein and tyrphostin A1 an inactive inhibitor used as negative control for tyrphostin A9 were tested. In presence of daidzein (Fig. 9A) and tyrphostin A1 (Fig. 9B), the extent of increase in proteoglycan synthesis due to IGF-1 remained unchanged. In these experiments, identical results were obtained when PG were labeled with [^{35}S]-sulfate (data not shown).

4. Discussion

In the present work, we have examined the regulation of peritubular cell PG synthesis by IGF-1, which is a growth factor widely produced in the rat testis [8]. We have found that IGF-1 enhanced the synthesis of the PG, visualized by the [^{35}S]-sulfate and the [^{3}H]-D-glucosamine incorporations by peritubular cells, in a time and dose-dependent manner. Since a wide variety of cell types demonstrate a mitogenic response to IGF-1 [27] and though the cells were used at confluence, a possible effect of IGF-1 on cell proliferation was not excluded. Indeed, IGF-1 did not modify the DNA content of the cell layer in our experimental conditions, proving that its action did not occur through cell proliferation. Pulse-chase experiments showed that the increased content of PG found in peritubular cells in the presence of IGF-1

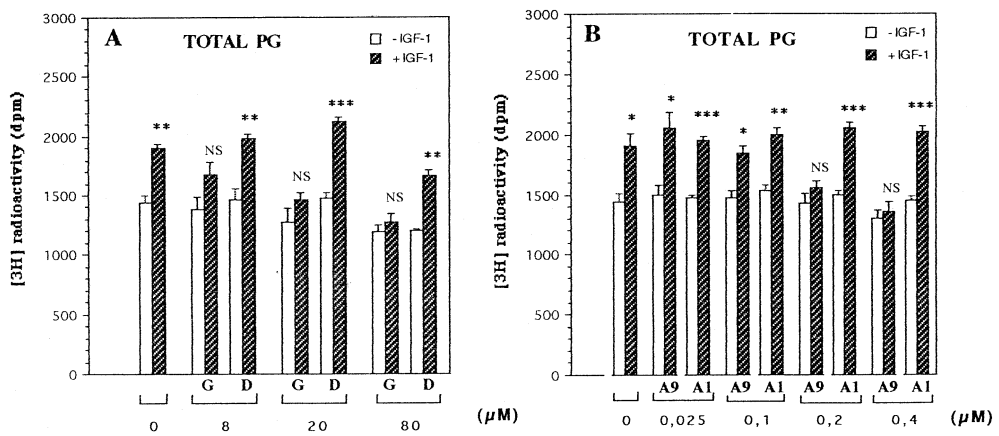

Fig. 9. Effect of genistein and tyrphostin A9 on the IGF-1 stimulated PG synthesis. Confluent peritubular cells were pretreated with increasing concentrations of genistein (A, left) or tyrphostin A9 (B, right) for 30 min. Then, IGF-1 (50 ng/ml) was introduced (hatched bars) or not (open bars) and cell cultures were labeled with [^{35}S]-sulfate and [^{3}H]-glucosamine. After 24 h of labeling, the synthesis of total PG was determined. Daidzein and tyrphostin A1 are used as negative control for genistein and tyrphostin A9 respectively. Only results concerning [^{3}H] incorporation were shown. Values are means ± SEM from 4 wells in 3 separated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared to their respective control. NS: not significant. G: genistein, D: daidzein, A9: tyrphostin A9, A1: tyrphostin A1.

(50 ng/ml for 24 h) was the result of an enhancement of PG synthesis rather than a decreased rate of degradation. The rate of PG which disappeared in the cell layer corresponded to an apparent $t_{1/2}$ of 11 and 24 h when estimated after prelabeling with [^{35}S]-sulfate and [^{3}H]-D-glucosamine, respectively. Since the [^{3}H]-D-glucosamine labeling reflected the PG amount while the [^{35}S]-sulfate labeling reflected the PG sulfation, it might be deduced that a desulfation process occurred before the release of PG from the cell layer, whatever the conditions (with or without IGF-1).

