Biochemical Pharmacology, Vol. 42, No. 10, pp. 1987–1995, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

QUESTIONING THE RELIABILITY OF *p*-NITROPHENYL-β-D-XYLOSIDE AS PROBE TO STUDY THE METABOLIC EFFECTS OF ABROGATED PROTEOGLYCAN SYNTHESIS IN CULTURED CELLS

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(*Received* 25 *April* 1991; *accepted* 27 *June* 1991)

Abstract—*p*-Nitrophenyl-β-D-xylopyranoside (PNP-Xyl) and similar aglycone derivatives of xylosides are proposed selective inhibitors of proteoglycan synthesis which are used frequently to analyse the metabolic and cellular effects of abrogated proteoglycan formation and, hence, tentatively, the functions of these complex molecules. Using rat liver fat storing cell (FSC) cultures as a model, the possibility was tested that *p*-nitrophenol (PNP), which might be generated by the enzymatic hydrolysis of PNP-Xyl, could mediate some of those effects ascribed previously to PNP-Xyl induced inhibition of proteoglycan synthesis. PNP-Xyl and PNP inhibited dose-dependently the proliferation of FSC reaching 50% inhibition at about 1.9 and 0.6 mM, respectively. The inhibition of proliferation was not accompanied by signs of toxic cell damage and was fully reversible after withdrawal of the drugs. After an initial 4-fold stimulation of the formation of [^{35}S]sulfate-labeled medium glycosaminoglycans (GAG) by PNP-Xyl at 0.1 mM, higher concentrations of this compound (about 0.5 mM) but also PNP decreased progressively the synthesis of sulfated medium GAG. A proliferation inhibiting concentration of PNP (0.75 mM) induced disorganization and reduced the expression of desmin- and smooth muscle iso-α-actin containing cytoskeletal filaments. These effects were similar to related effects reported previously for PNP-Xyl. Incubation of FSC with 5 mM PNP-Xyl resulted in a time-dependent increase of PNP in medium and cells; intracellular concentrations of PNP were reached sufficient to inhibit the mitotic activity of FSC. In lysates of FSC 0.65 nmol PNP/hr/μg DNA or 1×10^5 cells were generated from PNP-Xyl (5 mM) added as substrate. Exemplified with PNP-Xyl-treated FSC cultures, the results suggest for other cell and organ systems also that PNP, which is enzymatically cleaved from PNP-Xyl, might mediate at least some of the major effects attributed previously to the inhibition of proteoglycan synthesis. The aglycone may interfere with the effects of PNP-Xyl on proteoglycan metabolism and, therefore, could complicate in an unpredictable manner the interpretation of metabolic inhibitory studies using these compounds.

Proteoglycans comprise a heterogeneous group of protein–polysaccharide complexes consisting of a protein back bone (core protein) linked N- or O-glycosidically with highly polyanionic, sulfated carbohydrate polymers (glycosaminoglycans) [1–3]. They are distributed ubiquitously in the extracellular tissue matrix but are present also on the cell surface [4] and in intracellular structures and compartments [3, 5]. The functional roles of proteoglycans either in the supramolecular organization of the extracellular matrix or in the regulation of cell activities are not clearly defined. These molecules have been implicated repeatedly in the control of cell growth, regulation of cellular differentiation, migration and adhesion but also in the process of matrix assembly and morphogenesis [5, 6]. Since the demonstration in various cells and other biological systems (e.g. whole embryo cultures) of the ability of *p*-nitrophenyl-β-D-xyloside to compete with protein-bound xylose for the enzyme UDP-galactosyl transferase I, and to act thereby as an initiation site for the synthesis of galactose-linked chondroitin sulfate chains [7–12], this compound and similar sugar derivatives [13] have been suggested frequently for and used as powerful tools in the analysis of the roles of proteoglycans in the control of fundamental cellular and morphogenetic processes [6, 14–17]. Recently, it has been shown in a variety of cell types that *p*-nitrophenyl-β-D-xylopyranoside (PNP-Xyl†) and other xyloside-derivatives which have different aglycone residues, inhibit the proliferation of monkey aortic smooth muscle cells [18], rat aortic smooth muscle cells [19], human T-lymphocytes [20] and human monoblastic U-937-cells [21]. Inhibitory effectiveness was found to be dependent on the aglycone structure [21]. Furthermore, cultured rat liver fat storing cells, the principal proteoglycan synthesizing cell type in fibrotic liver [22], respond to PNP-Xyl with a dose-dependent inhibition of proliferation and disorganization of desmin and smooth muscle iso-α-actin cytoskeletal filaments [23]. Similar rearrangements of cytoskeletal structures

