Biosynthetic growth hormone increases the collagen deposition rate in rat aorta and heart

A Brüel and H Oxlund

Department of Connective Tissue Biology, Institute of Anatomy, University of Aarhus, Aarhus, Denmark


Disorders of the cardiovascular system often are associated with alterations in the metabolism of the collagens of these tissues. A method for in vivo determination of collagen deposition rate in small tissue samples is delineated and used for assessment of the effect of biosynthetic growth hormone (GH) injections on the collagen deposition rate in rat aorta and cardiac musculature. Rats were injected with GH, and the controls with saline, twice daily for 7 days. The in vivo collagen deposition rate was measured by injecting iv a large dose of [3H]-proline with a flooding dose of “cold” proline, followed by determination of the production of [3H]-hydroxyproline during a 4-hour labelling period. Extractable collagens that were not bound in the tissue and therefore do not contribute mechanical strength to it were removed from the samples. [3H]-Labelled- and “cold” amino acids were assessed by reversed-phase HPLC combined with simultaneous flow scintillation detection on the same sample. In the control group the deposition per hour was 0.13 ± 0.02% (mean ± SEM) in aortic intima media and 0.72 ± 0.09% in cardiac left ventricular musculature. Growth hormone induced a threefold increase (p < 0.001 and p < 0.01, respectively) in the collagen deposition rate: 0.45 ± 0.06% in aortic intima media and 2.43 ± 0.45% in cardiac left ventricular musculature. The method described enables a rapid and sensitive determination of collagen deposition per hour in small tissue samples from experimental animals. The collagen deposition rate of cardiac musculature is fivefold higher compared with that of aortic intima media. Biosynthetic GH induces a threefold increase in the collagen deposition rate of these tissues.

Hans Oxlund, Department of Connective Tissue Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark

Collagens are very important structural proteins in blood vessels (1, 2) and cardiac musculature (3). A wide range of pathological disorders of the cardiovascular system — atherosclerosis, hypertension, cardiac hypertrophy, ischaemia, infarct — are associated with fibrotic or degenerative alterations in blood vessels or cardiac musculature and disorders in the metabolism of the collagens of these tissues. The level or amount of collagens in the tissue is determined by the balance between synthesis and degradation of collagens. Studies of collagen metabolism may elucidate the mechanisms of such disorders and lead to improvements in diagnosis and treatment regimens. Studies of synthesis and degradation in vivo, however, in patients and experimental animals have been hampered because of lack of suitable methods (4, 5). The method developed by Laurent (6) and Mays et al. (7) has made it possible to estimate the synthesis rate of collagens in vivo in experimental animals. As shown by these investigators, a large part of synthesized collagen is degraded quickly and never deposited in the tissue: 57–96% of the collagens in rat heart (7). The collagen that is not cross-linked does not contribute mechanical strength to the tissue. We focused on that part deposited and bound in the tissue, and applied the technique of tissue preparation used when analysing newly synthesized collagen cross-links in order to determine the collagen deposition rate.

Repair and regeneration of aortic tissue and heart musculature are poor. Biosynthetic growth hormone (GH) has been found to stimulate wound healing in skin (8) and colon anastomoses (9) and may reduce aneurysm formation following an experimental cardiac infarct in rats (10). Recent observations in our laboratory indicate that GH induces a marked increase in the deposition of collagens and thereby enhances the capacity of tissue repair (11). The aim of the present study was to examine the effect of GH injections on the collagen deposition rate of rat aorta and heart musculature. A modification of the method of Laurent (6) is delineated for determination of the deposition rate of the collagen bound in the tissues.

Materials and methods

Experimental animals

Thirty-six female Wistar rats, 24 weeks old, with an
average body weight of 283 g at the start of the experiment, were used. The time course of a flooding dose of $[^3]H$-proline was assessed in 12 rats, allocated at random to four groups, three rats in each group, corresponding to measurements of the specific activity of $[^3]H$-proline 1, 2, 3 and 4 h after iv injection of $[^3]H$-proline. The collagen deposition rate was determined in 24 rats, allocated at random to two groups: a control group and a group injected with GH.