This enhancement of PG synthesis in the presence of IGF-1 has already been reported with other cell types such as glomerular cells [28], flexor tendon cells [29], mesangial cells [30] and particularly chondrocytes which have received much attention. In the last cell type, IGF-1 stimulated the synthesis of aggrecan whose physical and chemical characteristics were similar to the control [10]. In order to examine if IGF-1 could act in the same manner in our cell model, the hydrodynamic sizes of the proteoglycans secreted in the medium and associated to the cell layer as well as the nature of their GAG were studied.

In peritubular cell cultures, IGF-1 stimulated the proteoglycan synthesis from the medium and cell layer but did not alter the proteochondroitin sulfate/proteoheparan sulfate distribution in these two compartments. The biosynthesis of proteoglycans, such as proteochondroitin sulfate and proteoheparan sulfate, is a complicated process which consists of sequential formation of the core protein, xylosylation of specific serine residues of the core protein, elaboration of the tetrasaccharide protein-glycosaminoglycan linkage moiety, repeated addition of hexosamine residues alternating with glucuronic acid residues to form the large polymer glycosaminoglycan chains, additional modifications of these glycosaminoglycan chains (O-sulfation, deacetylation/N-sulfation, epimerization of glucuronic acid to iduronic acid) [31,32]. Proteoglycan biosynthesis may be dependent on the regulation of several steps of this process. IGF-1 increased incorporation of [^{3}H]-hexosamine and in lesser extent those of [^{35}S]-sulfate in glycosaminoglycan chains of peritubular cell proteoglycans. So, β-xyloside was used in order to test the effect of IGF-1 on proteoglycan glycosylation. β-xyloside act as exogenous acceptor for the addition of the first linkage region galactose by galactosyl transferase I. This diverts glycosaminoglycan chain elongation from the xylosylated core protein onto the exogenous xyloside and under these conditions, the availability of endogenous core proteins is not a restrictive factor for the glycosaminoglycan synthesis [33]. When IGF-1 was added together with PNPX, it enhanced [^{3}H]-hexosamine incorporation in the newly synthesized glycosaminoglycans to an extent higher than PNPX alone. At the same time, an increase in [^{3}H]-labeling with identical magnitude was found for the core protein pool. These observations indicated that one of the effects of IGF-1 was to stimulate glycosaminoglycan chain biosynthesis. Moreover, cycloheximide suppressed the IGF-1 effects suggesting that this factor could enhance glycosaminoglycan production by induction of glycosytransferase(s). In our work, IGF-1 modified neither the GAG chain length, nor the PG molecular weight. The most plausible explanation is that IGF-1 initially enhances the number of newly synthesized PG, whose characteristics remain unchanged. This phenomenon may result from an increase in core protein pool glycosylation, but we do not rule out that IGF-1 may also stimulate the expression of core proteins concomitant with those of glycosyltransferases. Further investigations are necessary to clarify this point.

In the presence of IGF-1, proteoglycan from the medium and cell layer appeared undersulfated, the ratio [^{35}S]-sulfate/[^{3}H]-hexosamine decreasing by 20–25% in heparan sulfate and chondroitin sulfate chains. Interestingly, similar changes in glycosaminoglycan sulfation were reported in vascular endothelial cells, smooth-muscle cells and fibroblasts [34] after treatment by bFGF. Sulfation involves the activation of inorganic sulfate as adenosine 3′-phosphate 5′ phosphosulfate (PAPS) and the subsequent transfer of the 'active sulfate' by specific sulfotransferases. It is generally accepted that sulfation occurs at the same time as, or immediately after glycosaminoglycan chain elongation [31,32]. Although sugar-chain formation and sulfation are spatially and temporally related, these two processes may be, at least in part, differentially affected by IGF-1 and bFGF but at present, the causes of this imbalance are not defined. Despite the half-life of [^{35}S] and [^{3}H]-labeled PG being highly different, it is unlikely that, after GAG chain catabolism, the difference in the re-use of