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†Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; DMEM, Dulbecco's modification of Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; FSC, fat storing cells; GAG, glycosaminoglycan(s); NEM, *N*-ethylmaleimide; PBS, phosphate buffered saline; PMSF, phenyl-methyl-sulfonyl-fluoride; PNP, *p*-nitrophenol; PNP-Xyl, *p*-nitrophenyl-β-D-xylopyranoside.

and of the deposition of extracellular matrix molecules were observed with smooth muscle cells treated in a comparable fashion [19]. The conclusions drawn from these and other experiments using β-D-xyloside derivatives to abrogate proteoglycan synthesis suggest a variety of fundamental, direct or indirect functions, which these macromolecules might fulfil in cell metabolism, tissue organization and morphogenesis. In this study using fat storing cells we present evidence that some of the effects of PNP-Xyl tentatively ascribed to abrogated proteoglycan synthesis are mimicked by PNP, which is cleaved enzymatically from PNP-Xyl. It is suggested also for other cell and organ systems that the aglycone interferes potentially with the effects of PNP-Xyl on proteoglycan metabolism and, therefore, complicates in an unpredictable manner the interpretation of metabolic inhibitory studies using these compounds.

MATERIALS AND METHODS

Materials. *p*-Nitrophenyl-β-D-xylopyranoside and 4-nitrophenol (spectrometric grade, crystalline) were obtained from the Sigma Chemical Co. (Munich, Germany). Both the xylopyranoside and *p*-nitrophenol were prepared as 50 mM stock solution in 1% DMSO and adjusted to final concentrations by appropriate dilutions with incubation medium. The sources of reagents for cell isolation and culture were as described previously [24, 25].

Isolation and culture of fat storing cells. Fat storing cells were prepared from 1-year-old male Sprague–Dawley rats (body weight 500–700 g, Lippische Versuchstierzucht, Extertal, Germany) which had free access to a standard laboratory chow diet containing 15,000 I.U. vitamin A/kg and tap water. Non-parenchymal liver cells were isolated by the pronase–collagenase method [26] with slight modifications as described elsewhere [27]. Fat storing cells were purified from the non-parenchymal cell suspension by a single step density gradient centrifugation with Nycodenz (Nyegaard Co. AS, Oslo, Norway), which was reported in detail previously [27]. The cells were identified by their typical light and electron microscopic appearance, immunofluorescent staining for desmin and vimentin [28], vitamin A-specific autofluorescence [27] and, negatively, by their inability to phagocytose latex beads (LB11 polystyrene beads, mean diameter 1.1 μm), stain for peroxidase and express Fc receptors [29]. The mean purity of freshly isolated cells was 89 ± 7% and cell viability checked by Trypan blue exclusion was 91 ± 6%. The cells were seeded at a density of 0.4×10^6 cells/10 cm^2 and maintained in 2 mL of DMEM containing 4 mmol/L L-glutamine, penicillin (100 I.U./mL), streptomycin (100 μg/mL) and 10% (v/v) FCS (all from Boehringer GmbH, Mannheim, Germany). The cells were cultured in a humidified atmosphere of 5% CO_2–95% air. The first medium change was carried out 16 hr after seeding, after which time the purity of fat storing cells was higher than 95%.

Determination of proteoglycan/glycosaminoglycan synthesis. Fat storing cells were exposed to different concentrations of PNP-Xyl or PNP, each dissolved in DMSO. Control cultures received adequate concentrations of the vehicle (DMSO). The synthesis of sulfated GAG was determined by the incorporation of [^{35}S]sulfate (18.5–22.2 GBq/mmol; 740 kBq/mL medium; NEN-DuPont, Dreieich, Germany) into the GAG during a labeling period of 24 hr in the presence of PNP-Xyl or PNP. Proteoglycans were determined in the medium (where normally about 80% of newly synthesized proteoglycans are found [27]) by a nonproteolytic isolation procedure [25]. The medium was removed and centrifuged immediately (6 min, 1000 *g*, 4°). Cell-free medium (700 μL) was mixed with 3 mL buffer A (7 M urea, 1 mM EDTA, 1 mM PMSF, 10 mM NEM, 0.1% CHAPS, 0.13 M Tris–Cl, pH 7.5) and unlabeled GAG (hyaluronan, heparin, chondroitin-4-sulfate, chondroitin-6-sulfate; all from Sigma) was added as carrier. Then the proteoglycans were bound to a batch of DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with the same buffer. After washing the resin with buffer B (= buffer A + 0.1 M NaCl, pH 7.5) total proteoglycans were eluted with buffer C (= buffer A + 0.8 M NaCl, pH 7.5). An aliquot of the eluate was counted for radioactivity and referred to the DNA content of the culture.

Measurement of [^{3}H]*thymidine incorporation into DNA.* DNA synthesis was determined by incubation of the cells with [6-^{3}H]thymidine (673 GBq/mmol, 18.5 kBq/mL medium, NEN-DuPont) during a labeling period of 24 hr. Radioactivity incorporated into DNA was measured as described previously [25].

