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THE DEPRESSOR EFFECT OF STREPTOZOTOCIN - INDUCED DIABETES IN SPONTANEOUSLY HYPERTENSIVE RATS: THE ROLE OF VASCULAR COLLAGEN SYNTHESIS

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ABSTRACT

ENHANCED VASCULAR COLLAGEN SYNTHESIS HAS BEEN IMPLICATED AS AN ETIOLOGIC FACTOR IN THE DEVEOLPMENT OF SYSTEMIC HYPERTENSION IN VARIOUS ANIMAL MODELS. WITHIN THE AORTA AND RESISTANCE VESSELS OF SPONTANEOUSLY HYPERTENSIVE RATS (SHR), ACCUMULATION OF FIBROUS PROTEINS (COLLAGEN, ELASTIN) AND SMOOOTH MUSCLE CELL HYPERPLASIA AND HYPERTROPHY ARE ASSOCIATED WITH AN INCREASE IN SYSTOLIC ARTERIAL PRESSURE (SAP). FURTHERMORE, AGENTS WHICH LOWER SAP ALSO REDUCE VASCULAR COLLAGEN SYNTHESIS. INITIAL OBSERVATIONS FROM OUR LABORATORY INDICATED THAT STREPTOZOTOCIN - INDUCED DIABETES (8 WEEKS DURATION) LOWERS SAP OF SHR WITHOUT AFFECTING SAP OF NORMOTENSIVE CONTROL WISTAR - KYOTO RATS (WKY). IN ORDER TO CHARACTERIZE THE COMPONENTS OF THIS DEPRESSOR EFFECT, I PROPOSED TO EXAMINE THE ROLE OF VASCULAR COLLAGEN BIOSYNTHESIS IN DIABETIC SPONTANEOUSLY HYPERTENSIVE RATS. THE RESULTS SHOWED THAT DIABETES LOWERED THE ACTIVITES OF MARKER ENZYMES FOR COLLAGEN BIOSYNTHESIS, PROLYL HYDROXYLASE AND LYSYL OXIDASE, IN THE SHR STRAIN AORTAE AND MESENTERIC ARTERIES WITHOUT AFFECTING WKY ENZYME ACTIVITIES. THESE RESULTS PROVIDED PRELIMINARY EVIDENCE THAT THE DEPRESSOR EFFECT OF DIABETES MAY BE RELATED IN PART TO A REDUCTION IN VASCULAR COLLAGEN SYNTHESIS. TO FURTHER CHARACTERIZE THE APPARENT INHIBITION OF COLLAGEN SYNTHESIS, MORE DIRECT IN VITRO MEASUREMENTS WERE NECESSARY. HYDROXYPROLINE ANALYSIS IS THE STANDARD METHOD FOR QUANTITATION OF COLLAGEN SYNTHESIS AND CONTENT, DUE TO ITS ABUNDANCE IN COLLAGEN (10%) AND ITS RELATIVELY INFREQUENT OCCURENCE IN OTHER PROTEINS. THE METHOD WE SELECTED FOR THE ANALYSIS OF HYDROXYPROLINE SPECIFIC ACTIVITY WAS

REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY, UTILIZING PRECOLUMN DERIVATIZATION WITH FLUORESCENT MARKER, 7-CHLORO-4-NITROBENZOFURAZAN (NBD-CL). NBD-CL PREFERENTIALLY LABELS SECONDARY AMINES UNDER APPROPRIATE EXPERIMENTAL CONDITIONS. QUANTITATION OF HYDROXYPROLINE AND PROLINE STANDARDS WERE LINEAR OVER A RANGE OF AMOUNTS (4 TO 45nmol). INCORPORATION OF ^{14}C-PROLINE INTO ^{14}C-HYDROXYPROLINE , REPRESENTING THE RELATIVE RATE OF DE NOVO COLLAGEN SYNTHESIS , WAS LINEAR UP TO 4 AND 10 HOURS IN NONVASCULAR TUMOR HOMOGENATES AND IN AORTIC TISSUE, RESPECTIVELY. THE METHOD WAS THEN APPLIED TO QUANTIFY COLLAGEN SYNTHESIS AND CONCENTRATION FROM DIABETIC SHR AND WKY AORTAE. DIABETES MARKEDLY REDUCED SHR MESENTERIC ARTERIAL PROLYL HYDROXYLASE ACTIVITY AND AORTIC ^{14}C-PROLINE INCORPORATION INTO ^{14}C-HYDROXYPROLINE , IN VITRO. DIABETES ALSO INCREASED AORTIC COLLAGEN CONCENTRATION IN THE SHR STRAIN. THESE RESULTS FURTHER SUPPORT THE HYPOTHESIS THAT CHRONIC DIABETES HAS STRAIN DEPENDANT EFFECTS ON VASCULAR COLLAGEN METABOLISM. WHETHER THE OBSERVED METABOLIC CHANGES ARE EFFECTS OR CAUSES OF THE REDUCTION IN ARTERIAL PRESSURE REMAINS TO BE DETERMINED.

ACKNOWLEDGEMENTS

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I would like to dedicate this thesis to my family. Without their years of love and encouragement , this would not have been possible. Finally, I would like to thank Dr. Clinton Chichester, and special friends, David Olsen and Linda Toy, for their support.

PREFACE

This thesis was prepared according to the manuscript format. Two manuscripts are included:

I. Quantitation of Collagen Synthesis by Reverse Phase High Performance Liquid Chromatography Utilizing 4-Chloro-7-Nitrobenzofurazan Derivatives

II. The Influence of Streptozotocin - Induced Diabetes on Vascular Collagen Synthesis in Spontaneously Hypertensive Rats

The first has been accepted for publication in the Journal of Chromatography (Biomedical Applications). The second manuscript is being submitted to the journal Diabetes.

