1988

THE EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES ON SYSTOLIC ARTERIAL PRESSURE AND AORTIC COLLAGEN BIOSYNTHESIS IN THE GOLDBLATT RENOVASCULAR HYPERTENSIVE RAT

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BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

1988

MASTER OF SCIENCE THESIS

OF

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APPROVED:

THESIS COMMITTEE

MAJOR PROFESSOR

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

1988

Abstract

Hypertension is associated with elevated rates of collagen synthesis and deposition in the arterial wall. Whether the increased synthesis and accumulation of vascular collagen is a cause or an effect of the elevated blood pressure in animal models of hypertension is still unresolved. Previous studies in this laboratory have indicated that chronic streptozotocin (STZ)-induced diabetes (8 weeks) reduces systolic arterial pressure (SAP) and vascular collagen synthesis in the spontaneously hypertensive rat (SHR) without affecting either measurement in the normotensive Wistar-Kyoto (WKY) strain. It was not apparent from those studies whether the reductions in SAP and vascular collagen synthesis in the diabetic SHR were causally or coincidentally related. Others have shown that diabetes does not affect SAP in the Goldblatt renovascular hypertensive (RVH) rat. Its effects on vascular collagen synthesis in the RVH model of hypertension has not been investigated. Aortic collagen synthesis was measured in diabetic and non-diabetic RVH and WKY rats in order to determine whether diabetes affects vascular collagen synthesis indirectly through changes in SAP, or whether diabetes affects vascular smooth muscle (VSM) collagen synthesis by a different mechanism. Vascular collagen synthesis was quantified by measuring the incorporation of [^{14}C]-proline into collagen-bound [^{14}C]-hydroxyproline in

rat aortic minces **in vitro**. Aortic prolyl hydoxylase (PH; a collagen biosynthetic enzyme) activity was also measured as an index of collagen biosynthesis. Compared to vehicle-injected controls, rats injected with STZ exhibited the weight-loss, elevated serum glucose, and reduced serum insulin and thyroid hormone levels characteristic of the diabetic state. Renovascular hypertension caused an increase in vascular PH activity and collagen synthesis, presumably through an increase in arterial wall tension. Diabetes significantly reduced vascular collagen biosynthesis and PH activity in the RVH and WKY rat without affecting SAP of either group. Diabetes and hypertension had no significant effects on aortic collagen concentration. These results suggest that experimental diabetes inhibits vascular collagen synthesis by a mechanism independent of a reduction in arterial pressure. The mechanism by which diabetes reduces VSM collagen synthesis in the rat is unknown, but may involve hypoinsulinemia, hypothyroidism, or autonomic neuropathy.

ACKNOWLEDGEMENTS

I am extremely grateful for the patience, concern, and remarkable insight of Robert L. Rodgers throughout the course of this study. Dr. Rodgers has done more to ensure the completion of this work than most will ever know. I am indebted to Amy Davidoff for her invaluable assisstance and endless encouragement from the beginning. I do not stand alone in my appreciation of Amy's willingness to help. I would like to thank special friends Michael Christe and Francine Pinnault for making the stresses of graduate study just that much more bearable. I would also like to thank Dr. Chichester for his contributions to this project. This acknowledgement would not be complete if I did not extend my heartfelt thanks to Bill Sessa and Linda Toy for their ever-present confidence and moral support in my endeavors.

I can not begin to express my gratitude for the love, support, and understanding I have received from my family, especially my father, Louis Mariani, and step-mother, Kathleen Mariani, throughout my lifetime.

DEDICATION

I wish to dedicate this thesis to the memory of my mother, Katherine W. Mariani, whose pride in this accomplishment would have been unsurpassed.

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Introduction

Experimental diabetes mellitus inhibits the synthesis of collagen by vascular smooth muscle (VSM). Both chemically-induced diabetes (streptozotocin; STZ) and genetic diabetes are characterized by reduced rates of aortic collagen synthesis in the rat (Schneir **et al.**, 1979; Bowersox and Sorgente, 1985). Administration of insulin **in vitro** to vascular tissue obtained from diabetic animals partially restores the depressed rates of collagen synthesis toward normal (Mikkonen **et al.**, 1966; Arnquist and Norrby, 1980). The evidence suggests that insulin regulates VSM collagen synthesis, but leaves open the possibility that other factors may be involved.

The rate of collagen synthesis by VSM is also influenced by arterial pressure, and the resultant stress on the arterial wall (Gilligan and Spector, 1984). Elevated wall stress produced by hypertension is consistently associated with increases in arterial VSM collagen synthesis and collagen content (Wolinsky, 1970, 1971; Foidart **et al.**, 1978), regardless of the experimental model of hypertension. Both prolyl hydroxylase (PH; a collagen biosynthetic enzyme) activity and the incorporation of radiolabelled proline into collagen (a more direct measure of collagen synthesis) are elevated in the desoxycorticosterone acetate (DOCA)-salt and

spontaneously hypertensive rat (SHR) models of hypertension (Iwatsuki et al., 1977a). Antihypertensive therapy usually prevents or reverses the elevated rates of VSM collagen synthesis (Ooshima et al., 1974), most likely by reducing arterial pressure rather than by a direct action on the vascular smooth muscle cell (Oshima et al., 1983).

Diabetes can affect arterial pressure, especially of hypertensive animals. In the absence of hypertension, diabetes produces small and variable changes in blood pressure (Cavaliere et al., 1980; Pfaffman, 1980; Sasaki and Bunag, 1982; Rodrigues and McNeill, 1986; Rodgers, 1986; Ganguly et al., 1987). However, in the SHR, chronic diabetes of several weeks duration consistently reduces systolic arterial pressure (SAP), occasionally restoring SAP to the levels of nonhypertensive controls (Somani et al., 1979; Cavaliere et al., 1980; Rodrigues and McNeill, 1986; Rodgers, 1986; Yamamoto and Nakai, 1988). This depressor (pressure-lowering) effect of diabetes may be dependent on the experiemntal model of hypertension. Diabetes does not affect SAP in the DOCA-salt (Sasaki and Bunag, 1982) or the renovascular hypertensive (RVH) rat (Factor et al., 1981; Fein et al., 1984). The mechanism for the selective effect of diabetes on SAP in the SHR strain remains to be clarified.