Immunofluorescent stainings of cytoskeletal proteins. At the end of the culture time cells were washed with PBS and fixed with ethanol/acetic acid (95:5, v/v) for 15 min at 4°. The cells were then washed again and incubated with fetal calf serum/PBS (50:50, v/v) for 15 min at 37°. After repeated washings the cells were incubated for 2 hr at 37° with appropriate dilutions of monoclonal mouse antibodies to human desmin, vimentin (Dakopatts, Glostrup, Denmark) and the α-actin isoform of smooth muscle cells (Boehringer GmbH). Then, the cells were washed with PBS and incubated for 90 min at 37° with biotinylated rabbit antimouse-immunoglobulin followed by FITC-conjugated streptavidine (Dakopatts), to demonstrate cytoskeletal proteins. Control cultures were processed similarly but without first incubation with antibody.

Determination of the concentration of p*-nitrophenol in cells and medium.* *p*-Nitrophenol was determined in medium and cells exposed for various lengths of time to PNP-Xyl or PNP. The cell layer was rinsed three times with PBS without calcium and magnesium and then detached by incubation for 10 min with 5 mL of a solution (in PBS, pH 7.4) of trypsin (0.5 g/L)–EDTA (0.2 g/L). Trypsin was blocked by addition of trypsin inhibitor (2 mg/mL, from soybean, type I-S; Sigma) and the cell suspension was centrifuged (2000 *g*, 5 min, 4°). The pellet was dissolved in DNA buffer (50 mM phosphate, 2 M NaCl, pH 7.4) of which an aliquot was used for the determination of DNA; the remainder was diluted with an equal volume of 1 M Na_2CO_3 and mixed vigorously. After centrifugation the absorption of

the clear supernatant was read at 405 nm and compared with a standard curve of PNP, treated identically. Medium was diluted with an equal volume of 1 M Na_2CO_3 and read at 405 nm against the respectve standard curve.

Enzymatic hydrolysis of p-*nitrophenyl-β-*D-*xylopyranoside in lysates of fat storing cells.* Confluent layers of 7-day-old primary cultures of fat storing cells in 75 cm^2 flasks were washed three times with PBS and detached with trypsin, as described above. The cell pellet was suspended in an ice-cold solution of 0.25 M sucrose in 0.1% Triton X-100 and sonicated repeatedly for a total of 15 min. After incubation for 20 min in ice the lysate was centrifuged and an aliquot of the supernatant containing 3.5 mg protein/mL was used for the hydrolysis of PNP-Xyl added at a final concentration of 5 mM. The incubation was performed at 37° in 0.1 M sodium acetate buffer, pH 5.0. The reaction was terminated by addition of an equal volume of 1 M Na_2CO_3 before the absorbance was read at 405 nm and compared with a standard curve of PNP.

General techniques. Cells were quantitated by fluorometric determination of DNA [30]. Viability was assayed by the Trypan blue exclusion test, measurement of LDH activity in the medium and fluorochromasia [31]. For the latter test, cells were incubated for 15 min at 37° in the incubator with 0.1 μg/mL fluorescein diacetate. Nonvital cells were identified by a negative staining in fluorescence microscopy [31]. Protein concentration of the cell extract was measured by a modification [32] of the method of Lowry *et al.* [33] using bovine serum albumin as standard.

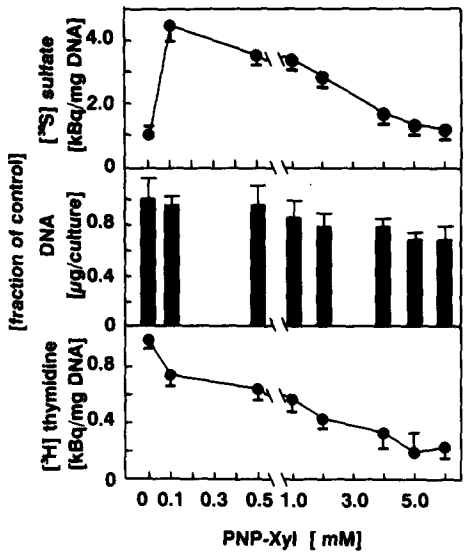

Fig. 1. Dose-dependent effects of PNP-Xyl on the synthesis of [^{35}S]sulfate-labeled medium GAG (upper graph), the DNA content per culture well (middle graph) and the incorporation of [^{3}H]thymidine into DNA (lower graph) of fat storing cells exposed for 3 days to increasing concentrations of PNP-Xyl. The exposure was started on the 2nd day after seeding. Medium was changed daily. Control cultures (0 mM PNP-Xyl) received 0.1% DMSO. Mean values ± SD of triplicate cultures are fractions of control (defined to be 1.0).