**Assessment of the time course of a flooding dose of $[^3]H$-proline**

The rats were anaesthetized lightly by ether and vena saphena magna was exposed through a 1-cm long skin incision in the right tibia. Subsequently the rats were injected iv with L-(5-$[^3]H$)proline (1.7 mCi/kg rat, Amersham International, Amersham, Bucks, UK) with a flooding dose of L-proline (12 mmol/kg, Sigma, St Louis, MO) in phosphate-buffered saline as outlined by Laurent (6). At the end of the time periods the rats were killed by an overdose of pentobarbital ip. The aorta and heart quickly were dissected free, frozen and stored at $-20^\circ$C. Within 1 month the aortas and hearts were thawed to room temperature, the adventitia was removed carefully from the aortas and the heart muscle of the left ventricle was dissected free from the hearts. The resulting preparations of aortic intima and heart muscle were minced thoroughly, suspended in 67% ethanol and extracted for 16 h ($5^\circ$C, constant stirring). The samples were centrifuged at 15 000 g for 30 min at 4°C. The supernatant was filtered (0.45 μm filter, Millipore, Harrow, Middlesex, UK), and then evaporated in a speedvac concentrator (Savant, Hicksville, NY). The $[^3]H$-proline and proline contents of the samples were determined by reversed-phase HPLC combined with simultaneous flow scintillation detection on the same sample as described below. The specific radioactivity (dpm/μmol proline) of each sample was calculated from these data.

**Assessment of the time course of collagen deposition**

The pellets left over after the ethanol extraction were washed thoroughly by three extractions with copious amounts of phosphate-buffered saline (0.15 mol/l, pH 7.4, 5°C, 24 h, constant stirring) in order to remove $[^3]H$-hydroxyproline from the intracellular and extracellular degradation of collagen, i.e. the $[^3]H$-hydroxyproline not incorporated in stable collagen fibrils and fibres. This method of preparation of biological tissues for studies of their collagen fibrils were adopted from studies of newly synthesized collagen cross-links (12–16). The washed pellets containing fibrillar collagen were hydrolysed in redistilled 6 mol/l HCl at 100°C for 16 h, followed by evaporation of the acid in the speedvac concentrator and determination of the specific radioactivity of $[^3]H$-hydroxyproline in the pellets.

**Determination of the specific radioactivity of proline and hydroxyproline**

The specific radioactivity of proline (dpm/μmol proline) in the ethanol extracts and specific radioactivity of hydroxyproline (dpm/μmol hydroxyproline) in the pellets were determined by reversed-phase HPLC combined with simultaneous flow scintillation detection on the same sample. The amino acids of the dried samples were derivatized with phenylisothiocyanate. The derivatized amino acids then were analysed by reversed-phase HPLC using an acetate/acetonitrile gradient, a C$_{18}$ PicoTag$^\text{®}$ column (Waters, Millipore Corporation, Milford, MA) at 50°C and an HPLC system (Kontron, AG, Zürich, Switzerland) with UV detection at 254 nm. The outlet of the UV detector was connected to a flow scintillation counter (Berthold, HPLC radioactivity monitor LB507A, Wildbad, Germany). The sample was mixed with scintillation fluid (Monolow 2, Manville, NJ) 1:3 by means of a pump and the dpm counted using a 2000-μl flow cell and a total flow rate of 4.0 ml/min. The data were processed by the Kontron MT450 data system, resulting in two chromatograms for each probe: the amino acids and the radioactive amino acids, respectively. From these chromatograms the specific radioactivities of proline and hydroxyproline were calculated. Calculation of the collagen deposition rate (k$_{cdr}$, %/hour) was calculated using the equation

$$k_{cdr} = \frac{S_B \times 100}{S_A \times t}$$

where $S_B$ is the specific radioactivity of hydroxyproline in the pellet, $S_A$ is the specific radioactivity of proline in the ethanol extract and $t$ is the time between $[^3]H$-proline injection and death of the rats. This method of collagen biosynthesis rate assessment is a modification of the collagen biosynthesis rate measurement developed by Laurent (6) and Mays et al. (7).

**Growth hormone injections**

The GH preparation was Norditropin$^\text{®}$ (Novo-Nordisk A/S, Gentofte, Denmark), which is human GH produced by recombinant technology using a non-pathogenic K12 strain of *Escherichia coli*. The specific activity of the biosynthetic human GH preparation was 3 IU/mg. For 7 days, the rats of the GH-injected group received 5.0 mg GH/kg actual body weight daily, divided into two subcutaneous injections given in the nape of the neck. The first injection was given at 09.00 h and the second at 15.00 h because the secretion of GH is pulsatile and the effects of GH depend on this pulsatile occurrence of GH. The control rats received a corresponding volume of vehicle, i.e.
sterile physiological saline. At the end of the GH injection period the rats were anaesthetized with ether and injected iv with $[^3]$H-proline and flooded with proline as described above. Four hours later, the rats were killed with an overdose of pentobarbital ip and the aorta and heart were dissected free and quickly frozen. Preparations of aortic intima media and heart musculature, ethanol extracts and samples for determination of the specific activity of $[^3]$H-proline and $[^3]$H-hydroxyproline were done likewise as described above.