Recent work in this laboratory indicates that diabetes exerts a much more profound effect on VSM collagen synthesis in the SHR than it does in the nonhypertensive WKY control strain. STZ-induced diabetes reduced SAP **in vivo**, and caused a depression in several indices of collagen synthesis by aortic segments **in vitro**, in the SHR (Rodgers **et al.**, 1985; Sessa **et al.**, manuscript in preparation). In contrast, diabetes of equal severity and duration had no effect on SAP or vascular collagen synthesis in the normotensive WKY control. These observations led to two alternative hypotheses. Diabetes may have reduced VSM collagen synthesis in the SHR indirectly through its influence on SAP. Alternatively, the reductions in VSM collagen synthesis and SAP in diabetes may have been coincidental and unique to the SHR strain.

The purpose of the present study was to provide additional evidence to distinguish between these possibilities. Arterial pressure and aortic PH activity and collagen synthesis were measured in diabetic and non-diabetic RVH and WKY rats. The lack of a depressor effect of diabetes in the Goldblatt RVH rat, in contrast to its depressor effect in SHR, provides a model system to determine whether diabetes reduces vascular collagen synthesis indirectly through a reduction in SAP, or whether diabetes reduces vascular collagen synthesis by a mechanism independent of this phenomenon. This study also enabled us

to determine whether the depressor effect of diabetes in the SHR was unique to that strain.

Experiemntal

Animal preparation

Male Wistar-Kyoto (WKY) rats were purchased from Charles River Breeding Labs (Wilmington, MA), housed five per cage, and maintained on standard rat chow (Agway Inc., Syracuse, NY) and tap water ad libitum. At 12 weeks of age, "two-kidney, one-clip" Goldbaltt renovascular hypertension was induced by placing a silver clip (internal diameter 0.20 mm) on the left renal artery and leaving the contralateral kidney intact (Goldblatt **et al.**, 1934). Sham-operated WKY animals served as normotensive controls. The clip size was chosen so as to yield maximal sustained hypertension (Murphy **et al.**, 1984). Systolic arterial pressure (SAP) was determined weekly by the standard tail cuff method after warming the animals in a thermoregulated chamber (32^{o}C x 20 min.). All SAP values are means of four to six measurements recorded after the blood pressure had stabilized. Three to four weeks after surgery (15-16 weeks of age), the clipped animals with an SAP of 160 mmHg or greater were included in the hypertensive group, while the remaining normotensive animals were excluded from the study. Hypertension was produced in approximately 65-70% of the clipped animals. A randomly selected subgroup of each of the hypertensive and sham-operated normotensive animals was made diabetic with a

single tail vein injection of streptozotocin (STZ; 50 mg/Kg in 0.10 M citrate buffer, pH 4.5) under light ether anesthesia. Prior to STZ injection, the mean SAP values of the two treatment subgroups within each group were not significantly different from each other. The respective controls received an equal volume of the citrate buffer alone. Thus, four experimental groups comprised the study: 1) renovascular hypertensive nondiabetic control, 2) renovascular hypertensive-diabetic, 3) sham-operated WKY normotensive nondiabetic control, and 4) sham-operated WKY normotensive diabetic. Diabetes was monitored weekly by urinary glucose measurement (Diastix^{R}). In addition, heart rate and body weight were also monitored weekly throughout the study. Groups 1-4 were sacrificed at 24 weeks of age following 8 weeks of diabetes. In a separate study, groups 1-3 were sacrificed at 18 weeks of age, 3 weeks after STZ injection. All animals were killed by rapid decapitation and blood was collected for the assay of serum glucose, T_3, T_4, and insulin levels.

Serum glucose, insulin, T_3, and T_4 determinations

Serum glucose was measured colorimetrically by the glucose oxidase method using a kit purchased from the Sigma Chemical Company (St. Louis, MO). Serum T_3, T_4, and insulin were measured by radioimmunoassay with kits purchased from

Cambridge Diagnostics (Cambridge, MA). All serum determinations were kindly performed by F. Pinnault in our laboratory.

Determination of collagen synthesis

Aortic collagen biosynthesis was characterized by measuring the extent of incorporation of U-^{14}C-L-proline into [^{14}C]-hydroxy-L-proline at a single time point *in vitro*. The activity of prolyl hydroxylase (PH) was measured in cell-free aortic homogenates. At sacrifice the thoracic aorta extending from the arch to the diaphragm was excised and cleaned of perivascular tissue. When prolyl hydroxylase activity was to be measured, the aorta was cut in half longitudinally and one half was immediately frozen for prolyl hydroxlase determination at a later time. The aorta was minced and incubated in 2mL of gassed (95% O_2, 5% CO_2) Krebs-Henseleit (K-H; Rodgers et al., 1985) buffer solution (pH 7.4) supplemented with U-[^{14}C]-L-proline (10uCi/mL; specific activity 268.1 uCi/umole) and ascorbate (0.50 mM) at 37^{o}C for 6 hours. It has been previously shown in this laboratory that aortic collagen rates are linear for up to 10 hours under these conditions (Sessa et al., 1986). After incubation, protein synthesis was halted by replacing the K-H buffer with 2 mL ice-cold saline containing cycloheximide (0.10mg/mL) and incubating on ice for 20 minutes.

An experiment was done to test the effect of carrier L-proline on the rate of aortic collagen synthesis in vitro, as Peterkofsky **et al.** (1982) have found that this treatment enhances the uptake of proline into the cell. This may in turn affect the rate of collagen synthesis by expansion of the intracellular proline pool. It was found, however, that carrier proline did not enhance collagen synthesis in the rat aorta. The results are presented and discussed in Appendix 1.

An aliquot of the whole homogenate was taken for the assay of NaOH-soluble protein by the method of Lowry **et al.** (1951) as follows. Fifty uL of the homogenate was added to 450 uL of 1N NaOH, allowed to stand 1 hour at room temperature, and then centrifuged for 15 minutes in a Beckman tabletop refrigerated centrifuge. Two 200 uL aliquots were removed from each sample and immediately frozen for future protein determination.