RESULTS

Dose-dependent effects of p-*nitrophenyl-β-*D-*xyloside and* p-*nitrophenol on the synthesis of sulfated medium glycosaminoglycans and on the proliferation of fat storing cells*

The exposure of non-confluent early primary cultures of FSC to increasing concentrations of PNP-Xyl resulted in a dose-dependent stimulation of the synthesis of sulfated medium GAG (Fig. 1). Maximum enhancement of [^{35}S]sulfate-labeled GAG formation greater than 4-fold above control was achieved at 0.1 mM PNP-Xyl; concentrations exceeding this value reduced progressively the degree of stimulation. PNP-Xyl also influenced in a dose-dependent manner the proliferation of FSC as shown by a reduction in the incorporation of [^{3}H]thymidine and by a decline in the DNA content per culture well (Fig. 1). At 0.1 mM PNP-Xyl, incorporation of [^{3}H]thymidine was reduced by 25%; maximum inhibition of about 80% was reached at 5 mM PNP-Xyl. The DNA content per well decreased by about 30% during an incubation period of 3 days with 5 mM PNP-Xyl, this was not due to cell detachment but rather to inhibition of cell multiplication. The solvent of PNP-Xyl, i.e. 0.1% DMSO, was without any effect on the incorporation rate of [^{3}H]thymidine into DNA and of [^{35}S]sulfate into GAG (data not shown). Experiments similar to those described for PNP-Xyl were performed with PNP (Fig. 2). This

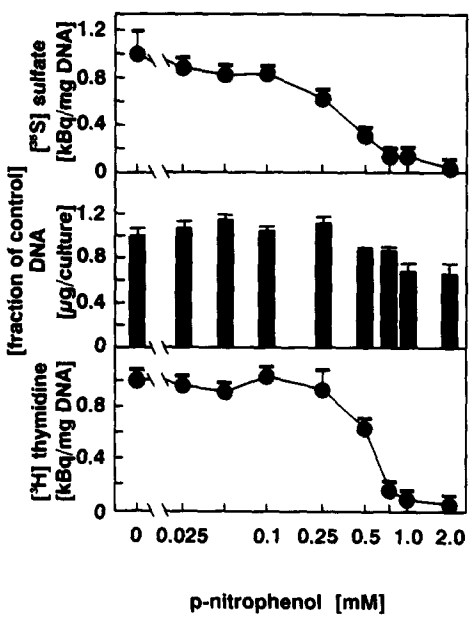

Fig. 2. Dose-dependent effects of PNP on the synthesis of [^{35}S]sulfate-labeled medium GAG (upper graph), the DNA content per culture well (middle graph) and the incorporation of [^{3}H]thymidine into DNA (lower graph) of fat storing cells exposed for 3 days to PNP. Culture conditions were similar to those described in the legend to Fig. 1. Results are expressed as fractions of control.

compound did not stimulate but decreased dose-dependently the formation of [^{35}S]sulfate-labeled medium GAG at concentrations exceeding 0.1 mM (Fig. 2). PNP concentrations of 0.5 and 0.75 mM caused reductions of about 75 and 89%, respectively, in labeled GAG synthesis. Similarly, a steep decline in the incorporation of [^{3}H]thymidine was measured at PNP concentrations higher than 0.25 mM (Fig. 2). Exposure of FSC to 0.75 mM PNP for 3 days resulted in an 84% inhibition of [^{3}H]thymidine incorporation which was paralleled by a 15% reduction of the DNA content per well (Fig. 2).

Neither PNP-Xyl nor PNP at the concentrations and under the conditions used affected the viability of FSC in early primary cultures. Using Trypan blue exclusion, positive fluorochromasia and LDH activity in the media as criteria, there were no significant differences between controls (around 95% viable), 5 mM PNP-Xyl. (90% viable) and 0.75 mM PNP-treated (87% viable) cultures. Furthermore, the inhibition of cell growth by 0.5 mM PNP was fully reversible upon removal of the drug (data not shown), a similar finding to that described previously for 5 mM PNP-Xyl [23].

Effects of p*-nitrophenol on the morphology and cytoskeletal organization of fat storing cells*

