Specific Inactivation of Prolyl 4-Hydroxylase and Inhibition of Collagen Synthesis by Oxaproline-containing Peptides in Cultured Human Skin Fibroblasts*

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The crucial role of collagen in fibrotic disorders has prompted attempts to develop drugs that inhibit collagen accumulation. Peptides containing the unphysiological amino acid 5-oxaproline (Opr) have recently been found to act as specific, syncatalytic inactivators of pure prolyl 4-hydroxylase, the enzyme that catalyzes the formation of 4-hydroxyproline in collagens. The present study indicates that oxaproline-containing peptides benzyloxycarbonyl-Phe-Opr-Gly-benzyl ester (I) and benzyloxycarbonyl-Phe-Opr-Gly-ethyl ester (II) inactivate prolyl 4-hydroxylase in cultured human skin fibroblasts, peptide I being about twice as potent as peptide II. Inactivation by 50% was observed after culturing with about 20–40 μM concentrations of peptide I for 48 h. The inactivation appears to be specific, as no changes were found in the activities of two other intracellular enzymes of collagen synthesis, lysis hydroxylase and galactosylhydroxylsyl glucosyltransferase. Synthesis of 4-hydroxyproline by the cells was markedly decreased, and 4-hydroxyproline-deficient procollagen accumulated intracellularly, and no changes were found in the incorporation of [14C]leucine into protein after culturing of the cells with a 30 μM concentration of peptide I for 48 h. No changes were seen in the viability of the cells or the release of lactate dehydrogenase from them into the culture medium. No significant changes were found in the steady-state levels of the mRNAs for the pro-α chains of type I and type III procollagens or for the α and β subunits of prolyl 4-hydroxylase or fibronectin after culturing with 75 μM peptide I for 48 h. The data indicate that inactivation of cellular prolyl 4-hydroxylase has marked effects on cellular 4-hydroxyproline formation and collagen secretion but no effects on the steady-state levels of mRNAs for type I and III procollagens or the two types of subunit of prolyl 4-hydroxylase.

Prolyl 4-hydroxylase (procollagen L-proline, 2-oxoglutarate: oxygen oxidoreductase, 4-hydroxylating, EC 1.14.11.2) catalyzes the formation of 4-hydroxyproline in collagens and other proteins with collagen-like amino acid sequences by the hydroxylation of proline residues in peptide linkages. The reaction requires Fe2+, 2-oxoglutarate, O2, and ascorbate and involves an oxidative decarboxylation of 2-oxoglutarate (for recent reviews, see Refs. 1 and 2).

Prolyl 4-hydroxylase plays a central role in collagen synthesis, as the 4-hydroxyproline residues formed are an absolute requirement for the folding of the newly synthesized procollagen polypeptide chains into triple helical molecules (1, 2). This crucial function of 4-hydroxyproline in collagens makes prolyl 4-hydroxylase a potential target for pharmacological modulation of the excessive collagen accumulation found in patients with various fibrotic disorders, as inhibition of this enzyme reaction will lead to a nonfunctional protein that is rapidly degraded (1–4). Potent competitive inhibitors with respect to 2-oxoglutarate and ascorbate have been described (5–7) and shown to suppress 4-hydroxyproline formation in cultured cells (8–11), and synthetically acting irreversible inactivators have also been developed recently which act as analogues of these two cosubstrates of the enzyme (12, 13). Nevertheless, the opportunities for using these compounds as antifibrotic agents depend on whether their action upon other 2-oxoglutarate- or ascorbate-dependent enzymes can be excluded or eliminated.

Very recently peptides containing the unphysiological amino acid 5-oxaproline (Opr)1 (Fig. 1) in the sequence R1-Xaa-Opr-Gly-OR2 have been found to act as synthetically inactivators of prolyl 4-hydroxylase (14), those with the aromatic substitutes R1 and R2 being particularly potent as compared with peptides with an aliphatic group or without a C-terminal blocking group. The most effective peptide tested had the structure of benzyloxycarbonyl-Phe-Opr-Gly-benzyl ester and inactivated pure prolyl 4-hydroxylase by 50% in 1 h at a 0.8 μM concentration (14). The present paper investigates the effect of two such peptides, benzyloxycarbonyl-Phe-Opr-Gly-benzyl ester (I) and benzyloxycarbonyl-Phe-Opr-Gly-ethyl ester (II), on prolyl 4-hydroxylase activity and collagen synthesis in cultured human skin fibroblasts. Special emphasis is laid on the specificity of inhibition with respect to other enzymes of collagen synthesis and on possible feedback effects on the steady-state levels of mRNAs for the major types of collagens produced by these cells and the two types of subunit of prolyl 4-hydroxylase.