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MANUSCRIPT I

QUANTITATION OF COLLAGEN SYNTHESIS BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY UTILIZING 4-CHLORO-7 NITROBENZOFURAZAN DERIVATIVES

ABSTRACT

A reverse-phase high performance liquid chromatography method was used to quantify hydroxyproline as an index of collagen content and synthesis from different tissue types. The procedure utilizes precolumn derivatization of amino acids with 4-chloro-7-nitrobenzofurazan for fluorimetric detection. Analysis of hydroxyproline from rat aorta or human tumors indicated a strong correlation with values obtained by this method and by a standard colorimetric assay. Incorporation of ^{14}C-proline into tumor and aortic hydroxyproline permitted the quantitation of collagen synthesis in these tissues. Linear rates of incorporation were found up to 4 and 10 hours for tumors and aortae, respectively. This method provides accurate measurements of collagen synthesis in normal and pathologic tissues.

INTRODUCTION

Hydroxyprolines, trans-4-hydroxy-L-proline (t-4-Hyp) and trans-3-hydroxy-L-proline (t-3-Hyp), are widely used markers for the quantitation of collagen content and synthesis in a variety of tissues. The hydroxylated imino acids are formed via post-translational modifications of peptidyl bound proline residues by specific hydroxylase reactions. Because of its relative infrequent occurrence in other proteins, t-4-Hyp is considered a marker for interstitial collagens, whereas t-3-Hyp is an indicator of basement membrane collagen [1,2].

Several methods are available to quantify various isomers of hydroxyproline. The most common are amino acid analysis, ion exchange chromatography and the colorimetric assays [3]. All of these methods are time consuming, especially when large numbers of samples are involved.

Recently, reverse phase high performance liquid chromatography (RP-HPLC) was applied to imino acid analysis with and without precolumn derivatization [4,5,6,7]. Prior treatment of the sample with 4-chloro-7-nitrobenzofurazan (NBD-Cl) allows preferential labelling and sensitive fluorometric detection of secondary amines. This approach has been used to assay hydroxyproline in urine, plasma and purified collagen standards [4,8,9]. However, it has not been employed to quantify collagen levels or synthesis rates in tissues.

The present report describes the application of RP-HPLC of NBD derivatives to determine tissue collagen levels and synthesis rates in two tissue types: rat aorta and human xenograft tumors. The values for collagen content obtained with this method correlate closely with those

using the Juva and Prockop procedure [10]. The method also allows the determination of specific activities of hydroxyproline and proline in each tissue sample.

MATERIALS AND METHODS

Reagents

All reagents were of the analytical grade. Materials and their sources are as follows: t-4-hydroxy-L-proline, cis-4-hydroxy-L-proline, L-proline L-glycine and NBD-Cl from Sigma (St. Louis, Mo). Acetonitrile and methanol (HPLC grade) were purchased from J. T. Baker (Medford, MA). Isotopes, ^{14}C-U-L-proline (specific activity = 273 mCi/mmol) and ^{3}H-hydroxy-L-proline (specific activity = 5.9 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

Sample Preparation

Radiolabelled vascular tissue and tumors: thoracic aortae (approximately 40 mg) from 15 week old male Wistar rats (Charles River Breeding Labs) were placed into 2 ml of gassed (95% oxygen; 5% carbon dioxide) Krebs-Henseleit (K-H) solution and allowed to incubate for 30 minutes at 37^{o}C. The ionic composition of the K-H solution was: NaCl 113 mM; KCl 4.7 mM; $CaCl_2$ 2.5 mM; Mg SO_4 1.2 mM; $NaHCO_3$ 36 mM; KH_2PO_4 6.2 mM; sodium ascorbate 0.05 mM and glucose 11.5 mM. At 30 minutes, the above solution was removed and replaced with fresh K-H solution (2 ml) supplemented with ^{14}C-L-proline (10 uCi/ml) and incubated up to 10 hours. At each time point, aortae were washed with phosphate buffered saline (PBS) pH 7.4, and subsequently homogenized in 1 ml of PBS. Proteins were precipitated with 50% TCA (0.1 ml), centrifuged and washed 6 times with 5% TCA to remove unincorporated label. Residual TCA was extracted via 2 ether washings (4.0 ml each). Labelled aortae were then hydrolyzed in 2 ml of 6M constant boiling HCl for 24 hours at 110^{o}C. Hydrolysates were filtered, evaporated to dryness and neutralized with potassium tetraborate, 0.2 M, pH 9.5.

Human pancreatic adenosquamous carcinoma xenograft tumors were produced by injecting COLO 357 cells grown in culture into the flanks of 6 week old nude mice as described [11]. When the tumors reached 400 mg to 600 mg the tumors were removed from mice, washed with PBS, minced and placed into 2 ml of K-H buffer supplemented with ^{14}C-L-proline (10 uCi/ml) and incubated at 37^{o}C for up to 4 hours. At each time point, samples were treated as described above.

Unlabelled vascular and tumor tissue: thoracic aortae (approximately 40 mg) from 15 week old male Wistar rats and aliquots of lyophilized tumor samples were hydrolyzed for 24 hours at 110^{o}C. After filtration, samples were neutralized with KOH and analyzed by HPLC and by the method of Juva and Prockop.