A striking phenomenon observed previously by us using PNP-Xyl (5 mM)-treated FSC cultures, but also by others using xyloside-exposed vascular smooth muscle cells [19] concerns the disorganization and reduced expression of desmin- and smooth muscle iso-α-actin-containing cytoskeletal filaments, important markers of transforming FSC [34–36]. Therefore, we exposed FSC for 6 days under similar conditions to an inhibitory concentration of PNP (0.75 mM) to see whether PNP might mimic the effects of PNP-Xyl on the expression of cytoskeletal filaments. The expression and organization of α-actin and desmin were affected greatly by the treatment of FSC for 6 days with PNP (Fig. 3). Thick α-actin bundles were expressed in about 50% of untreated cells at this stage of culture. They were either absent or stained very faintly in more than 90% of all FSC exposed to the compound. Similarly, desmin-containing filaments were found in about 70% of control FSC but this fraction was reduced to nearly 30% in cultures kept in the presence of PNP. The nitrophenol-induced changes were completely reversible within 3 days of withdrawal of the drug, as seen in Fig. 3C. Typical structures of α-actin- and desmin-containing filaments were observed in almost 70 and 80% of FSC, respectively. The morphology of vimentin-positive filaments was also changed by treatment with PNP, however, the fraction of vimentin-positive cells (nearly 100%) remained unchanged. The vimentin structure appeared to be condensed during treatment but soon after removal of PNP the typical filamentous network of vimentin reappeared (data not shown).

The phase contrast microscopic appearance of FSC was changed by exposure to PNP (0.75 mM) for 6 days (Fig. 4). The drug decreased cell density considerably; number and length of cellular extensions were reduced; extensions were thinner than in control cultures, cell–cell contact was found

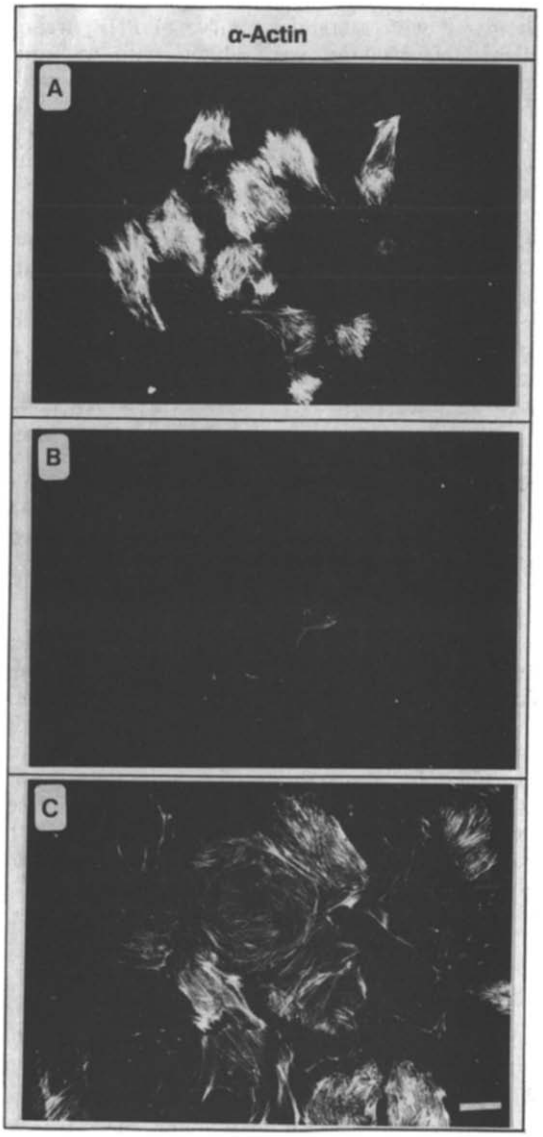

Fig. 3. Effect of PNP on immunofluorescent staining patterns of smooth muscle iso-α-actin in primary cultures of fat storing cells. Beginning on the 1st day after seeding FSC were exposed for 6 days to 0.1% DMSO (control, A) or 0.75 mM PNP (B). Some cultures exposed previously to PNP for 6 days were washed with fresh medium and cultured for a further 3 days in the absence of PNP before immunostainings were performed (C). Representative stainings are shown. Bar = 100 μm.

rarely and the number and size of lipid droplets was decreased by a smaller extent than in control cultures (Fig. 4B). Upon removal of the drug the normal phenotype of FSC reappeared within 3 days and the cell density increased strongly (Fig. 4C). Morphological evidence of toxic cell damage was not obtained in cultures treated with PNP.

Generation of p*-nitrophenol from* p*-nitrophenyl-β-*D*-xyloside in cell culture and cell extracts*

