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CHLORATE INHIBITS TYROSINE SULFATION OF HUMAN TYPE III PROCOLLAGEN WITHOUT AFFECTING ITS SECRETION OR PROCESSING

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Sodium chlorate, a potent inhibitor of sulfation reactions, completely inhibits the formation of tyrosine-O-sulfate in type III procollagen in human fibroblasts, when used in concentrations that do not affect the incorporation of radioactive amino acids into protein. The unsulfated type III procollagen is secreted into the medium at a rate comparable to those of sulfated type III procollagen and type I procollagen, which normally does not undergo sulfation. The enzymatic cleavage of the aminoterminal propeptide of type III procollagen is incomplete in fibroblast cultures, irrespective of the sulfation status of the protein.

Tyrosine sulfation is a wide-spread posttranslational modification of proteins (1). The reaction takes place in the trans Golgi and is catalysed by a transferase, an integral membrane protein of this cellular compartment (1,2). In a constitutively secreted protein sulfation of tyrosine is most probably the last modification occurring before the exit of the protein (2). Specific inhibitors of sulfate incorporation, including chlorate, which inhibits the enzyme sulfate adenylyltransferase, provide means for testing the biological significance of this modification in proteins (3).

Two collagenous proteins, type III procollagen (4,5) and partially processed forms of type V procollagen (6,7), contain sulfated tyrosine residues, which are the reason for the low isoelectric points of these proteins (4,6). In both cases, the anion is located in the aminoterminal part of the molecule, the exact locations being still unknown. Interestingly, both of these precursors of interstitial collagens undergo only a slow and partial processing at the aminoterminus, which is in contrast to the situation at the carboxyterminal end of the same molecules and to that at the aminotermini of other interstitial procollagens, such as

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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types I and II. However, the biological significance of tyrosine-O-sulfate has not yet been cleared for any collagenous protein.

The aim of the present study was to find out whether tyrosine sulfation of human type III procollagen can be modified by chlorate and whether this affects the rates of synthesis or secretion of this protein in fibroblasts.

MATERIALS AND METHODS

Cell culture and metabolic labeling

Human skin fibroblasts were cultured as described previously (5). For metabolic labeling, confluent cultures were incubated for the times indicated in fresh Dulbecco's medium without serum but containing β-aminopropionitrile fumarate (50 µg/ml) and with one of the following radioactive substances: L-[2,3-^{3}H]proline (10 µCi/ml), L-[^{3}H]tyrosine (25 µCi/ml) or ^{35}S-sulfate (0.3 mCi/ml). Sulfate-free medium, containg only 1 % of the normal concentrations of cystine and methionine, and tyrosine-free medium were used in the experiments with radioactive sulfate and tyrosine, respectively. Sodium chlorate was used in the concentrations given; the chlorate-treated cultures were preincubated in chlorate-containing medium for 1 h before adding the radioactive isotope.

Processing of culture medium and pericellular matrix

After incubation, protease inhibitors were added to the medium (5), which was exhaustively dialyzed against the immunoprecipitation buffer. Pericellular matrix free of cells was prepared with the deoxycholate procedure (8).

Immunoprecipitation, SDS PAGE and fluorography

Immunoprecipitation was carried out essentially as described by Cooper et al. (9). The antibodies against the aminoterminal propeptide of human type III procollagen were affinity-purified (5). SDS-PAGE was carried out according to Laemmli (10) with 6.5 % or 12.5 % acrylamide in the separating gel. After electrophoresis, the gels were processed for fluorography and exposed to Kodak-X-Omat autoradiographic films.

Radioimmunoassays

The concentration of the aminoterminal propeptide of type III procollagen was determined by radioimmunoassay, as presented previously (11). For the radioimmunoassay measuring the carboxyterminal propeptide of type I procollagen, the antigen was purified from the culture medium of human fibroblasts and antibodies were raised in rabbits (12,13).

Statistical method

The statistical significances of the differences between control and chlorate-treated cultures were tested with Student's t test.

RESULTS

An overnight incubation of confluent fibroblast cultures in the presence of 10 mM sodium chlorate did not affect the viability of the cells or the incorporation of radioactive proline or tyrosine into medium proteins (Table 1). In contrast, the incorporation of radioactive sulfate into medium macromolecules was dramatically reduced (Table 1,

Table 1: Effect of 10 mM sodium chlorate on incorporation of radioactive precursors into medium macromolecules in fibroblasts

Precursor	Control	Chlorate	
	dpm/10^6 cells		(% of control)
[^{3}H]Proline	657 000	701 000	(107)
[^{3}H]Tyrosine	10 300 000	10 600 000	(103)
[^{35}S]Sulfate	11 000 000	415 000	(4)

The labeling was carried out for 20 h. For experimental details, see Materials and Methods.