Proteins were precipitated by the addition of 50% ice-cold trichloroacetic acid (TCA) to yield a final concentration of 5%, and washed 6 times with ice-cold 5% TCA to remove unincorporated [^{14}C]-proline. Residual TCA was removed with 3 ethanol-ether washings (3:1, v/v) followed by 3 washings with anhydrous ether. The protein pellet was hydrolyzed in 6N HCl for 20 hours at 110^{o} C. The hydrolysates were filtered, and the filtrate was evaporated

to dryness, resuspended in distilled water and evaporated to dryness again to remove residual HCl. The dried residue was was stored at -10^{o}C until the time of analysis by high performance liquid chromatography (HPLC).

HPLC sample preparation

The sample residue was resuspended in 0.40 mL of 0.10 M potassium tetraborate (pH 9.5) at which time a thick whitish precipitate would form. A 100 uL aliquot of the suspension was placed in a microcentrifuge tube and centrifuged at 6000 x g for 5 minutes. The supernatant fluid was removed and saved, the pellet was washed twice with 150 uL of the tetraborate buffer, and all the washings were added to the supernatant fluid. The volume of the combined supernatant fluid and washings was adjusted to 480 uL with 0.10 M tetraborate, and 20 uL of 5 mM cis-4-hydroxy-L-proline was added as an internal standard. Cis-4-hydroxy-L-proline (cis-hypro) was chosen as the internal standard because of its absence from naturally occuring proteins and its convenient elution pattern between trans-4-hydroxy-L-proline (trans-hypro) and L-proline during separation by HPLC. An epimerization reaction does occur during hydrolysis in which approximately 10% of the trans-hypro is converted to the cis form of the molecule (Berg, 1982), but this was taken into account in the calculation of collagen synthetic rates (see

Data Analysis). The identity of the precipitate remains unknown; however it was determined that neither proline nor hydroxyproline were components of the precipitate itself but, rather, were trapped within the matrix of the pellet. Greater than 99% of the radioactivity could be removed from the pellet by 4-5 washings, and in the present experiments approximately 3-5% of the total radioactivity remained in the pellet after the two tetraborate washes. Values for incorporation of proline into collagen were not corrected for the 3-5% of radioactivity remaining in the washed pellet.

Imino acid derivatization and HPLC analysis

4-chloro-7-nitrobenzofurazan (NBD-Cl) is a derivatizing agent used to label secondary amines. The reaction of NBD-Cl with secondary amino acids such as hydroxyproline and proline occurs at a rate 10 times faster than that for primary amino acids (Ahnoff **et al.**, 1981). The resulting fluorescent derivatives can be seperated by reversed-phase HPLC and quantified by fluorescence spectroscopy with a high degree of sensitivity. The derivatization and chromatographic conditions were those of Ahnoff **et al.** (1981) and Lindblad and Dieglman (1984) as modified by Sessa **et al.** (1986) for the quantification of collagen biosynthetic rates in vascular tissue. An equal volume of

NBD-Cl (20 mM) was added to 500 uL of the resuspended, washed hydrolysate and incubated in the dark at 60^{o}C for 3 minutes. The reaction was stopped by immersion in an ice-bath and the addition of 0.10 mL 6N HCl. A 100 uL aliqot was taken for separation and analysis by reversed-phase HPLC.

The HPLC system consisted of a Waters M-45 constant flow pump, an ISCO Isis autosampler, and a Gilson Spectraglow flourimeter connected to a Shimadzu CR-3A chromatopac integrator. Separation was performed on a 30 cm x 3.9 mm i.d. 10 micron silica uBondpak C_{18} column preceded by a guard column with C_{18} pellicular packing. The mobile phase consisted of 24.5% acetonitrile and 0.08% phosphate (pH 2.30) and was degassed by vacuum suction for 30 minutes. Separation was performed isocratically with a flow rate of 1.6 mL/min. at room temperature. Fractions (0.80 mL) were collected and radioactivity was measured by liquid scintillation spectroscopy in a Packard Tri-Carb^{R} 4000 Series liquid scintillation counter. Quenching caused by the yellow fluorescent color was corrected for by using the quenching index parameter function of the Packard counter, but in most cases the amount of quenching was minimal. The counting efficiency averaged 88-90%.

Data Analysis

Collagen synthesis was determined by the amount of radioactivity collected under the trans- and cis-hypro peaks (any radioactive cis-hypro arises from trans-hypro due to epimerization during hydrolysis), and expressed as disintegrations per minute (dpm) hypro formed per umole hypro (specific activity) per 6 hour incubation. Collagen concentration was expressed as nmole hypro per mg. protein. Total umoles of hydroxyproline were quantified by comparing the ratio of of trans- and cis-hydroxyproline peak heights of each sample to those generated from standards. A separate standard curve was generated for each set of samples analyzed by HPLC on any given day. The underivatized proline and hydroxyproline eluted in the first few fractions of each sample, and the percent derivitization was calculated by dividing the underivatized radioactivity by the total radioactivity recovered from each sample. All samples were corrected for percent derivatization (mean 90 $\pm$ 5%) in the calculation of collagen synthetic rates.

Prolyl hydroxylase assay

The assay of aortic proline hydroxylase (proline, 2-oxo-glutarate dioxygenase) was a modification of the method of Hutton **et al.** (1966) employing a 3,4-^{3}H-proline

labelled substrate prepared from 8 day-old chick calvaria bones. Aortic tissue was thawed after approximately one week at -10°C and homogenized in 0.50 mL of a buffer containing 0.25M sucrose, 50mM tris-HCl, 10mM EDTA, 10mM dithiothreitol, and 0.01% Triton X-100, pH 7.4. The homogenate was centrifuged at 15000 x g. for 20 minutes. A 0.20 mL aliquot of the supernatant fluid was incubated with substrate (denatured at 45°C for 30 minutes; approximately 400,000 cpm per tube) and cofactors necessary for proline hydroxylation for 30 minutes at 30°C. The cofactors and their final concentrations are as follows: $Fe(NH_4)_2(SO_4)\cdot 6H_2O$ (0.1mM), sodium ascorbate (0.1mM), alpha ketoglutarate (0.1mM), and bovine serum albumin (0.2%). The reaction was buffered at pH 7.4 with Tris-HCl (0.05M).