**Statistical analyses**

Comparison between parameters of the two groups was performed by the non-paired two-sample Mann–Whitney U-test (17). Differences were considered significant if $p < 0.05$.

**Results**

The experiment lasted for 7 days and during this period the GH-treated group increased their body weight by 14% ($p < 0.001$), whereas there were no changes in the body weight of the control group. The specific radioactivity of $[^3]$H-proline in the injection fluid was 6.26 ± 0.06 × 10^6 dpm/μmol proline (mean ± SEM), in the tissue-free proline pool of aortic samples it was 5.57 ± 0.25 × 10^6 and in the cardiac samples it was 5.50 ± 0.96 × 10^6 dpm/μmol proline.

The relationships between $[^3]$H-proline and total proline in the tissue-free proline pool and time describe an exponential decrease during the 4-h labelling period. At the end of the labelling period the proline concentration of the tissue-free proline pool was only three times higher compared with that of animals that had not received an injection of proline. The specific radioactivity of $[^3]$H-proline in the tissue-free proline pool of both aortic and cardiac samples was constant during the labelling period (Fig. 1).

The incorporation of $[^3]$H-hydroxyproline into collagen deposited in cardiac musculature during the 4-h labelling period was approximately linear. The collagen deposition was 0.063 ± 0.015 dpm/nmol (mean ± SEM) after 1 h, 0.147 ± 0.042 dpm/nmol after 2 h, 0.297 ± 0.093 dpm/nmol after 3 h and 0.430 ± 0.035 dpm/nmol after 4 h. Data of collagen deposition for aortic samples are not given, because amounts of $[^3]$H-hydroxyproline deposited after 1 and 2 h were too small to be assessed by the present procedure.

The collagen deposition rate in per cent per hour was more than five times higher in cardiac left ventricular musculature compared with aortic intima media (Table 1). The GH injections induced a threefold increase in the collagen deposition rate of both aortic and cardiac tissue (Table 1).

**Discussion**

The in vivo collagen deposition rate in aortic intima media and cardiac musculature was measured in rats by injecting $[^3]$H-proline intravenously with a flooding dose of "cold" proline, followed by determination of the production of $[^3]$H-hydroxyproline. The large flooding dose of proline was used in order to keep the specific radioactivity of proline (dpm/μmol proline) of the tissue fluid approximately constant during the 4-h labelling period, and in order to minimize effects of reutilization of $[^3]$H-proline and de novo synthesis of proline (6, 7). The hydroxyproline of these tissues stems predominantly from collagen. Only small amounts may originate in elastin (10% in rat aorta (2)) and traces from acetylcholinesterase and Clq component of complement.

In order to be able to calculate the collagen deposition...
rate it is necessary to keep the specific activity of $[^3\text{H}]$-proline constant during the 4-h incorporation period. On the basis of the investigation of McAnulty and Laurent (18), who found increases in this parameter compared with the injection fluid, we studied the specific activity of $[^3\text{H}]$-proline in the ethanol extracts in relation to time and found no significant changes (Fig. 1). One explanation may be that the rats of the present experiment were older and their metabolism slower than that of the rats used by McAnulty and Laurent (18).

After the ethanol extraction the samples were extracted with large amounts of phosphate-buffered saline in order to remove $[^3\text{H}]$-hydroxyproline in peptides originating from degradation of newly synthesized collagen and collagen molecules that are not bound in the tissues by intermolecular cross-links. Ultrasound treatment of the samples in order to disintegrate cell membranes and further extractions only removed an additional 0.5–1.0% of the total $[^3\text{H}]$-hydroxyproline from the samples. On the other hand, we examined the phosphate-buffered saline extracts by SDS-PAGE and found only traces of collagen molecules. Assessment of the time course of $[^3\text{H}]$-hydroxyproline incorporation in the collagen showed that the incorporation approximated a linear relationship with respect to time. Therefore, these data indicate that the $[^3\text{H}]$-hydroxyproline of the samples at this step represents collagen synthesized and bound in the tissue by collagen cross-links during the 4-h incorporation period.

The described method for determination of collagen deposition rate by reverse-phase HPLC combined with simultaneous flow scintillation detection on the same sample is rapid, sensitive and reproducible. The C$_{18}$ PicoTag column provides excellent separation of the amino acids with no interference from other amino acids on the peaks of hydroxyproline and proline. Three samples per hour are processed and run through the HPLC system. This makes it possible to increase the number of measurements and increase the precision.