EXPERIMENTAL PROCEDURES

Materials—The synthesis of the oxaproline peptides has been described previously (14, 15). [U-14C]Proline, [U-15N]leucine, [6-3H]

1 The abbreviations used are: Opr, 5-oxaproline; peptide I, benzyloxycarbonyl-Phe-Opr-Gly-benzyl ester; peptide II, benzyloxycarbonyl-Phe-Opr-Gly-ethyl ester; protocollagen is a biologically prepared protein consisting of nonhydroxylated pro-α chains with molecular weights of about 150,000 (see Ref. 16).

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lysin, uridine diphosphate [14C]glucose, [3H]dATP, [3H]dCTP, [3H]dGTP, and [32P]dUTP were obtained from Amersham International, Amersham, Bucks, United Kingdom.

Cell Cultures—Human skin fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2% glutamic acid, 50 μg/ml ascorbic acid, 100 units/ml penicillin, and 100 μg/ml streptomycin. The medium was replaced daily. The cells were used in passages 3-10. Confluent cultures were incubated with the 5-oxaprolin-containing peptide I or II in concentrations of 0-100 μM for various time periods of up to 96 h.

Assays of Enzyme Activities—Prolyl 4-hydroxylase, lysyl hydroxylase (EC 1.14.11.4), and galactosylhydroxylysyl glucosyltransferase (EC 2.4.1.66) activities were measured in the fibroblasts after incubation with the oxaprolin peptide. The cell layers were washed with 8 ml of 0.14 M NaCl containing 0.01 M sodium phosphate, pH 7.4, the cells harvested by trypsinization, and the tryps inactivated with soybean trypsin inhibitor (16). The cells were centrifuged at 500 g for 5 min, washed twice with the above NaCl/phosphate solution, and homogenized with a Teflon-glass homogenizer (1600 rpm, 60 strokes) into 1.5 ml of a buffer containing 0.2 M NaCl, 0.1 M glycine, 0.1% (w/v) Triton X-100, 0.01% (w/v) soybean trypsin inhibitor, and 0.02 M Tris-HCl, pH 7.5, at 4°C (16). An aliquot of the homogenate was taken for protein determination (17), and the rest was centrifuged at 15,000 X g for 30 min at 4°C. Aliquots of the supernatant were taken for the enzyme assays. Prolyl 4-hydroxylase was determined using [14C]proline-labeled collagen and lysyl hydroxylase using [6-3H]lysine-labeled collagen prepared in isolated chick embryo tendon cells as substrates (16). Galactosylhydroxylysyl glucosyltransferase was assayed by determining the amount of radioactive glucosylgalactosyl hydroxylysine formed from uridine diphosphate [14C]glucose in a gelatinized calf skin collagen substrate (16). Lactate dehydrogenase activity in the cell medium was measured using a commercially available kit (F. Merck, Darmstadt, Federal Republic of Germany).

Collagen and Noncollagen Protein Synthesis—Confluent cell cultures were labeled with 1.5 μCi/ml [14C]proline or [14C]leucine for the last 6 h of each incubation period together with various concentrations of the oxaprolin peptide. After labeling, the medium was collected, and protease inhibitors were added to final concentrations of 20 mM EDTA, 10 mM N-ethylmaleimide, and 1 mM phenylthioureylsulfonflul fluoride. The cell layers were scraped into a buffer containing 0.2 M NaCl, 50 mM Tris-HCl, pH 7.4, and protease inhibitors in the concentrations indicated above. Both the medium samples and the cell sonicates were heated at 80°C for 15 min and dialyzed extensively in the cold against a buffer containing 0.2 M NaCl and 50 mM Tris-HCl, pH 7.4. Samples of both dialysates were taken for trichloroacetic acid-precipitable protein measurements and for hydroxyproline analyses.

The trichloroacetic acid-precipitable protein in the dialysates was filtered through 0.45-μm Millipore filters, which were then washed in 5 ml of 2-methoxyethanol and counted. [14C]Hydroxyproline and [14C]leucine incorporation experiments (see below) suggested nonspecific effects on protein synthesis in some experiments after culturing of the cells with the oxaprolin peptide for 96 h. A culture time of 48 h with the oxaprolin peptide was therefore used in most of the subsequent experiments.

In the experiments in which fibroblasts were cultured with oxaprolin peptide I for 48 h, a significant decrease by about 35% was found in the cellular prolyl 4-hydroxylase activity with a 10 μM concentration of the peptide (Fig. 2). Inactivation of the cellular enzyme by 50% was usually obtained with a 30 μM concentration (Fig. 2), but this value ranged from about 20 to 40 μM in the various experiments. This variability may reflect in part minor differences in the state of confluence of the cultures and in part the known heterogeneity of fibroblasts.
blast populations with respect to their procollagen synthesis (see Ref. 11). Peptide II gave results basically similar to those obtained with peptide I, except that the potency of peptide II was found to be about half of that of peptide I.