Amino Acid Derivatization

Neutralized hydrolysate (0.1 ml) was placed into a test tube containing 0.4 ml of 0.2 M potassium tetraborate (pH 9.5). Cis-4-hydroxy-L-proline (C-4-Hyp; 5 mM, 0.020 ml) was added to each sample as an internal standard due to its convenient elution pattern between t-4-Hyp and proline. Derivatizing agent, NBD-Cl (0.5 ml of 16 mM in methanol) was added to the above mix and incubated at 60^{o}C for 3 minutes in the dark [8,9]. The reaction was halted by immediate immersion into an ice bath and quenched by the addition of 0.05 ml of 3M HCl. A 0.1 ml portion of the reaction mix was injected onto the column for analysis. Total hydroxyproline was determined by comparing the ratio of peak heights of t-4-Hyp/C-4-Hyp of hydrolysates to those generated from standards. Collagen synthesis was quantified as the amount of radioactive hydroxyproline collected under the corresponding

peak. Derivatization was expressed as a percentage of the total radioactivity recovered under the hydroxyproline and proline peaks. The mean derivation of radiolabelled proline and hydroxyproline in tissue samples was 90 $\pm$ 5%. All samples were corrected to 100% for calculations of collagen content and synthesis.

Instrumentation and Chromatographic Conditions

The equipment used consisted of a Waters M-45 constant flow pump and Gilson fluorimeter (excitation filter 330-380 nm, emission filter 460-600 nm) connected to a Linear chart recorder. Radioactive eluents (0.8 ml) were collected into 5 ml liquid scintillation vials using an Alpha 200 fraction collector. Radioactivity was quantified by liquid scintillation spectroscopy at 85% efficiency for C-14. Analytical separation was performed on a 30 cm x 3.9mm 10um silica uBondapak C-18 column (ASI) preceded by a direct connect precolumn (ALTECH) with pellicular C-18 packing. The mobile phase consisted of 21.75% acetonitrile, 0.015% phosphate (pH 2.85) with a flow rate of 1.2 ml/minute at room temperature. The mobile phase was filtered and degassed by vacuum suction and sonication for 30 minutes.

RESULTS AND DISCUSSION

Characterization of the Procedure

Two parameters were taken into consideration for optimizing the conditions of derivatization: 1) NBD-Cl concentration; and 2) the time of incubation. We sought to provide maximal derivatization of Hyp without sacrificing the selectivity of NBD-Cl for secondary amines.

Increasing concentrations of NBD-Cl increased the % derivatization of ^{14}C-proline and ^{3}H-Hyp in the presence of four amino acids (Figure 1). As NBD-Cl concentration approached 8 mM, the % derivatization plateaued at approximately 90%. The magnitude of t-Hyp peak height was proportional to the concentration of NBD-Cl added. The ratio of peak heights and linearity of response remained consistent throughout a range of concentrations of standards, confirming previous studies [9,12]. Based on the above observations, 8 mM NBD-Cl was used throughout the remainder of the study.

Increasing the time of incubation at 60°C beyond 3 minutes did not significantly increase the extent of derivatization of ^{14}C-proline and ^{3}H-Hyp (Table I). However, the selectivity of NBD-Cl for t-4-Hyp diminished as time of incubation increased beyond 5 minutes. These data support previous studies, suggesting that shorter reaction times favor the selective derivatization of secondary amines [4,8,9]. Therefore, all subsequent incubations were carried out for 3 minutes at 60°C.

Standard curves for t-4-Hyp and proline were linear (r=0.996 and 0.997, respectively) up to approximately 5 ug (38 and 43 nmoles, respectively per 0.1 ml injection; data not shown). The lower limit for reliable detection was approximately 0.5 ug/0.1 ml injection, (4nmoles) at the fluorimeter

setting used (10 x 0.5). Higher (picomolar) sensitivities could be obtained; however, this was not necessary for tissue analysis.

The separation of amino acid peaks from aortic hydrolysates was comparable to that of the corresponding standards (Figure 2). Therefore, prior treatment of the tissue samples with Dowex columns [13] or with o-opthalaldehyde [9,14] was not necessary.

Quantification of Collagen Content and Collagen Synthesis From Tissue Hydrolysates

Collagen content

Aorta and tumor hydroxyproline values obtained with RP-HPLC were well correlated with those from the standard colorimetric method of Juva and Prockop (Figure 3). The data from aortic samples also show that the method is reproducible when samples are assayed on different days. The mean value for all samples (9.31 ug Hyp/mg wet weight) is similar to previously reported values for thoracic aorta [15,16,17].

Collagen synthesis

The rates of incorporation of ^{14}C-proline into aortic and tumor collagen *in vitro* are shown in Figure 4. The rates were linear for at least 10 and 4 hours for aortae and tumors, respectively. The slopes of the lines represent the rate of net *de novo* collagen biosynthesis, assuming: 1) a constant pool size of intracellular proline; and 2) relatively low rates of degradation of newly formed collagen. Specific activities of hydroxyproline can be determined at each time point.

This method provides accurate and reproducible quantitation of collagen content and synthesis without the necessity for prior sample preparation or gradients.

Table I. Effect of incubation time on peak height ratios of t-4-hydroxy-L-proline and glycine, and % derivatization of ^{3}H-Hyp and ^{14}C-proline*

	Peak Height Ratio		% Derivatization	
Incubation Time (min)	t-4-Hyp	Glycine	^{3}H-Hyp	^{14}C-Proline
1	1.38	0.16	48.5	65.0
3	1.78	0.65	90.1	90.0
5	1.56	1.02	94.0	95.9
7	1.38	1.15	94.1	95.4
10	1.34	1.21	94.0	96.0
20	1.45	1.21	92.8	96.0

*Each reaction mix contained 0.1 ml of t-4-Hyp, 0.03 ml c-4-Hyp; 0.1 ml L-proline (5 mM); and 0.02 L-glycine (10 mM) in 0.2 M potassium tetraborate buffer, pH 9.5. Each sample was spiked with 0.01 uCi of ^{3}H-Hyp and ^{14}C-proline and derivatized with 0.5 ml of 16 mM NBD-Cl (8 mM final concentration) at 60°C.