The activity of prolyl hydroxylase was quantified by determining the amount of tritium released during incubation and collected as tritiated H_2O by vacuum distillation. The amount of protein in the 15,000 x g. supernatant fluid was determined by the Lowry method, and prolyl hydroxylase activity was expressed as cpm per mg. protein.

Statistical anylysis

Multiple comparisons between groups and treatments were made using analysis of variance (ANOVA) followed by Student Neuman-Keuls (Statistical Analysis Systems, Cary, NC). Single comparisons were made using the unpaired Student's t test. Significance was restricted to $p < 0.05$.

Results

The effects of streptozotocin (STZ)-induced diabetes of 8 weeks duration on serum measurements and body weight of renovascular hypertensive and normotensive rats are summarized in Table 1. Injection of STZ increased the serum glucose levels of both the hypertensive and normotensive groups to approximately the same extent. The serum insulin levels were similar in both of the nondiabetic control groups, and these levels were reduced to a similar degree by diabetes in both the hypertensive and normotensive diabetic animals. By these criteria, then, the degree of diabetes induced by STZ in the hypertensive group was similar to that of the normotensive animals. Serum thyroxine and triiodothyronine were also measured and found to be significantly reduced in the diabetic animals, indicating that STZ-induced diabetes is accompanied by hypothyroidism. Interestingly, serum T_3 in the renovascular hypertensive nondiabetic control was slightly but significantly lower than the corresponding WKY control group, while serum T_4 levels in these control groups were not significantly different from each other. The body weights of the hypertensive and normotensive controls were not significantly different from each other 12 weeks after surgery. Eight weeks of diabetes significantly reduced body weight in the RVH and WKY groups by 20% and 16%

Table 1. Serum glucose, insulin, T_3, and T_4, and body weight of renovascular hypertensive (RVH) and normotensive Wistar-Kyoto (WKY) rats following 8 weeks of experimental diabetes. Values are means $\pm$ S.D. Diabetes was induced 3-4 weeks after surgery (15-16 weeks of age) with a single tail vein injection of streptozotocin (STZ; 50 mg/Kg) and the animals were sacrificed 8 weeks thereafter.

Table 1

Group	Treatment	n	Glucose (mg/dL)	Insulin (uU/mL)	T_3 (ug/mL)	T_4 (ug/mL)	Body weight (grams)
RVH	control	7	127 ± 11	53.0 ± 22.6	70.2 ± 15.0[1]	5.22 ± 1.62	344 ± 47
	diabetic	7	426 ± 55[2]	1.8 ± 2.8[2]	41.9 ± 10.3[2]	2.59 ± 0.60[2]	274 ± 33[2]
WKY	control	5	116 ± 34	49.0 ± 9.5	86.0 ± 15.5	4.9 ± 0.13	378 ± 32
	diabetic	7	395 ± 62[2]	1.9 ± 3.0[2]	41.3 ± 6.5[2]	2.72 ± 0.36[2]	319 ± 26[2]

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

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

respectively. The mean body weights of the four groups studied were not significantly differerent from each other at the time of STZ or citrate buffer injection.

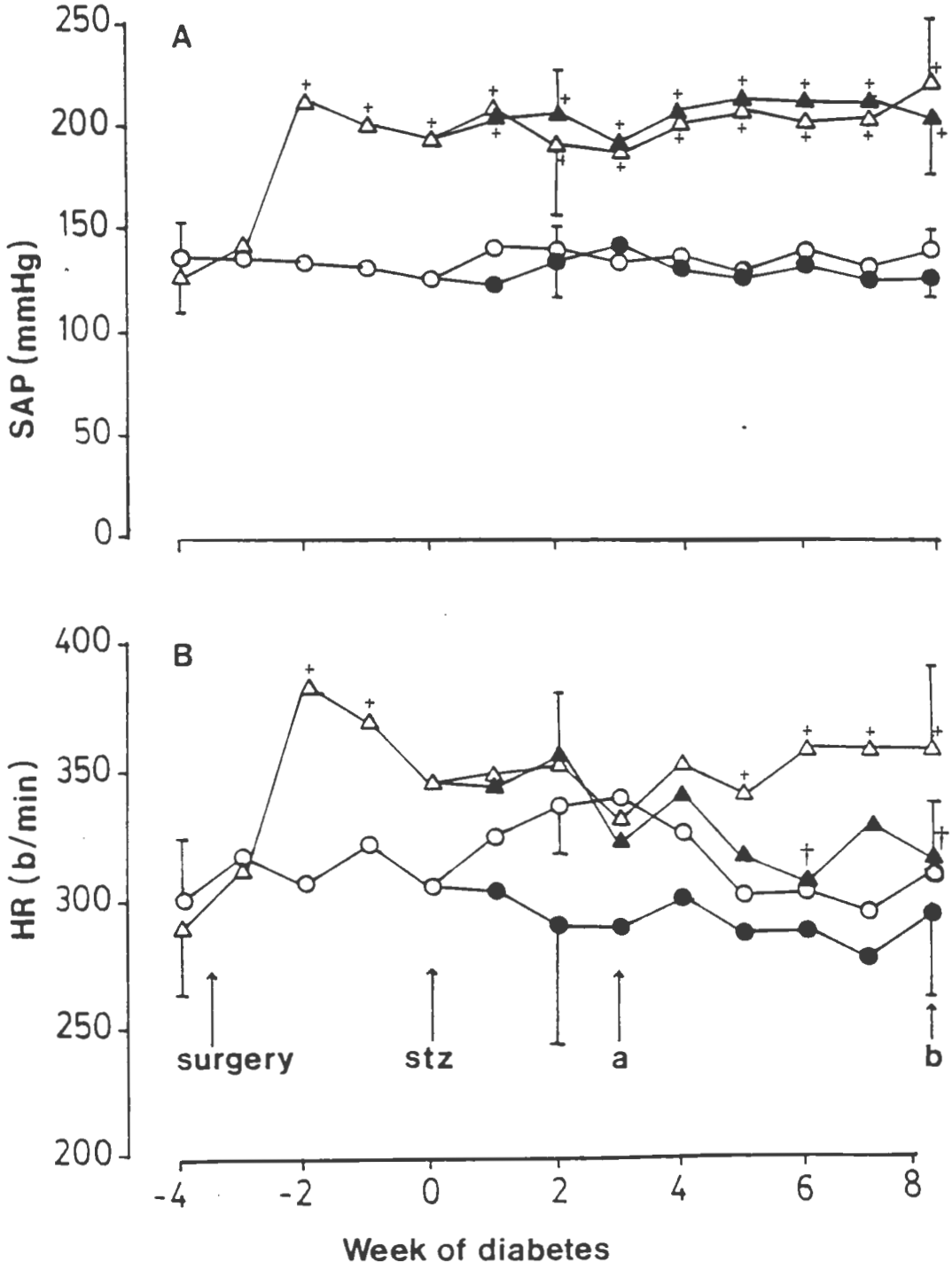
Figure 1 depicts the effects of diabetes on systolic arterial pressure (SAP) and heart rate (HR) in RVH and WKY rats. The SAP rose rapidly following surgery in the clipped animals and remained significantly elevated over the sham-operated controls for the duration of the study. Diabetes had no effect on SAP at any time point in either the hypertensive or normotensive groups. The SAP in the nondiabetic and diabetic RVH animals following 8 weeks of diabetes (12 weeks post-surgery) was 221 $\pm$ 29 and 200 $\pm$ 30 mmHg, respectively. Following 8 weeks of diabetes the SAP in the nondiabetic and diabetic WKY groups were 137 $\pm$ 4 and 126 $\pm$ 7 mmHg, respectively. The changes in heart rate observed in the hypertensive and diabetic state were more varied than the changes seen in systolic arterial pressure. The heart rate rose in parallel with SAP following surgery in the RVH animals and was significantly elevated over the normotensive WKY 2 and 3 weeks after renal artery clipping. However, the HR in the RVH control and diabetic groups was not significantly different from the normotensive control during the following 5 weeks, due in part to a transient elevation in the HR of the WKY nondiabetic control group. Heart rate in the RVH control group was significantly greater than the WKY control during the last four weeks of the study (9-12