[^{35}S]-sulfate and [^{3}H]-hexosamine may explain undersulfation. Since the PAPS pool is small and the intracellular sulfate pool is rapidly equilibrated with the exogenous sulfate (0.4 M in our culture medium), isotopic dilution of [^{35}S]-sulfate is considerable and will remain practically constant even if [^{35}S]-sulfate is re-used. Similarly, the specific activity of [^{3}H] in the metabolic hexosamine pool is often several hundred-fold lower than that of the [^{3}H]-glucosamine added to the cell culture medium due to a large contribution of glucose to the intracellular UDP-*N*-acetylhexosamine pool [35].

The cell surface receptor for IGF-1 is composed of two α- and two β-subunits. Whereas the α-subunits contain ligand-binding domains, the cytoplasmic domain of the β-subunits possess tyrosine kinase activity that is required for cellular signaling [36]. IGF-1 binding to the receptor results in activation of the receptor tyrosine kinase [37] and subsequent phosphorylation of both the receptor and intracellular substrate proteins that are direct substrates of the IGF-1 receptor kinase such as the insulin receptor substrate-1 (IRS-1) [38] and the Shc proteins [39]. Since genistein and tyrphostin A9, two potent and specific inhibitors of tyrosine kinase, totally abolished IGF-1-induced PG synthesis stimulation, we suggest that genistein and tyrphostin A9-sensitive putative tyrosine kinases are involved in the signal transduction pathways mediating the action of IGF-1 on PG production. In peritubular cell cultures, we have found that IGF-1 enhanced hyaluronic acid production approximately two fold. Hyaluronic acid synthesis depends on a plasma membrane-bound enzyme, the hyaluronate synthetase [40,41]. Phosphorylation of synthetase was required for activity and multiple kinases could phosphorylate it [42] among them the tyrosine kinase of pp60^{src} from Rous sarcoma virus [23]. Previous work showed that genistein pretreatment inhibited IGF-1-induced hyaluronic acid synthesis and hyaluronate syntethase activity [43]. This, therefore, raised the possibility that the IGF-1 stimulation of hyaluronic acid synthesis, in our model, might also be triggered by protein tyrosine kinase activation.

The physiological significance of the modifications induced by IGF-1 on proteoglycan biosynthesis and particularly the undersulfation is still unknown. It has been shown that the biological activity of IGF-1 is modified by IGF-binding proteins (IGFBPs) [44], the binding of IGF-1 to the cell surface or extracellular matrix associated IGFBPs resulting in potentiation of cellular actions of this growth factor [45,46]. IGFBP-2, mainly produced by peritubular cells [7], interacted with glycosaminoglycans, such as heparan sulfate, in which the presence of O-linked sulfate groups in the 2 or 3 position of the iduronic acid ring was essential for the binding [47]. Thus, the reduction in sulfation of heparan sulfate could result in a decrease in the heparan sulfate-IGFBP-2 binding and consequently in a lower IGF-1 activity, constituting a short negative local regulatory loop. It has also been shown that proteoglycans, mainly proteoheparan sulfate, serve as tissue stores or carrier systems for a large variety of important effector molecules and are implicated in receptor-growth factor interactions, in cell–cell recognition systems and in cell–matrix adhesion processes [48,49]. Within glycosaminoglycan chains, saccharide sequence(s) of defined structure is (are) required to specifically bind one particular ligand [32] and alterations in these sequences can disturb the binding. In testis, the multiple interactions between peritubular cells and Sertoli cells is of importance to preserve the structural and functional integrity of seminiferous tubules [50]. The undersulfation induced by IGF-1 of proteoglycans synthesized by peritubular cells could modify their influence on Sertoli cells and thus represent an additional control mechanism.

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