The results presented so far indicate that PNP

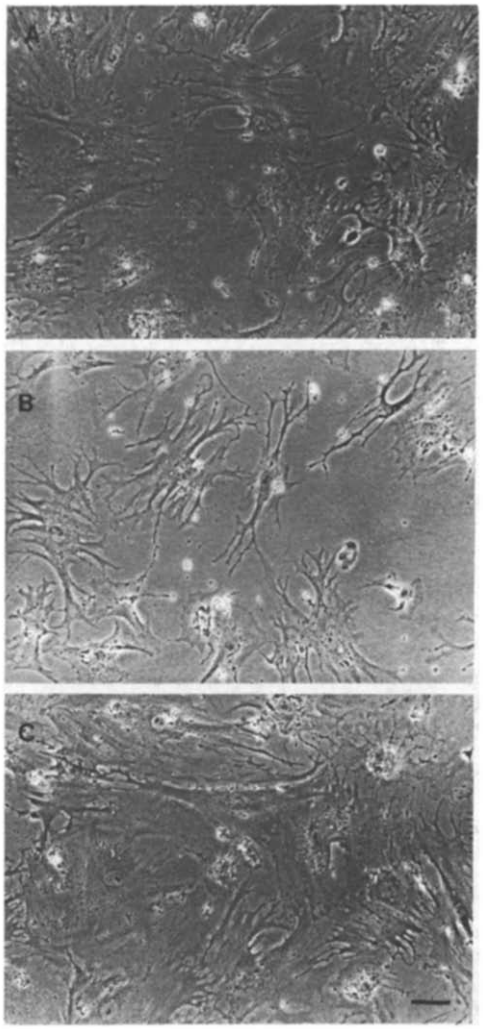

Fig. 4. Phase contrast microscopy of rat liver fat storing cells cultured, as described in the legend to Fig. 3, for 6 days in the presence of 0.1% DMSO (A, control) or 0.75 mM PNP (B). A culture exposed to PNP for 6 days and then for a further 3 days to PNP-free medium is shown in C. Bar = 50 μm.

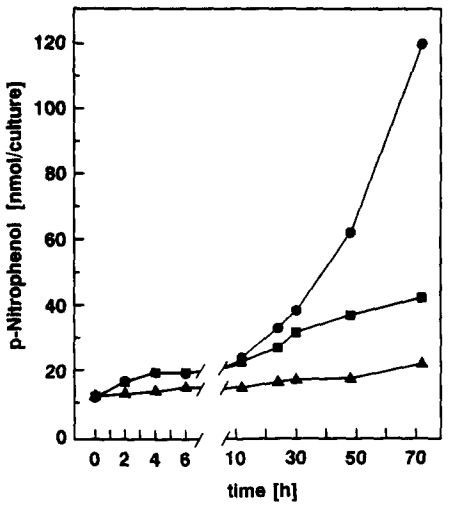

Fig. 5. Time course of the generation of PNP from PNP-Xyl in the medium of cultured fat storing cells (●), culture medium (DMEM + 10% FCS) without cell contact (■) and culture medium which has been pre-conditioned by incubation with fat storing cells (▲). Starting on the 2nd day of primary culture, FSC in 2 mL medium were exposed to 5 mM PNP-Xyl and, at the times indicated (0–72 hr), medium was aspirated, diluted with 1 M Na_2CO_3 and absorbance was read at 405 nm. Culture medium which was not in contact with cells or conditioned was analysed similarly. Conditioned medium was prepared from confluent primary cultures of FSC (3×10^6 cells/75 cm^2) kept for 3 days in 15 mL medium. Mean values of duplicate determinations are shown.

imitates dose-dependently some of the major effects which PNP-Xyl exerts on FSC functions. This finding suggests that the aglycone moiety cleaved intracellularly from PNP-Xyl could be the mediator of some of those effects previously ascribed tentatively to the inhibition of proteoglycan formation. In fact, during culture of FSC in the presence of 5 mM PNP-Xyl the concentration of free PNP in the medium increased time-dependently (Fig. 5): between 2 and 72 hr of incubation it increased from 17 to 120 nmol/culture. In contrast, in complete incubation medium without any cell contact only a 2.5-fold elevation of the concentration of PNP was measured during the same time period (at 72 hr 42.5 nmol/culture). Medium conditioned by incubation with FSC before PNP-Xyl was added, in the absence of cells, was ineffective in the generation of PNP, ruling out the release of substantial amounts of catalytically active enzymes hydrolysing PNP-Xyl (Fig. 5).

The intracellular concentration of PNP in PNP-Xyl-treated (5 mM) cultures rose time-dependently 1 day after addition of the compound (Fig. 6). On average, 0.95 nmol PNP/day/μg DNA was generated in early FSC cultures. Interestingly, the addition of 0.75 mM PNP (a proliferation inhibiting concentration) to FSC resulted in a time-course of intracellular PNP concentrations very close to that generated by 5 mM PNP-Xyl. This is shown also in large scale cultures of FSC exposed for 2 days to PNP-Xyl or PNP (Table 1). Under both conditions the amount of PNP associated with the cell layer was very similar, clearly indicating that the addition of PNP-Xyl to FSC results in the intracellular liberation of PNP, to reach concentrations which are sufficient to inhibit the proliferation of FSC and the expression of cytoskeletal filaments.

Enzymatic hydrolysis of PNP-Xyl was also demonstrated in lysates of cultured FSC (Fig. 7) which generated, by incubation with PNP-Xyl (5 mM), 7.7 nmol PNP/hr/190 μg protein. Based on a protein/DNA ratio of about 16 for FSC [37], 0.65 nmol PNP/hr/μg DNA or 1×10^5 cells were produced *in vitro*. These data demonstrate the ability of FSC lysates and of intact cultures to split the aglycone–xyloside binding in a dose- and time-dependent fashion.