Figure 1. The effect of streptozotocin (STZ)-induced diabetes on systolic arterial pressure (SAP; A) and heart rate (HR; B) of renovascular hypertensive (RVH) and Wistar-Kyoto (WKY) rats. Animals were injected with STZ 3-4 weeks after surgery (15-16 weeks of age) and sacrificed following 8 weeks of diabetes (b). In addition, a group of RVH and WKY nondiabetic control animals was sacrificed 6 weeks following surgery (18 weeks of age; a). Vertical brackets indicate 1 S.D. The groups and their symbols are as follows: RVH nondiabetic, open triangles; RVH diabetic, closed triangles; WKY nondiabetic, open circles; WKY diabetic, closed circles.

*Significantly different from the WKY nondiabetic control.

tSignificantly different from the control of the same group.

Figure 1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weeks after surgery). Heart rate was significantly reduced in the RVH diabetic versus nondiabetic 6 and 8 weeks following STZ injection, while diabetes reduced HR in the WKY animals only at week 3 following injection. In general, 8 weeks of diabetes reduced HR in the RVH animals, while it had no effect on SAP at any point during the study. In the WKY animals, diabetes affected heart rate at only a single early time point, while, similar to the RVH animals, diabetes had no effect on SAP throughout the eight week period.

Aortic collagen synthesis after 8 weeks of streptozotocin-induced diabetes is presented in Table 2. Collagen synthesis was also measured in hypertensive diabetic and nondiabetic animals 3 weeks after STZ or vehicle injection (6 weeks after surgery), but the data was not included in Table 2 because the severity of diabetes was in question (see Appendix 3). Aortic collagen synthesis is expressed as dpm hydroxyproline incorporated into collagen per umole total hydroxyproline (specific activity) during the 6 hour incubation period. Aortic collagen synthesis was significantly elevated in the RVH control 12 weeks after renal artery clipping. Eight weeks of diabetes significantly reduced collagen synthesis in the RVH and WKY groups by 55% and 72%, respectively.

Table 2. Aortic collagen synthesis in renovascular hypertensive (RVH) and Wistar-Kyoto (WKY) rats 6 and 12 weeks following surgery. Values are means ± S.D.

Group[a]	Treatment	n	Collagen Synthesis (dpm hypro/ umole hypro x 10^{-3})
RVH	control	10	72.5 ± 28.5[1]
	diabetic	9	32.7 ± 11.6[2]
WKY	control	8	58.3 ± 27.9
	diabetic	7	16.2 ± 3.9[2]

[a]The RVH and WKY animals were sacrificed 12 weeks following surgery and 8 weeks following STZ injection

[1]significantly different from WKY control.

[2]significantly different from control of the same group.

Collagen synthesis has also been expressed as dpm hydroxyproline per mg. protein (Ooshima **et al.**, 1977; Iwatsuki **et al.**, 1977a); however, this expression of the data was not included in Table 2 because diabetes also affects total protein content of various tissues (see Appendix 2).

The concentration of collagen in the aortas of 8 week diabetic RVH and WKY rats are shown in Table 3. Collagen concentration is expressed as nanomoles hydroxyproline per mg. total protein. Although there were no statistically significant differences between any of the groups, certain trends are suggested. The aortic collagen concentration of the 3 week diabetic group is presented and discussed in Appendix 3.

Table 4 shows the effects of diabetes of 8 weeks duration on aortic prolyl hydroxylase (PH) activity in the hypertensive (RVH) and normotensive (WKY) rat. The activity of this collagen biosynthetic enzyme paralleled the overall

Table 3. Collagen concentration in aortas of renovascular hypertensive (RVH) and Wistar-Kyoto (WKY) rats 6 and 12 weeks following surgery. Values are means ± S.D.

			Collagen concentration (nMole hypro/mg. protein)		
Group	Treatment	n	6 weeks	n	12 weeks[a]
RVH	control	8	385 ± 68	7	430 ± 91
	diabetic	–	———	7	601 ± 220
WKY	control	7	444 ± 80	5	588 ± 108
	diabetic	–	———	7	591 ± 171

[a]The 12 week RVH and WKY rats were diabetic for 8 weeks.

Table 4. Aortic prolyl hydroxylase (PH) activity in renovascular hypertensive (RVH) and Wistar-Kyoto (WKY) rats after 8 weeks of diabetes. Values are means ± S.D. and expressed as cpm per mg. protein.