Fig. 1). No sulfate-labeled type III procollagen could be immunoprecipitated after this treatment, whereas the unsulfated form of this protein could easily be visualized if labeled with radioactive proline (Fig. 1).

The secretion of type I and III procollagens was quantified with specific radioimmunoassays for their precursor-specific propeptide sequences. Incubation in the presence of chlorate slightly reduced the amounts secreted into the medium during 20 h, with no difference between the two procollagen types (Table 2).

The secretion kinetics of type I and III procollagens were assessed during shorter incubations in the presence of 1 mM and 10 mM chlorate

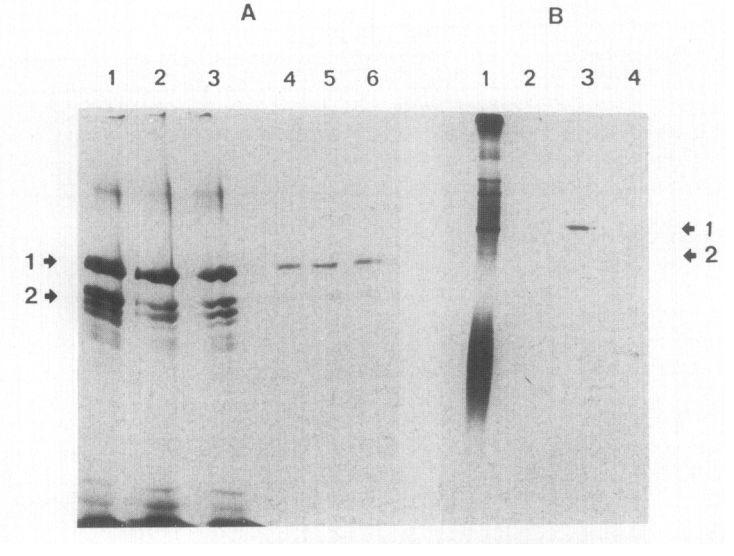

Fig. 1. Analysis of labeled macromolecules secreted into the medium during 20 h of culture (6.5 % gel). A: [^{3}H]proline labeling; B: [^{35}]sulfate labeling. Lanes A1, A4, B1 and B3 represent control cultures, lanes A2 and A5 cultures incubated in the presence of 1 mM sodium chlorate, and lanes A3, A6, B2 and B4 cultures incubated in the presence of 10 mM sodium chlorate. Lanes A1-A3, B1 and B2 unfractionated medium; lanes A4-A6, B3 and B4 immunoprecipitates with antibodies against the aminoterminal propeptide of type III procollagen. The markers are the proα1(III) chain (1) and the pNα1(III) chain (2) of human type III procollagen.

Table 2: Effect of 10 mM sodium chlorate on secretion of procollagens into the medium during 20 h

Procollagen type	Control	Chlorate	
	$\mu g/10^6$ cells		(% of control)
Type I*	17 ± 3	14 ± 3	(82)
Type III**	0.121 ± 0.018	0.091 ± 0.02	(75)

*Expressed as concentration of carboxyterminal propeptide.
**Expressed as concentration of aminoterminal propeptide.
For experimental details, see Materials and Methods.

(Fig. 2). In a short-term experiment, lasting for up to 2 h, the secretion rate of unsulfated type III procollagen was comparable to that of the sulfated protein. During longer incubations, for up to 23 h, there was a tendency for the chlorate-treated cells to secrete less of both