**Lack of Inactivation of Cellular Lysyl Hydroxylase and Galactosylhydroxylsyl Glucosyltransferase**—To study the specificity of prolyl 4-hydroxylase inactivation, the activities of two other post-translational enzymes of collagen synthesis were also measured. Both these enzymes, like prolyl 4-hydroxylase, are located within the cisternae of the rough endoplasmic reticulum (1, 2, 16). Lysyl hydroxylase requires the same cosubstrates as prolyl 4-hydroxylase and appears to have an identical reaction mechanism (2, 16), whereas galactosylhydroxysyl glucosyltransferase differs from the two hydroxylases with respect to its cosubstrates and mechanism (26). No changes were found in the cellular activities of lysyl hydroxylase or galactosylhydroxysyl glucosyltransferase even after culturing the fibroblasts for 48 h with a 75 μM concentration of peptide I (Fig. 3), a concentration that reduced prolyl 4-hydroxylase activity by about 65% (Fig. 2).

**Reduced Synthesis of 4-Hydroxyproline**—Fibroblasts were cultured for 48 h with varying concentrations of oxaproline peptide I or II and labeled with [14C]proline during the last 8 h of culture. A marked decrease was found in the synthesis of [14C]hydroxyproline when assayed both in the cell layer and in the medium fractions (Fig. 4, A and B). A distinct decrease was also found in the ratio of [14C]hydroxyproline to total 14C radioactivity recovered in a nondialyzable form (Fig. 4, A and B), which fell from 11.28 ± 1.10% (mean ± S.D.) to 6.16 ± 0.28% in the cell layer protein fraction and from 20.75 ± 0.62% to 13.59 ± 0.67% in the medium fraction with 100 μM peptide I. Peptide II again gave similar results but was about half as potent as peptide I.

**Lack of General Nonspecific Effects on Protein Synthesis and Cell Viability**—No significant changes were found in the incorporation of [14C]leucine into the cell layer or medium protein with a 30 μM concentration of peptide I, whereas a decrease by about 20–25% was found in both fractions with 75–100 μM concentrations (Fig. 5). Nevertheless, a decrease of up to 50% in [14C]leucine incorporation was found in some experiments after culturing the cells with 75 μM peptide I for 96 h, suggesting that nonspecific effects on protein synthesis may occur after prolonged incubation.

Culturing of the cells with the oxaproline peptides caused no changes in their viability, and no increase was found in the leakage of lactate dehydrogenase activity (27) into the cell medium even after culturing with a 100 μM concentration of the peptide (details not shown).

**Intracellular Accumulation of the 4-Hydroxyproline-deficient Procollagen**—Fibroblasts were cultured with a 100 μM concentration of the oxaproline peptide II for 16 h and then stained with a polyclonal antibody to the N-terminal propeptide of type III procollagen. A marked accumulation of staining in the cells was found in the treated cultures (Fig. 6A), whereas diffuse staining of the cells and extracellular matrix...
Inhibition of Collagen Synthesis by Oxaproline Peptides

The data reported here indicate that the peptides benzylxycarbonyl-Phe-Opn-Gly-benzyl ester and benzylxycarbonyl-Phe-Opn-Gly-ethyl ester inactivate prolyl 4-hydroxylase in cultured human skin fibroblasts. This inactivation appears to be highly specific as no decrease was found in the activities of two other intracellular enzymes of collagen synthesis, even though lysyl hydroxylase requires the same cosubstrates as prolyl 4-hydroxylase and probably has an identical reaction mechanism (1, 2, 16). The oxaproline peptides thus differ from all the inhibitors and inactivators of prolyl 4-hydroxylase which have been studied so far in cultured cells or tissues (2, 8–11), as the other compounds may also act on lysyl hydroxylase and some other 2-oxoglutarate-coupled dioxygenases.

The concentration of the oxaproline peptide required to inactivate cellular prolyl 4-hydroxylase by half was much lower than concentrations of 2-oxoglutarate and ascorbate analogues reported to be needed to inhibit the cellular enzyme activity to the same extent (8–11), although it was in turn more than 1 order of magnitude higher than that needed for 50% inactivation of pure prolyl 4-hydroxylase (14). There may be several explanations for this difference. One is that the peptide may be poor at penetrating cell membranes, another that it may be partially degraded in cell cultures, and a third that the presence of large quantities of the peptide substrate for prolyl 4-hydroxylase in the cells may protect the enzyme from inactivation, as has been observed in the case of the pure enzyme (14). The present peptides will therefore be unsuitable for attempts to inhibit collagen accumulation in vivo, but they may nevertheless serve as a valuable tool for studies on the effects of specific inactivation of cellular prolyl 4-hydroxylase on some of the critical questions related to in vivo studies.