Figure 1. Effect of NBD-Cl concentration on percent derivatization of ^{3}H-Hyp and ^{14}C-proline. Each reaction mix contained 0.1 ml of t-4-Hyp (5mM), 0.02 ml c-4-Hyp (5mM), 0.02 glycine (10mM) and 0.1 ml proline (5mM) in 0.2 M potassium tetraborate buffer, pH 9.5. Each sample was spiked with 0.01 uCi of ^{3}H-Hyp and ^{14}C-proline and subsequently derivatized with 0.5 ml NBD-Cl (in 70% methanol) for 3 minutes at 60^{o}C. 0.1 ml of each sample was injected for quantification of percent derivatization. Recovery of radioactivity was 97%.

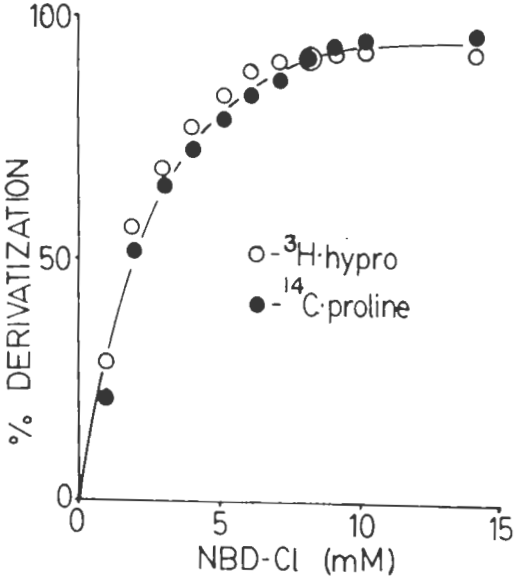

100
50
0
% DERIVATIZATION
o - ^{3}H·hypro
● - ^{14}C·proline
0
5
10
15
NBD-Cl (mM)

Figure 2. Typical chromatogram of standards (A) and 0.1 ml of aortic hydrolysate (B). The rank elution profile (from left to right) is as follows : t-4-Hyp (5.7 min), c-4-Hyp (7.8 min), glycine (8.1 min), and proline (17.5 min). Peaks a and b in the chromatogram are unknown.

A
B
a
b
Inj.
Inj.
0
6
12
18
24
Retention Time (min)
Retention Time (min)
RELATIVE FLUORESCENCE

Figure 3. Quantitation of aortic (A) and tumor (B) hydroxyproline by RP-HPLC and the Juva and Prockop colorimetric assay. The four symbols (●, ○, ▲, △) represent four aortae (A) or four treatment groups (B). The individual points represent single determinations within groups on three different days (A) or on the same day (B). Hydrolyzed aorta were resuspended in 0.4 ml potassium tetraborate (0.2 M, pH 9.5) and an aliquot taken for each assay. Lyophillized tumors were hydrolyzed, filtered and neutralized with KOH, and an aliquot taken for each assay. ^{14}C-proline was added to each sample for determination of percent derivatization. The mean percent derivatization was 89 ± 4 and 90 ± 2 for tumors and aortae, respectively. The lines were obtained by regression : $y = 0.90x + 96.0$ ($r=0.978$) for A; $y = 1.05x = 0.08$ ($r=0.968$) for B.

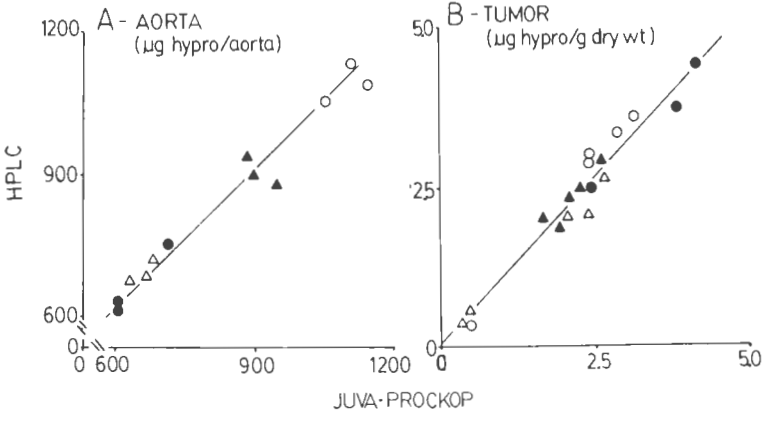
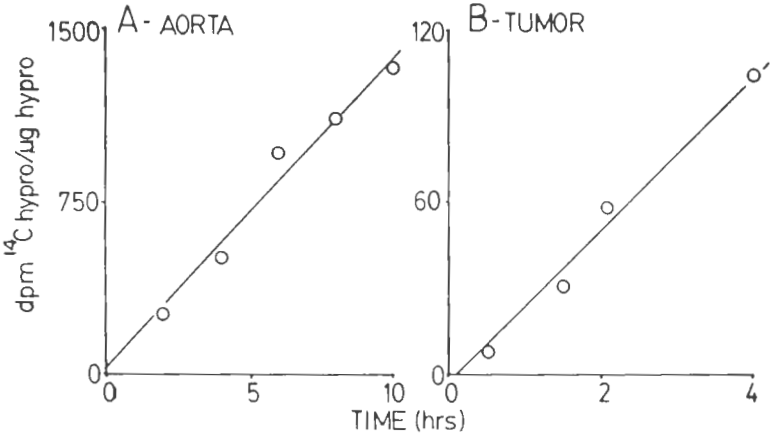

A - AORTA
(µg hypro/aorta)
B - TUMOR
(µg hypro/g dry wt)
HPLC
JUVA-PROCKOP
1200
900
600
0
600
900
1200
5.0
2.5
0
8
9

Figure 4. Time course of ^{14}C-proline incorporation into aorta (A) and tumor (B) ^{14}C-hydroxyproline. Aortic and tumor samples were metabolically labelled with ^{14}C-proline (10 uCi/ml) for the above specified times at 37^{o}C. At each time point, the specific activity of ^{14}C-Hyp eluted under its corresponding peak, divided by the the ratio of peak heights for the sample, compared to those generated from standards. The lines were obtained by regression : $y = 130.0x + 33.0$ ($r=0.989$) for A; $y = 27.5x + 3.0$ ($r=0.990$) for B.