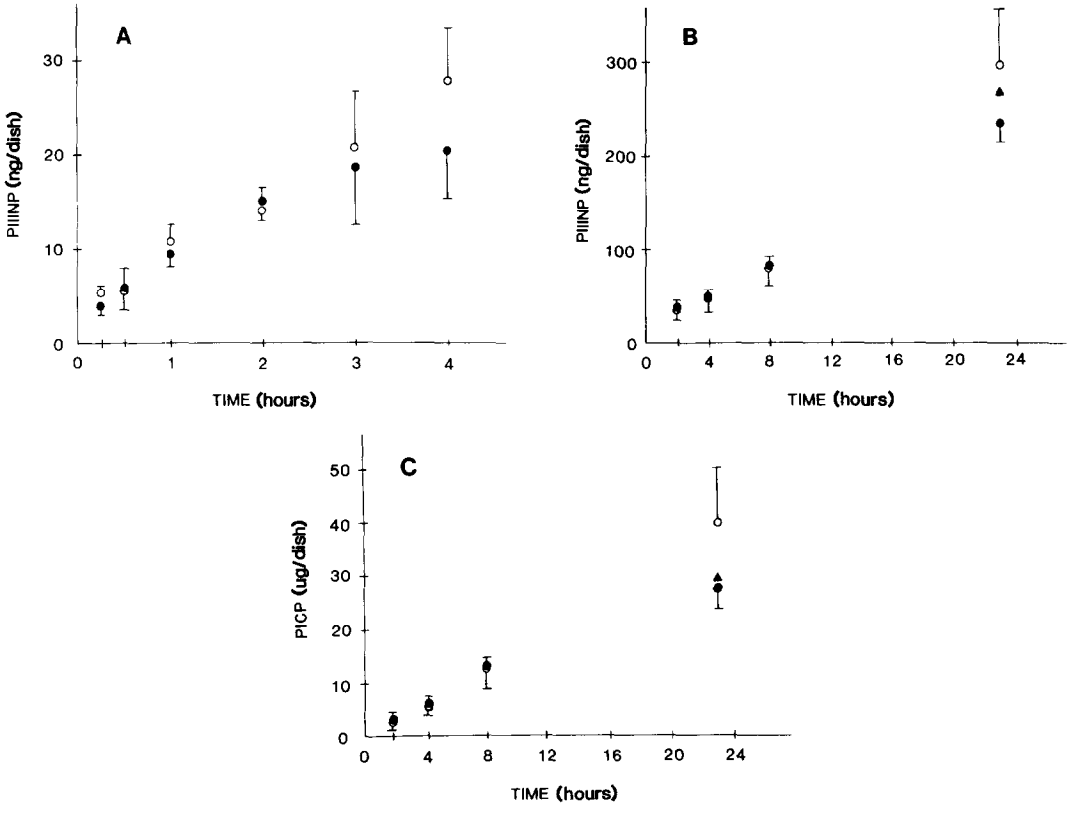

Fig. 2. Secretion of type I and type III procollagens by fibroblast cultures. The concentrations were assessed in cell culture medium with radioimmunoassays for the carboxyterminal and aminoterminal propeptides of these procollagens, respectively. Open symbols: control cultures; closed symbols: chlorate treated cultures (● = 10 mM, ▲ = 1 mM). Each value represents mean±SD of four cell culture dishes. A. Type III procollagen (first 2 h); B. Type III procollagen (up to 23 h); C. Type I procollagen (up to 23 h). In B and C, the values obtained with 1 mM chlorate at 2, 4 and 8 h were identical to those obtained for the controls.

type I and type III procollagens than did the control cells, but the difference was not statistically significant at any point in time.

Immunoprecipitation with monospecific antibodies against the amino-terminal propeptide of type III procollagen did not indicate the presence of any free propeptide either in the medium or in the pericellular matrix (not shown). In gel filtration of cell culture medium on Sephacryl S-300, the majority of this antigen was recovered in the elution position of procollagen, with no difference between the cultures incubated with or without chlorate (not shown).

DISCUSSION

Sulfated tyrosine occurs in several different kinds of proteins, the structures of which have otherwise relatively little in common (1). In the extracellular matrix, this modification is present at least in precursors of type III and V collagens, in fibronectin and in nidogen/entactin. According to our present results, the presence or absence of tyrosine-O-sulfate does not affect the synthesis and secretion of type III procollagen in human skin fibroblasts. Thus this protein behaves in a manner identical to a major proteoglycan (PG II) of human fibroblasts (14), in which the sulfation of the glycosaminoglycan side chains can be inhibited without affecting its secretion rate. We assessed the synthesis rates of type III procollagen, which is normally tyrosine sulfated, and type I procollagen, which normally does not undergo sulfation, with specific radioimmunoassays. This approach is superior to measuring the incorporation of radioactive amino acids into the proteins, since it is not sensitive to any changes in the specific radioactivities of the amino acid pools, possibly induced by the chemical modifier.

Tyrosine sulfate is found in the aminoterminal parts of two precursors of interstitial collagens (types III and V) which are characterized by their slow processing at this end. In the present experiments, no change in the rate of cleavage of the aminoterminal propeptide could be brought about by inhibiting the sulfation, however. This may mean that the presence or absence of the negatively charged sulfated residue has no effect on the process whereby a specific N-proteinase removes the aminoterminal propeptide from the type III collagen proper. Another explanation could be lack of a sufficient concentration of this enzyme in the vicinity of the substrate under cell culture conditions (15); if this is the case, the removal of any inhibitory effect of the sulfate would go unnoticed.

An important function of the propeptide parts of interstitial procollagens is thought to be to prevent premature aggregation and fibril

formation. This function is probably greatly enhanced by a strong negative charge in a propeptide, a situation analogous to that in fibrinogen, where the fibrinopeptides fulfill a similar function. Interestingly, in these such a charge can be provided either by sulfate (16) or by phosphate (17), depending on species.

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