The inactivation of cellular prolyl 4-hydroxylase was found to lead to a marked decrease in 4-hydroxyproline formation and to a definite, though somewhat lower, decrease in the ratio of [14C]hydroxyproline to total 14C radioactivity. There was also a substantial intracellular accumulation of the newly synthesized 4-hydroxyproline-deficient collagen similar to that well documented previously for other situations leading to deficient 4-hydroxylation of proline residues and therefore impaired triple helix formation of the newly synthesized procollagen (28). An additional consequence of the inactivation of prolyl 4-hydroxylase was a decrease in the total protein-bound [14C]radioactivity of the oxaproline peptide-treated cultures in both the cell layer and medium fractions after labeling with [14C]proline (as evidenced by the larger decrease in [14C]hydroxyproline radioactivity than in the ratio of 4-hydroxyproline radioactivity to total radioactivity in Fig. 4). On the other hand, no significant change was found in the incorporation of [14C]leucine into total protein, except for a small decrease in the case of 75–100 μM oxaproline peptide concentration. The decrease in the protein-bound [14C]radioactivity after labeling with [14C]proline was thus probably not due to a decrease in protein synthesis but was due to an increased intracellular degradation of the nontriple helical procollagen, as has been reported previously (29–31). It can be calculated from the ratios of [14C]hydroxyproline to total [14C]radioactivity that about two-thirds of the total [14C]proline-derived radioactivity in the medium fraction and one-third in the cell layer fraction of the control cultures was present in procollagens, and thus an enhancement of intracellular procollagen degradation can be expected to produce a substantial decrease in the synthesis of collagen.
in total protein-bound [14C]proline-derived radioactivity. Due to the abundance of proline in procollagen, the increased intracellular procollagen degradation can be expected to influence the [14C]leucine radioactivity only to a minor extent. It is significant that the inactivation of prolyl 4-hydroxylase and its consequences on intracellular collagen accumulation over 48 h caused no changes in the viability of the cells or the release of lactate dehydrogenase from them.

Prolyl 4-hydroxylase is an \( \alpha_2 \beta_2 \) tetramer (1, 2). The \( \beta \) subunit is present in cells also in large amounts as the monomer (1, 2, 28). The \( \beta \) subunit has recently been found to be a multifunctional polypeptide that is identical to the enzyme protein disulfide isomerase (22, 32) and may further act as a cellular thyroid hormone-binding protein (33) and a glycosylation site-binding polypeptide of oligosaccharid transferase (34). The \( \alpha \) subunit probably contains the major parts of the catalytic sites of the prolyl 4-hydroxylase tetramer, and regulation of the amounts of active hydroxylase tetramer appears to occur mainly through regulation of the amounts of the \( \alpha \) subunit (see Ref. 2). Indirect evidence has been reported previously suggesting that inhibition of prolyl 4-hydroxylase in cultured cells and in vivo may lead to increased production of the enzyme (35, 36). No direct data are available on this aspect, however. Such an increase in enzyme production could crucially influence any attempts to inhibit collagen formation in fibrotic disorders by means of prolyl 4-hydroxylase inhibitors, as the increased enzyme production would tend to overcome the inhibition.

The present data indicate that inactivation of prolyl 4-hydroxylase for 48 h caused no increase in the levels of mRNA for either the \( \alpha \) or the \( \beta \) subunit of the enzyme, since these levels showed a slight tendency to decrease rather than increase. It thus seems unlikely that the production of prolyl 4-hydroxylase by the cells was increased under our experimental conditions. The ratio of \( \alpha \) subunit mRNA to \( \beta \) subunit mRNA was 0.13 in both control and oxaprin-treated fibroblasts. This ratio agrees well with the fact that the \( \beta \) subunit is produced in a similar excess in cultured fibroblasts (37).

No changes were found in the level of mRNA for fibronectin, and a small but nonsignificant decrease was found in mRNA for the pro-\( \alpha 1 \) chains of type I and type III procollagens at high peptide concentrations. Decreases in procollagen mRNA levels have previously been reported in cells cultured in the absence of ascorbate and attributed to feedback inhibition of the transcription of the collagen genes by the intracellular procollagen accumulation (38). The small decrease in procollagen mRNA levels found here may have a similar explanation and may also contribute to the decrease in cellular collagen formation in the oxaprin peptide-treated cultures.

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