A - AORTA
B - TUMOR
1500
750
0
120
60
0
0
5
10
0
2
4
TIME (hrs)
dpm ^{14}C hypro/µg hypro

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MANUSCRIPT II

THE INFLUENCE OF STREPTOZOTOCIN - INDUCED DIABETES ON VASCULAR COLLAGEN SYNTHESIS IN SPONTANEOUSLY HYPERTENSIVE RATS

ABSTRACT

Recently, we reported that streptozotocin - induced (STZ) diabetes (8 weeks) , reduced the systolic arterial pressure and the activities of collagen biosynthetic enzymes , prolyl hydroxylase and lysyl oxidase, in arteries of spontaneously hypertensive rats (SHR). The purpose of this study was to further characterize the effect of diabetes on aortic collagen synthesis of SHR using more direct methods. Collagen synthesis was quantified by the extent of incorporation of ^{14}C - hydroxyproline into proline by thoracic aortic segments, in vitro. The results demonstrated that STZ - induced diabetes markedly reduced aortic collagen synthesis of SHR, but had no effect on collagen synthesis on aortae from normotensive Wistar - Kyoto rats (WKY). These data lend further support to the hypothesis that the depressor effect of diabetes in SHR may be related to reduced rates of arterial collagen synthesis.

INTRODUCTION

Enhanced vascular collagen synthesis may be implicated as an etiologic factor in the development and maintenance of systemic hypertension in various animal models (1). In prehypertensive spontaneously hypertensive rats (SHR), ^{14}C-lysine incorporation into vascular proteins is increased prior to the onset of hypertension, suggesting a causal relationship between vascular protein synthesis and blood pressure elevation (2). Markers of collagen biosynthesis, prolyl hydroxylase, lysyl oxidase and ^{14}C-proline incorporation into collagenase digestible proteins are elevated in SHR and desoxycorticosterone-salt (DOCA-salt) hypertension. Diuretic and sympatholytic antihypertensive agents prevent or reduce elevated systolic arterial pressure (SAP) and concommitantly lower indices of collagen synthesis (3-7).

Experimental diabetes, of several weeks duration, lowers SAP of SHR without significantly influencing blood pressure of normotensive controls (8-10). A component of the depressor effect in SHR may be secondary to reductions in cardic output or pressure development (11). However, diabetes also disrupts vascular smooth muscle metabolism (12,13). We recently reported that streptozotocin (STZ) induced diabetes reduced aortic and mesenteric arterial prolyl hydroxylase and lysyl oxidase activity in SHR, and hypothesized that the depressor effect in this strain is associated with a reduction in collagen biosynthesis. However, more direct evidence was required to support or refute the hypothesis.

The purpose of the present study was to characterize the effects of diabetes on de novo collagen synthesis of SHR and normotensive Wistar Kyoto (WKY) rat aorta in vitro. The results show that diabetes selectively reduces vascular collagen synthesis of SHR, but not WKY rats, supporting earlier observations. Whether or not these metabolic changes contribute to, or simply coincide with, the reductions in arterial pressure remains to be determined.

METHODS

Experimental Groups

Male weanling spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY) were obtained from Charles River Breeding Labs, housed 6 per cage and fed food and tap water ad libitum. At 14 to 15 weeks of age, diabetes was induced, via a single tail vein injection, with streptozotocin (STZ; 50 mg/kg), dissolved in citrate buffer (0.1 M, pH 4.5). Non-diabetic controls received an equal volume of citrate vehicle alone. The duration of diabetes was eight weeks. Diabetes was monitored weekly by urinary glucose measurements (Diastix^{R}).

Measurement of Systolic Arterial Pressure (SAP)

Systolic arterial pressure (SAP) was determined by the standard tail cuff method after rats were acclimated in a thermoregulated chamber for 20 minutes at 32°C. Pressures were taken in triplicate the week prior to, and 8 weeks after post diabetogen injection (10). At the end of eight weeks the animals were sacrificed by rapid cervical dislocation and aortae and mesenteric artery removed for subsequent biochemical determinations.

Quantitation of Arterial Prolyl Hydroxylase Activity and Collagen Synthesis

Prolyl hydroxylase (PH) activity was quantified by the method of Hutton et al. (14) employing a 3,4-^{3}H-proline labelled peptidyl substrate. Each mesenteric artery was homogenized in 0.5 ml of 0.25 M sucrose buffer containing 0.05 M Tris-HCl (pH 7.4), 10^{-5} M EDTA, 10^{-4} M dithiothreitol and 0.01% triton X-200. The homogenates were centrifuged (15,000 x G for 15 minutes) and 0.2 ml aliquot of the supernatant was

incubated with substrate (0.1 ml/tube, 400,000 dpm) and cofactors necessary for proline hydroxylation for 30 minutes at 30°C. Enzymatic activity was determined by the amount of tritiated water formed after vacuum distillation.