Group	Treatment	n	PH Activity
RVH	control	14	5213 ± 2592[1]
	diabetic	12	2355 ± 1449[2]
WKY	control	14	3306 ± 1671

All animals were sacrificed 12 weeks after renal artery clipping (RVH) or sham operation (WKY).

[1]Significantly different from the nondiabetic WKY control

[2]Significantly different from the nondiabetic control of the same group.

rate of collagen synthesis measured by the incorporation of ^{14}C-proline into ^{14}C-hydroxyproline (Table 3). Twelve weeks after renal artery clipping, aortic PH activity in the RVH control was significantly greater than that in the WKY control. Eight weeks of diabetes reduced PH activity in the aortas of RVH rats to, and even below, normotensive values. Aortic PH activity was not determined in the WKY diabetic rat.

Discussion

One prominent finding in this study is that the rate of aortic collagen biosynthesis in the renovascular hypertensive (RVH) rat is significantly elevated over that in the normotensive WKY control, 12 weeks after renal artery clipping (Table 2). Collagen synthesis was not significantly elevated in the RVH rats 6 weeks after the induction of hypertension ($p = 0.07$). This may have been due to the high variability in the synthetic rates in both the RVH and WKY groups. The aortic prolyl hydroxylase (PH) activity paralleled the rate of aortic collagen biosynthesis in the hypertensive and normotensive rats 12 weeks following surgery (Table 3). These findings are in line with the large body of evidence indicating that elevated arterial pressure is associated with an increase in PH activity and collagen biosynthesis in the blood vessel wall (Gilligan and Spector, 1984). It is still unclear, however, whether the elevated arterial pressure is a cause or an effect of the elevated synthesis and accumulation of vascular collagen. There is evidence in the literature supporting both of these possibilities. Both vascular collagen and noncollagen protein synthesis are increased in SHR before blood pressure is significantly elevated (4-5 weeks of age; Yamabe and Lovenberg, 1974; Deyl et al., 1987). Iwatsuki et al. (1977b) examined the role of vascular collagen in the development and maintenance of elevated arterial pressure in the

DOCA-salt hypertensive rat using the lysyl oxidase inhibitor beta-aminoproprionitrile (BAPN). Lysyl oxidase (LO) is involved in the crosslinking of newly synthesized collagen fibrils. Inhibition of this enzyme reduces the number of lysine-derived crosslinks, which increases the solubility of collagen and renders it more susceptible to proteolytic degradation (Vater **et al.**, 1979). Iwatsuki **et al.** reported that BAPN delayed the onset and partially reversed the elevated arterial pressure in DOCA-salt hypertensive rats. This was accompanied by a reduction in vascular collagen content in these animals. This study suggests that vascular collagen accumulation may be responsible in part for the development and maintenance of elevated blood pressure in the DOCA-salt model of hypertension. The findings of Gilligan **et al.** (1982) partially support this hypothesis. They showed that 8 weeks of clonidine (a sympatholytic antihypertensive agent) treatment reduced arterial pressure and vascular collagen synthesis and content in the DOCA-salt hypertensive rat to normotensive levels. Arterial pressure and collagen synthesis remained normal after discontinuation of clonidine treatment, suggesting that the restructuring of the arterial wall in itself may be sufficient to halt the progression of hypertension in the DOCA-salt model. There are studies, however, which report a dissociation between arterial pressure and vascular collagen synthesis or content in several models of hypertension. Both reserpine and captopril prevent the increase in blood pressure in the

Goldblatt one-kidney, one-clip (1K-1C) RVH rat, but only reserpine prevented the increase in aortic collagen deposition in this model (Rorive **et al.**, 1980). Treatment of DOCA-salt hypertensive rats with the prolyl hydroxylase inhibitor P-1894B for 6 weeks prevented the rise in PH activity and collagen synthesis in the thoracic aorta and mesenteric artery without affecting the development of elevated arterial pressure (Fukumoto **et al.**, 1984). Franklin **et al.** (1982) examined the effect of BAPN and 3,4 dehydroproline (DHP; substitutes for proline and results in the synthesis of defective collagen) on arterial pressure and aortic collagen content in the spontaneously and DOCA-salt hypertensive rat. In the SHR, BAPN reduced arterial pressure without reducing aortic collagen content. Both BAPN and DHP prevented the increase in aortic collagen content in the DOCA-salt rats, but only BAPN prevented the rise in blood pressure. These results suggest that BAPN may have a depressor effect in hypertensive animals independent of its effect on vascular collagen accumulation, and therefore the reductions in arterial pressure and vascular collagen content in BAPN treated hypertensive animals may be coincidental. BAPN also reduces vascular collagen content in normotensive rats without affecting arterial pressure (Iwatsuki **et al.**, 1977b). Gilligan **et al.** (1982) showed that clonidine treatment also reduced arterial pressure and vascular collagen synthesis and content in the SHR. However, discontinuation of clonidine treatment resulted in an

increase in both blood pressure and vascular collagen synthesis, suggesting that arterial pressure is a regulator of vascular collagen biosynthesis in this model. To directly examine the role of arterial pressure in regulating vascular collagen biosynthesis, Iwatsuki **et al.** (1977a) measured collagen synthesis in arteries and veins of SHR and DOCA-salt hypertensive rats. They found that PH activity, collagen synthesis, and collagen content were elevated in the arterial, but not the venous, vessels of both models. It appears that the elevations in vascular collagen synthesis and content are limited to the portion of the vasculature that is subjected to increased hemodynamic stress. Hume and Bevan (1978) measured collagen synthesis in blood vessels from rabbits with abdominal coarctation hypertension, with sham-operated, normotensive animals serving as controls. Collagen synthesis was increased in the arteries proximal to the aortic constriction (thoracic aorta, ear and carotid arteries), while collagen synthesis in arteries and veins on the normotensive side of the ligature was not elevated. There is also **in vitro** evidence supporting a regulatory influence of arterial pressure on vascular collagen synthesis. Leung **et al.** (1976) showed that collagen synthesis of cyclically stretched arterial smooth muscle cells (SMCs) in culture was much greater than collagen synthesis in non-stretched control cells. They speculated that the cyclic elevations in tension were the stimulus for the elevated synthesis of collagen by the arterial SMCs. The

majority of evidence, then, indicates that the elevated vascular collagen synthesis and content observed in experimental hypertension represents an adaptative response to the elevated arterial wall stress caused by the increasing blood pressure. Although accumulation of mural fibrous protein could further exacerbate hypertension by reducing the lumen diameter of resistance vessels (thereby increasing peripheral vascular resistance), it is probably not the primary cause of elevated arterial pressure in experimental hypertension.