The collagen fractional biosynthesis rate in hearts of 6–15-month-old rats was found to be 9–15% (7). Most of this newly synthesized collagen was reported to be degraded rapidly. In our study the collagen deposition rate of cardiac musculature was 0.72 × 24 = 17%/day, which is reconcilable with the investigation of Mays et al. (7).

The GH injections were given daily for 7 days, resulting in a threefold increase in the collagen deposition rates of the aortic intima media and cardiac musculature. This is in agreement with a previous study (2), where GH treatment for 80 days was shown to increase the hydroxyproline content (µg/mm$^2$) of the aortic intima media by approximately 10%. The GH-induced increase in collagen deposition rate is much higher than would be expected from the increase in hydroxyproline content after long-term treatment with GH. Therefore, the GH treatment seems to increase the turnover of collagenous structures, as shown by Aer et al. (19).

The dose of GH, 2.5 mg/kg given twice daily, is a high dose. The peak levels of endogenous GH of 24-week-old female rats is 300–600 µg/l serum. Between peaks the level is 20–100 µg/l serum (20). A subcutaneous injection of 1 mg of biosynthetic human GH to rats of this age increases the serum level of GH to 420–760 µg/l (21) and subcutaneous injections of 4 mg or 8 mg of biosynthetic human GH per kg rat may increase the serum level of GH to 1200 and 2700 µg/l, respectively (unpublished data). In a previous investigation we found that the present treatment, biosynthetic human GH 2.5 mg/kg twice per day, injected subcutaneously for 80 days resulted in a twofold increase in serum IGF-I, an increase in body weight by 100% and an increase in bone lengths and muscle mass in the rats (22). The effects of this GH treatment regimen on rat aorta were increased diameter, a relative increase in the amount of collagen type I and a reduction in the concentration of elastin, all alterations that may influence the elasticity and recoiling properties of aorta (2). Using in vitro cultures of rabbit aortic media, Ledet (23) showed that supplementation of the medium with human GH, 1–5 µg/l, resulted in an enhancement of the growth of cell cultures and an increase in cell proliferation. Another study showed that human GH enhanced the production of procollagen type I and fibronectin (24). These findings are in agreement with the data of the present study.

Patients with acromegaly are known to have an increased mortality, and 25–45% die of cardiovascular diseases (25–27). The causes of death include myocardial infarction, cerebrovascular insults, cardiac failure, arrhythmias and aortic aneurysms. Enlargements of the heart have been found to be greater than the generalized organomegaly, and histological studies of the myocardium have shown cellular hypertrophy, patchy fibrosis and myofibrillar degeneration (28). The findings of the present study of marked increases in collagen deposition rate in the myocardium of GH-injected rats are in accordance with these reports concerning alterations in the myocardium of acromegalic patients.

Biosynthetic human GH is used for treatment of GH deficiency. The availability of biosynthetic GH permits a more extended use of GH therapy for short stature not caused by the classic GH deficiency, i.e. idiopathic short stature, renal failure or Turner’s syndrome. The capability of biosynthetic human GH to reduce net protein loss in critically ill surgical patients (29) and experimental results on the healing of colonic anastomoses (11), skin wounds (8) and bone fractures (21) may provide further indications for the use of biosynthetic GH in the future. The doses of biosynthetic human GH used for treatment of patients with GH deficiency are around 0.1 mg/kg and therefore lower than the doses used in treatment of the rats of the present study. The level of serum GH in rats, however,
appears to be tenfold higher compared with that of human beings. Therefore, doses given to human beings and to rats are not directly comparable. Short-term biosynthetic GH therapy for short stature has not produced significant side effects. Long-term effects of biosynthetic human GH therapy are not yet available, and it is not known if long-term side effects will occur.

The described method enables a rapid and sensitive in vivo determination of collagen deposition in small tissue samples from experimental animals, and opens up new opportunities for studies of collagen metabolism in tissue repair and regeneration. The collagen deposition rate of cardiac musculature was fivefold higher compared with that of aortic intimamedia. Biosynthetic GH induced a threefold increase in the collagen deposition rate of both these tissues.

Acknowledgments. The authors wish to thank C Kneibus, R Kofoed and M Mortensen for skilled technical assistance and M Fischer for linguistic revision. This study was supported by grants from The Danish Medical Research Council (no. 12-0361), Novo-Nordisk A/S, Thomas og Elisabeth Frølund Nielsens Fond, Nordisk Insulinfonds Komité, Henny og Helge Holgersens Mindelegat, Novo Nordisk Fondien and Musikforhæggerne Agnes og Knut Mørks Fond.

References


Received February 1st, 1994
Accepted September 26th, 1994