Collagen synthesis was quantified by hydroxyproline analysis as described recently (15). Briefly, thoracic aorta were quickly excised and placed into 2 ml of gassed (95% oxygen, 5% carbon dioxide) Krebs-Henseleit buffer, supplemented with 0.5 mM solution of ascorbate and 20 μCi of ^{14}C-U-L-proline (273 mCi/mmol) and incubated at 37°C for 6 hours. After incubation, protein synthesis was arrested by the addition of cyclohexamide (100 μg/ml) for 20 minutes. Aortae were washed and homogenized in 1 ml phosphate buffered saline (pH. 7.4). Proteins were precipitated with 5% cold TCA and centrifuged. Pellets were washed (5% TCA), ether extracted and hydrolyzed in 6 N HCl for 20 hours at 110°C. Hydrolysates were filtered, evaporated to dryness and neutralized with 0.4 ml potassium tetraborate (0.2 m, pH 9.5). An aliquot (0.1 ml) of the sample was placed into a test tube with 0.02 ml of cis-4-hydroxyproline (internal standard) and potassium tetraborate and derivatized with 0.5 ml of 4-chloro-7-nitrobenzofurazan (NBD-Cl; 8 mM final concentration) for 3 minutes at 60°C in the dark. The reaction was halted by immediate immersion into an ice bath and quenched by the addition of 0.05 ml of 3N HCl. Derivatized amino acids were separated by reverse-phase high performance liquid chromatography, utilizing a 30 cm X 3.9 mm 10 μm silica μBondapak C-18 column (ASI) preceded by a direct connect precolumn (ALTECH) with pellicular C-18 packing and detected with a Gilson fluorimeter (excitation filter 330-380 nm, emission filter 460-600 nm). Total hydroxyproline was determined by

comparing the peak height ratio of determined by comparing the peak height ratio of trans-4-hydroxyproline/cis-4-hydroxy-proline of hydrolysates to those generated from standards. Collagen synthesis was quantified as the amount of radioactive hydroxyproline collected under the corresponding peak. Recovery of radioactivity was uniformly between 90 and 100%. Radioactive eluents were quantified by liquid scintillation spectroscopy (85% efficient for C-14).

Determination of Protein, Serum Glucose and Serum Thyroxine

Proteins were quantified by the method of Lowry et. al (16), using bovine serum albumin as a protein standard.

Serum glucose was assayed colorimetrically using a glucose-oxidase kit provided by Sigma(R) Chemical Company (17). Serum thyroxine (T_4) was measured by radioimmunoassay (Amersham-Searle^R).

Statistics

Mean values between groups within strains were compared using the unpaired Student's t test. Mean values between strains and other multiple comparisons were made using a non-parametric Wilcoxon sum test. Significance was restricted to $p < 0.05$.

RESULTS

The effects of STZ-induced diabetes of 8 weeks duration (henceforward referred to as "diabetes") on final body weight, serum glucose and thyroxine and SAP are shown in Table 1. Diabetes reduced body weights of SHR and WKY by 48 and 36%, respectively, compared to controls of each strain. In both strains, diabetic animals were hyperglycemic and hypothyroid. Diabetes significantly reduced SAP of SHR rats, but did not affect pressures of WKY rats.

Mesenteric arterial prolyl hydroxylase (PH) activity of SHR was higher than that of WKY controls (Table 2). Diabetes significantly reduced PH activity to 56% of control in the SHR strain without influencing WKY enzyme activity.

The concentration of hydroxyproline in aortic protein from SHR and WKY rats were comparable (Table 3). Diabetes increased hydroxyproline concentration in SHR, but not in WKY aortae.

The incorporation of ^{14}C-proline into aortic ^{14}C-hydroxyproline, expressed as the specific activity of hydroxyproline and the dpm hydroxyproline/total TCA insoluble protein, was elevated in SHR compared to WKY. Diabetes markedly reduced the formation of ^{14}C-hydroxyproline in SHR aortae (66%) without significantly altering incorporation into WKY aortae. The de novo hydroxyproline synthesis rate of diabetic SHR aorta was below that of WKY controls.

Table 1. The effects of STZ-induced diabetes (8 weeks) on final measurements in spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). Values are mean ± S. D.

Strain	Group	n	Body Weight[1] (G)	Serum Glucose (mg/dl)	Serum Thyroxine (μg/100 ml)	SAP (mm Hg)
SHR	Control	6	316 ± 10	180 ± 37	5.16 ± 0.64[3]	202 ± 22[3]
	Diabetic	5	167 ± 30[2]	604 ± 147[2]	2.72 ± 0.63[2]	148 ± 24[2]
WKY	Control	5	330 ± 10	190 ± 28	3.84 ± 0.52	129 ± 13
	Diabetic	6	209 ± 32[2]	629 ± 29[2]	1.84 ± 0.32[2]	131 ± 15

[1]The weight of rats (14 weeks old) prior to STZ injection were 259 ± 9 and 204 ± 6 for SHR and WKY, respectively.

[2]Significantly different from the control of the same strain ($p < 0.05$).

[3]Significantly different from the WKY control group.

Table 2. Effects of diabetes on mesenteric arterial prolyl hydroxylase (PH) activity. Values are mean ± S.D., n=5 for each group.

Strain	Group	PH Activity (cpm/mg protein)
SHR	Control	6546 ± 1364[1]
	Diabetic	2881 ± 1458[2]
WKY	Control	4500 ± 1148
	Diabetic	3612 ± 726

[1]Significantly different from the WKY control group ($p < 0.05$).

[2]Significantly different from the control of the same strain ($p < 0.05$).

Table 3. Effects of diabetes on hydroxyproline concentration and on ^{14}C-proline incorporation into SHR and WKY aortic proteins. Values are means ± S.D.