Chronic streptozotocin-induced diabetes (8 weeks) significantly reduced aortic PH activity and the incorporation of ^{14}C-proline into aortic collagen in the RVH rat (Tables 2 and 3), and reduced aortic collagen synthesis in the normotensive WKY (Table 2). The activity of prolyl hydroxylase ia a reliable and consistent marker for the rate of collagen biosynthesis regardless of tissue type (Cardinale and Udenfriend, 1974), and although PH activity was not measured in the WKY aorta, it is likely that diabetes reduced vascular PH activity in this group as well. This contention is supported by both Yue **et al.** (1987) and McClellan **et al.** (1988) who showed that STZ-diabetes reduced PH activity in granulation (neovascular) tissue recovered from cyclinders implanted subcutaneously in normotensive rats. In general, the results of the present study suggest

that diabetes does reduce net collagen synthesis in both the hypertensive and normotensive rat aorta.

Diabetes may affect collagen synthesis through an inhibition of amino acid transport. Although this possibility can not be entirely excluded, there is circumstantial evidence suggesting that it does not significantly contribute to the reduction in collagen synthesis observed with diabetes. It was shown in this laboratory (unpublished) that chronic STZ diabetes did not reduce aortic proline pools in the SHR or WKY rat. Pain and Garlick (1974) found that experimental diabetes did not change the ratio of tissue to plasma free tyrosine specific activity in the rat heart **in vivo**, indicating that diabetes did not interfere with the transport of the amino acid into the tissue. Furthermore, the rate of amino acid incorporation into protein was reduced in cell-free skeletal muscle preparations derived from diabetic rats (Wool **et al.**, 1968; Pain, 1973). These results seem to indicate that diabetes reduces collagen biosynthesis by a mechanism independent of a reduction in amino acid uptake by the vascular smooth muscle cell.

The argument could be raised that the apparent reduction in collagen synthesis caused by diabetes is simply an artifact of an underhydroxylation of collagen (Schneir **et al.**, 1985; Yue **et al.**, 1987), since the accumulation of

radiolabelled hydroxyproline in collagen is used to quantify collagen synthetic rates. However, expressing collagen synthesis as the specific activity of hydroxyproline (dpm hypro per umole hypro) in collagen (Table 2) tends to rule out any artifact due to underhydroxylation.

Collagen synthesis as measured in this study is actually "net" collagen synthesis, or total synthesis minus degradation. It is therefore possible that part of the reduction in vascular collagen synthesis caused by diabetes is due to increased collagen catabolism. Experimental diabetes increases collagen degradation (i.e. reduces content) in skin, tendon, and gingival tissue of the rat (Schneir **et al.**, 1982; Schneir **et al.**, 1984a; Leung **et al.**, 1986). Conversely, collagen content is increased, and collagen turnover is reduced in the diabetic rat heart, suggesting that the degradation of myocardial collagen is reduced by diabetes (Reddi, 1988). Schneir **et al.** (1979) found that although STZ-diabetes (20 days) reduced the specific activity of hydroxyproline in aortic collagen of the normotensive rat, diabetes did not affect the degradation of collagen in this tissue. In this same study, diabetes significantly reduced the specific activity of hydroxyproline in collagen (i.e. collagen synthesis) as well as collagen content in rat skin. Therefore, enhanced collagen catabolism is likely to contribute modestly, if at all, to the reduction in net vascular collagen synthesis

observed with diabetes. It is reasonable to conclude, then, that the reductions in aortic PH activity and net collagen synthesis observed in this study represent a true reduction in the rate of collagen biosynthesis in the diabetic rat.

Aortic collagen concentration (nmole hydroxyproline per mg. Lowry protein) was also determined 6 and 12 weeks following surgery (Table 4). Although there were no statistically significant differences among any of the means, certain trends are suggested by the data. Renovascular hypertension (6 and 12 weeks) tended to reduce aortic collagen concentration. This initially may seem paradoxical, but aortic noncollagen protein content was shown to increase at a greater rate than aortic collagen content in short-term (<10 weeks) hypertension (Wolinsky, 1972; Falcy **et al.**, 1985). Under these circumstances, vascular collagen concentration could decrease despite elevations in collagen synthesis and absolute content. Vascular collagen content was not measured in this study, so this relationship could not be examined. Diabetes tended to increase collagen concentration in the RVH aorta without affecting concentration in the WKY vessel. A diabetes-induced increase in collagen concentration may also seem paradoxical, but recent information can explain this effect. The hyperglycemia of diabetes increases non-enzymatic glycosylation of aortic collagen which enhances the formation of glucose-derived crosslinks, or,

ketoamine Amadori products (Brownlee **et al.**, 1986; Andreassen and Oxlund, 1987). These crosslinkages mature over time by the late-stage Maillard browning reaction forming highly stable, enzyme-resistant advanced glycosylation collagen adducts (Monnier **et al.**, 1988). Diabetes reduces the synthesis (Arnquist and Dahlquist, 1979; Pain and Garlick, 1974) and increases the degradation (Williams **et al.**, 1980; Dice **et al.**, 1978) of noncollagen protein, so it is very likely that diabetes disproportionately increases the degradation of noncollagen protein relative to the highly crosslinked collagenous protein, resulting in an increase in aortic collagen concentration. These results merely illustrate that diabetes can affect the composition of the arterial wall by indirectly modifying collagen structure.