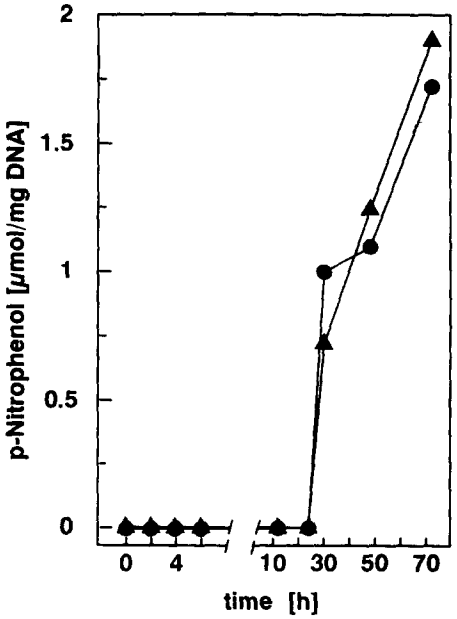

Fig. 6. Time-course of the intracellular concentration of PNP in fat storing cells exposed to PNP-Xyl (▲) or PNP (●). Beginning on the 2nd day, FSC were cultured in the presence of 5 mM PNP-Xyl or 0.75 mM PNP. At the times indicated (0–72 hr) cells were washed free of medium, detached and lysed, and the concentration of PNP was determined spectrophotometrically and referred to the DNA content of the well. Mean values of triplicate determinations are shown.

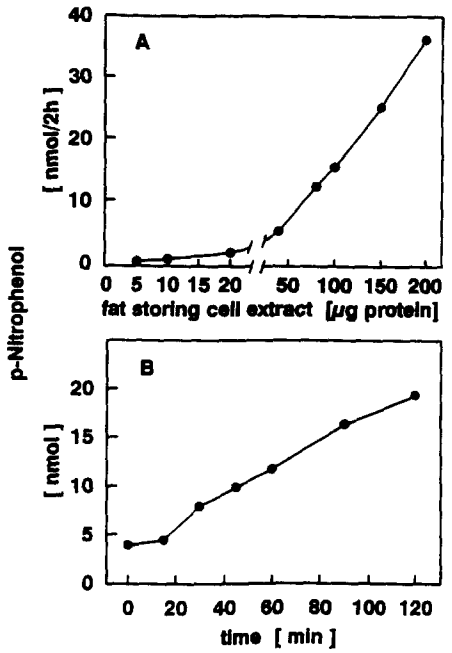

Fig. 7. Generation of PNP from PNP-Xyl *in vitro* by extracts from fat storing cells. Lysate (3.5 mg protein/mL) from 7-day-old confluent FSC was prepared and incubated in 0.1 M Na-acetate, pH 5, at 37° with 5 mM PNP-Xyl. Reaction was terminated by addition of an equal volume of 1 M Na_2CO_3 and absorbance was read at 405 nm. The dependency of PNP generation on the amount of cell lysate (A) and the incubation time (B) is shown. The time-course was performed with 190 μg lysate protein/assay.

DISCUSSION

Cultured fat storing cells, the major cell type synthesizing proteoglycan [27, 37, 38] and other extracellular matrix components [39] in liver, are sensitive to the treatment with *p*-nitrophenyl-β-D-xyloside, a proposed selective inhibitor of proteoglycan synthesis [6]. As we have shown recently [23], the major cellular effects of PNP-Xyl were found to be dose-dependent stimulation of [^{35}S]sulfate-labeled GAG synthesis; abrogation of proteoglycan formation; dose-related and reversible inhibition of proliferation which was not accompanied by signs of toxic cell damage; diminution and

Table 1. Determination of PNP in media and cell layers of primary cultures of fat storing cells exposed to proliferation inhibitory concentrations of PNP (0.75 mM) or PNP-Xyl (5 mM)

Culture conditions	DNA (μg/culture)	PNP (μmol/mg DNA)	
		Cells	Medium
Control (0.1% DMSO)	31.2	0	0
PNP (0.75 mM)	28.8	0.84	378
PNP-Xyl (5 mM)	29.7	0.79	60

Each 4×10^6 fat storing cells were seeded in 75 cm^2 flasks and cultured under standard conditions with 15 mL medium. On the 3rd day cells received medium containing either 0.75 mM PNP, 5 mM PNP-Xyl or 0.1% DMSO. After 48 hr medium was aspirated and cells were washed three times with PBS and trypsinized for 10 min followed by addition of 2 mg/mL soybean trypsin inhibitor. The cell suspension was centrifuged and the pellet resuspended in DNA-buffer (50 mM phosphate, 2 M NaCl, pH 7.4) of which a 500 μL aliquot was mixed with an equal volume of 1 M Na_2CO_3 to read absorbance at 405 nm. The amount of PNP was related to the DNA content of the culture. Similarly, an aliquot of the medium was mixed with Na_2CO_3 to determine PNP.