Strain	Group	n	Collagen concentration (μmol hypro/ mg protein)	Collagen Synthesis (dpm hypro/ μmol hypro)	Collagen Synthesis (dpm hypro/ mg protein)
SHR	Control	6	0.279 ± 0.042	20,472 ± 10,775[2]	6020 ± 3154[2]
	Diabetic	5	0.456 ± 0.194[1]	5157 ± 2504[1]	2001 ± 644[1]
WKY	Control	5	0.280 ± 0.110	9951 ± 2517	2447 ± 881
	Diabetic	6	0.261 ± 0.086	6544 ± 4621	1599 ± 990

[1]Significantly different from the control of the same strain ($p < 0.05$).

[2]Significantly different from the WKY control group.

DISCUSSION

In a recent report (10), we showed that the depressor effect of diabetes was associated with reductions in the activities of marker enzymes of collagen synthesis in arteries of SHR. The results of the present study extend the earlier observations, demonstrating more directly that chronic diabetes reduces the rate of SHR aortic collagen synthesis *in vitro*, as indexed by reduced incorporation of ^{14}C-proline into ^{14}C-hydroxyproline. However, diabetes also increased the concentration of collagen in the aorta of SHR, as measured by the amount of hydroxyproline per mg of total protein. These apparently conflicting results probably reflect at least two phenomena: 1) a diabetes-induced reduction in the rate of total protein synthesis by vascular smooth muscle (13) which is proportionally greater than the reduction in collagen biosynthesis, or 2) the relatively low rate of collagen breakdown compared to the catabolism of non-collagenous protein in the diabetic state (18). The hyperglycemia of diabetes may accelerate the non-enzymatic glycosylation of arterial collagen (19), making it more resistant to degradation (20,21). Consequently, diabetic collagen is less suseptable to collagenase digestion (22). In any case, the effects of diabetes on vascular collagen metabolism were strain-dependent; i.e., diabetes had no effect on vascular collagen synthesis or concentration in the normotensive WKY strain.

The mechanism for the reduction in vascular collagen biosynthesis in SHR by diabetes is not known, but may be similar to its effects on skin, which are better characterized. Diabetic rat skin collagen is underhydroxylated, and perhaps more susceptible to intracellular degradation (23,24). Recently, Schneir *et al.* (25) suggested that the

reduced rate of hydroxylation may be related to a diabetes-induced deficiency in ascorbic acid, a cofactor for prolyl hydroxylase (26). Alternatively, either hypoinsulinemia (27) or hyperglycemia (28) may adversely affect collagen biosynthesis.

Circumstantial evidence supports a functional relationship between vascular collagen synthesis and hypertension; however, whether the former is an effect or a cause of the latter is not known (29). For example, the rates of vascular collagen synthesis are elevated in various models of hypertension (30,31,32; Table 3). Antihypertensive agents often reduce the rates of vascular collagen synthesis (1,3-7). Conversely, ß-aminoproprionitrile (BAPN), which inhibits lysyl oxidase-dependent collagen crosslinking reactions, also prevents the development of hypertension and lowers the established blood pressure in various animal models (5,6,32).

Accordingly, a causal relationship between the depressor effect of chronic diabetes and the reductions in vascular collagen biosynthesis in SHR remains to be determined. Interestingly, chronic treatment of SHR with the antihypertensive agent methyldopa, like chronic diabetes, reduces aortic collagen synthesis but increases its concentration (7). The reduced arterial pressure of diabetic SHR might also be related to an attendant autonomic neuropathy (33), undernutrition or hypothyroidism (34,35,36; Table 1). Thyroidectomy prevents the development of hypertension in SHR (35,36). Preliminary results from our laboratory (unpublished) indicate that methimazole treatment lowers arterial pressure and aortic prolyl hydroxylase activity of SHR, but not to the same extent as diabetes does. In addition, hyperglycemia can cause vasodilation and reduced vascular resistance. (37).

The results of this study support the hypothesis that diabetes selectively reduces vascular collagen synthesis of SHR but not of WKY rats. The reduced arterial collagen synthesis may, in part, be responsible for the depressor effect of diabetes in SHR. Further studies examining the effects of hypothyroidism and other hormonal imbalances on vascular collagen metabolism in SHR remain to be elucidated.

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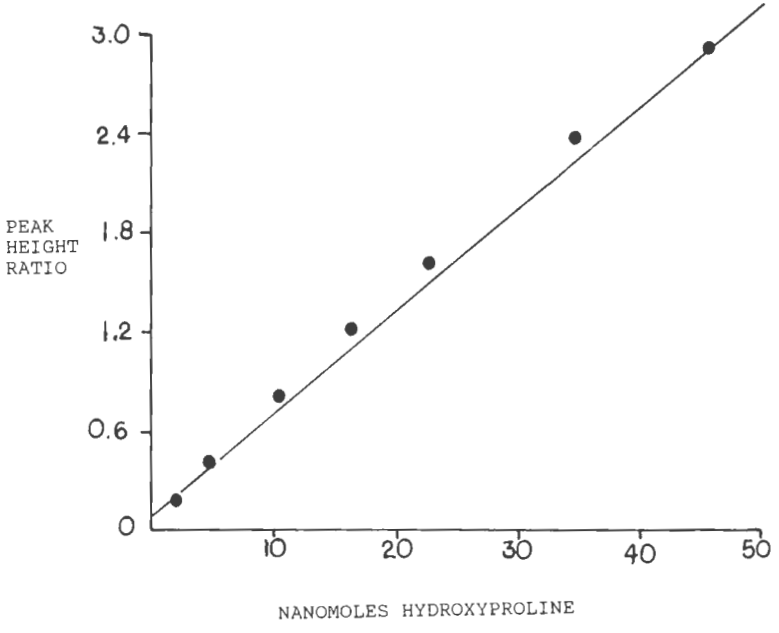
APPENDIX 1. RESULTS ANCILLARY TO MANUSCRIPT I

Figure 2. Standard curve for hydroxyproline obtained by RP-HPLC of the corresponding NBD - derivative. Peak height ratios represent the ratio of the standard peak height to internal standard (c-4-Hyp) peak height. Assay conditions were with 16mM NBD-Cl (8mM final concentration) for 3 minutes at 60^{o}C.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1.8
1.2
0.6
0
PEAK
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RATIO
10
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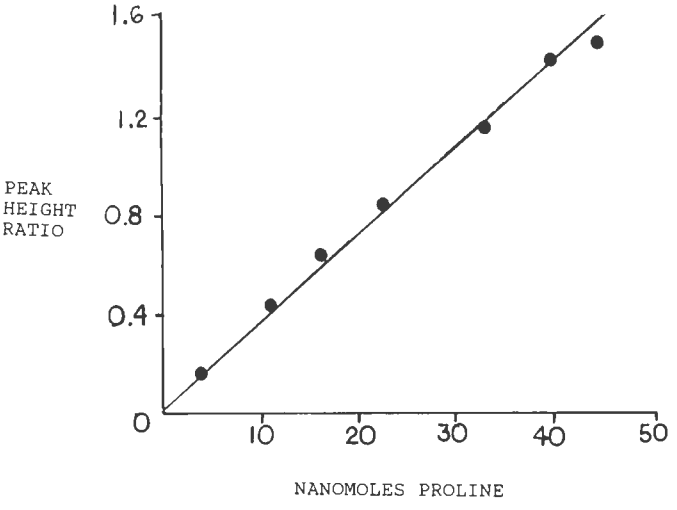
40
50
NANOMOLES HYDROXYPROLINE

Figure 3. Standard curve for proline obtained by RP-HPLC of corresponding NBD - derivative. Peak height ratios represent the ratio of the standard peak height to internal standard (c-4-Hyp) peak height. Assay conditions were 16mM NBD-Cl (8mM final concentration) for 3 minutes at 60°C.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1.2
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NANOMOLES PROLINE

APPENDIX 2. RESULTS ANCILLARY TO MANUSCRIPT II

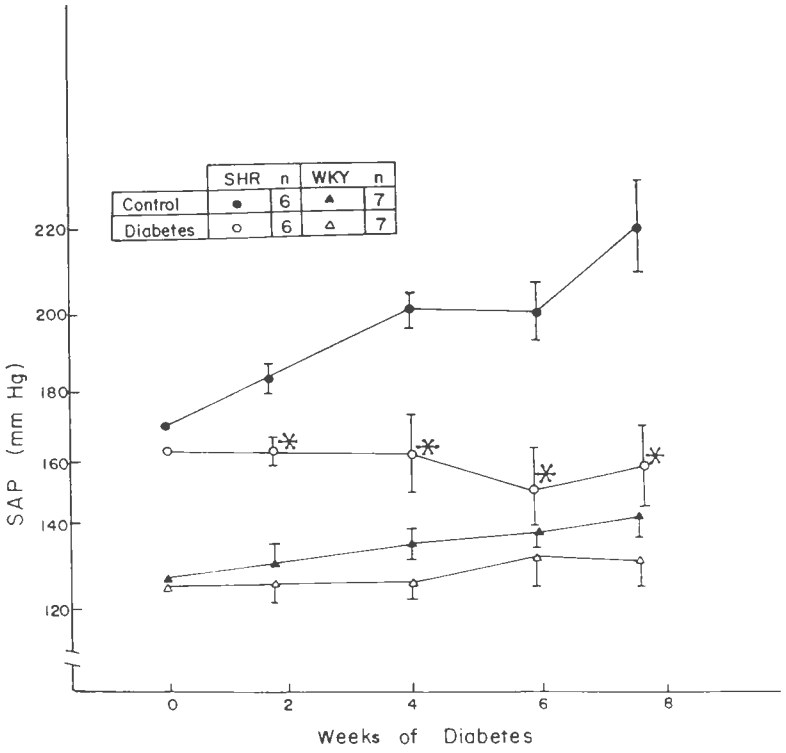

Figure 1. Effects of STZ - induced diabetes on systolic arterial pressure (SAP) of Spontaneously Hypertensive and Wistar - Kyoto rats. SAP was determined on conscious animals using the standard tail cuff method. Values are means ± S.D.

TABLE 4.

Effect of STZ-Induced Diabetes (8 weeks) on Mesenteric Arterial Prolyl Hydroxylase and Lysyl Oxidase Activities of Spontaneously Hypertensive (SHR) and Wistar-Kyoto (WKY) Rats. Values are Means ± S. D., in cpm/mg protein.

Strain	Group	n	Prolyl Hydroxylase Activity	n	Lysyl Oxidase Activity
SHR	Control	5	2261 ± 137*	5	4247 ± 900*
	Diabetic	7	1241 ± 241**	7	1718 ± 1090**
WKY	Control	6	1473 ± 310	6	2085 ± 830
	Diabetic	5	1160 ± 280	4	1780 ± 430

* Significantly different from the nondiabetic WKY group ($p < 0.05$).

** Significantly different from the control of the same strain ($p < 0.05$).

TABLE 5.
Effect of STZ-Induced Diabetes (8 weeks) on Mesenteric Arterial Prolyl Hydroxylase and Lysyl Oxidase Activities of Spontaneously Hypertensive (SHR) and Wistar-Kyoto (WKY) Rats. Values are Means ± S. D., in cpm/mg protein.

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