disorganization of cytoskeletal filament structures, mainly of the desmin- and smooth muscle α-actin type; and reduction of medium hyaluronan production. Taking all these effects together, PNP-Xyl appeared to slow down the transformation of FSC into myofibroblast-like cells, which occurs spontaneously during culture of these cells on plastic surfaces [36, 40] but also takes place at sites of necroinflammation in human and experimental liver injury [35, 41]. Since β-D-xyloside derivatives are used frequently as one approach toward studying proteoglycan function, by determining the functional consequences during inhibition of proteoglycan synthesis, the results suggest tentatively an involvement of these molecules in fundamental cell reactions such as FSC transformation [23]. In applying this strategy, results similar to those described above have been found with a variety of cultures of other cell types, whole organ cultures and embryo cultures. The xyloside-induced changes imply distortions of cytoskeletal structures [19], reduced accumulation of extracellular matrix [19], inhibition of cell proliferation [11, 18–21, 42] and modulations of embryonic growth, morphogenesis, tissue development and cytodifferentiation [13, 15–17, 43–45]. An additional finding compatible with that observed in our studies was reported for T-cells in which the PNP-Xyl-induced stimulation of [^{35}S]sulfated GAG synthesis was reversed progressively by further enhancement of the drug [20]. This phenomenon was explained hypothetically by a dose-dependent stimulation of the degradation of PNP-Xyl-initiated GAG [20]. Up until recently, the role of the aglycone moiety in determining the specificity and effectiveness of β-xylosides went largely unappreciated. Only a few reports are available which compare systematically the biological effectiveness of and the metabolic changes in GAG/proteoglycan synthesis with the chemical derivation of the aglycone residue [13, 21]. These studies indicate clearly that the cellular effects (e.g. on growth activity) [21], alteration of chicken embryonic development [23] and modulation of sulfated GAG synthesis [13, 21] are dependent on the nature of the non-carbohydrate structure of the xyloside. In this report we show that, for FSC which was used as a model cell culture system, during treatment with PNP-Xyl a significant fraction of PNP is liberated enzymatically. Intracellular concentrations of free PNP were reached which were found, by comparison with the effects of authentic PNP, to be sufficient to mimic some of those effects (on cytoskeletal structures and inhibition of proliferation) otherwise ascribed to the inhibition of proteoglycan synthesis. It is proposed, at least for FSC but also likely to be so for other cell and organ culture systems, that some of the functional consequences related to abrogated proteoglycan synthesis might be interfered with by the effects of the aglycon residue. This is of particular importance for PNP for which a general toxicity is acknowledged [46, 47]. The doses of PNP-Xyl used here are significantly higher than that needed for the uncoupling of the synthesis of core protein and glycosaminoglycans. Therefore, the magnitude of the effect which PNP might exert could be overestimated. However, the concentration of PNP-Xyl at which interference by liberated PNP might become relevant is hard to determine and, probably, can not be generalized because the rate of enzymatic cleavage may depend on the cell type, culture conditions, time of PNP-Xyl exposure and treatment of cells with growth factors and other modulators. In addition, the efficiency of the cellular mechanisms of detoxification will also be relevant for the control of the steady state level of free intracellular PNP. We found that even in fresh medium containing 10% fetal calf serum a small fraction of PNP-Xyl was cleaved but, as shown with the conditioned medium, FSC did not secrete (catalytically active) enzymes which hydrolysed PNP-Xyl extracellularly. The magnitude of the generation of PNP from PNP-Xyl becomes uncontrollable in more complex biological systems such as tissue, organ, or whole body cultures. The results lead us to suggest that careful consideration of the potentially interfering effects of PNP is necessary before the cellular consequences observed under conditions of PNP-Xyl-induced abrogated proteoglycan synthesis can be interpreted in terms of the functional roles which proteoglycans may fulfil within and outside a cell. Alternatively, the functions of these macromolecules might be studied by using glutamine analogues, which are potent inhibitors of glycosaminoglycan synthesis [48]; by transfection of cells with cDNA constructs encoding specific core proteins [49] and by blocking the translation of core protein mRNAs using antisense oligonucleotides. The latter two approaches offer the major advantage of being highly selective among the increasing number of structurally and functionally diverse species of individual proteoglycans detected.

Acknowledgements—The skilful technical support of Ms Birgit Lahme, Mrs Brigitte Heitmann and Mr. Lothar Scheckel is gratefully acknowledged.

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