The finding in this study that chronic experimental diabetes significantly reduced aortic collagen biosynthesis in both the renovascular hypertensive (RVH) and normotensive WKY rat without lowering systolic arterial pressure in either group (Table 2 and Figure 1) tends to refute the central hypothesis of this study. Earlier evidence from this laboratory had indicated that diabetes reduced arterial pressure of SHR and effected parallel reductions in vascular collagen biosynthesis as well as PH and lysyl oxidase (LO) activities. It was also shown that diabetes had no effect on SAP, collagen synthesis, or collagen biosynthetic enzymes of

normotensive rats (Rodgers **et al.**, 1985; Sessa **et al.**, in preparation). This led to the hypothesis that diabetes reduced these indices of vascular collagen synthesis through a reduction of systolic arterial pressure, and not by a direct mechanism. The results of the present study, however, in which diabetes lowered vascular collagen synthesis without lowering SAP in the RVH or WKY rats, suggest that diabetes inhibits vascular collagen biosynthesis independent of any associated changes in blood pressure.

There is some discrepancy between earlier findings in this laboratory (Sessa **et al.**, in preparation) and the present study regarding the effect of diabetes on aortic PH activity and collagen synthesis in the WKY rat. The 20% reduction in PH activity in the WKY reported by Sessa **et al.** was not statistically significant perhaps because of the relatively small sample size (n=5) combined with a high degree of variability within groups. The differences between these two studies regarding aortic collagen synthesis in the diabetic WKY may be explained in part by the optimization of the incorporation conditions (i.e. Krebs-Henseleit incubation medium). The collagen synthetic rates in the WKY (as well as the RVH) aorta were several-fold higher in our study compared to those reported previously. This may have served to unmask diabetes-induced changes in collagen synthesis that, in the prior study, went undetected because of low basal synthetic rates. Regardless of previous

findings, the results of the present study provide strong evidence that diabetes reduces collagen biosynthesis in the normotensive WKY rat aorta.

Although experimental diabetes has consistently been shown to reduce collagen and noncollagen protein synthesis in VSM, the mechanism for this effect has not been determined. Schneir and coworkers (1979) first reported that STZ diabetes (20 days) reduced aortic collagen synthesis in the normotensive rat **in vivo**. There also appears to be a defect in vascular collagen synthesis in a genetic model of diabetes. Aortic explant-derived SMCs from the spontaneously diabetic BB rat synthesize less collagen and contain lower amounts of extracellular matrix collagen than aortic SMCs from the non-diabetic control strain (Bowersox and Sorgente, 1985). Experimental diabetes has a duration-dependent inhibitory effect on the incorporation of ^{14}C-leucine into noncollagen protein in the rat aorta **in vitro**. Noncollagen protein synthesis was unaffected after one week, moderately reduced after 2 weeks, and severely impaired after 5 weeks of STZ-diabetes (Arnquist and Dahlquist, 1979). It has been observed in this laboratory (unpublished) that diabetes also reduces the incorporation of ^{3}H-leucine into total protein in the rat aorta, but to a lesser degree than the incorporation of ^{14}C-proline into collagen. This suggests that the effect of diabetes on vascular collagen synthesis is more pronounced than its effect on noncollagen protein

synthesis in the vasculature, but the mechanism for this selectivity has not been investigated in this tissue.

The derangements in collagen and noncollagen protein biosynthesis caused by diabetes are not confined to the vasculature, but are observed in non-vascular tissue as well. Williams et al. (1980) found that alloxan-induced diabetes reduced the incorporation of ^{14}C-phenylalanine into total protein in both the isolated working rat heart as well as isolated rat cardiac myocytes. Similarly, Reddi (1988) reported that diabetes (4-6 weeks) reduced **in vivo** myocardial collagen and total protein synthesis in the rat The incorporation of ^{3}H-leucine into protein is also reduced in dorsal root afferent neurons of STZ-diabetic rats (Sidenius and Jakobien, 1982). Collagen synthesis in the skin of streptozotocin diabetic rats is reduced compared to that of non-diabetc rat skin (Schneir **et al.**, 1979). However, diabetes has variable effects on the synthesis of collagen in skin fibroblasts **in vitro**. Collagen synthesis in dermal fibroblasts from human diabetics was found to be either elevated (Rowe **et al.**, 1977; Smith and Silbert, 1981), reduced (Seibold **et al.**, 1981), or unchanged (Kjellstrom and Malmquist, 1984). Both collagen and noncollagen protein synthesis in dermal fibroblasts isolated from the diabetic rat were no different than non-diabetic control fibroblasts (Spanheimer **et al.**, 1987). These variable findings in skin fibroblasts help to explain the

poor wound healing and skin thickening (scleroderma) sometimes observed in diabetic patients, and may merely represent the heterogeneity of the diabetic complications observed in these subjects. More recently, Spanheimer **et al.** (1988a) showed that the effect of diabetes on collagen and noncollagen protein synthesis in rat bone and cartilage depends upon its severity. Moderate diabetes (STZ 35-65 mg/Kg, i.v.) reduced collagen synthesis by approximately 50% in both tissues, whereas severe diabetes (>65 mg/Kg STZ) produced only a 30% reduction in noncollagen protein synthesis in both bone and cartilage. Furthermore, diabetes decreased collagen synthesis to a greater degree than noncollagen protein synthesis regardless of the severity of diabetes. Umpierrez **et al.** (1987; 1988) found that collagen synthesis was decreased in articular cartilage from streptozotocin diabetic rats, and that the magnitude of this effect was dependent on the severity of diabetes. Although noncollagen protein synthesis was reduced in the cartilage from severely diabetic animals, this effect was less marked than the effect on collagen synthesis. Overall, diabetes appears to have a more specific inhibitory effect on collagen versus noncollagen protein synthesis in both vascular and nonvascular tissue, suggesting that the widespread reductions in protein synthesis caused by diabetes are more selective for collagen and therefore may share a common etiology.

There are a number of possible mechanisms for the inhibition of vascular collagen synthesis by diabetes which are independent of hemodynamic changes. The hormonal imbalances and metabolic aberrations of diabetes, especially the lack of insulin, would appear to be the most logical explanation for the changes observed in the diabetic vasculature. The effect of insulin on collagen synthesis in vascular SMCs **in vitro** has remained largely unexplored, while the effect of insulin on noncollagen protein synthesis in these cells has been more thoroughly investigated. Mikkonen **et al.** (1966) reported that insulin markedly increased the incorporation of ^{14}C-proline into collagen in granulation (neovascular) tissue of the rat **in vitro.** However, Jarvelainen **et al.** (1987) showed that physiological concentrations of insulin (0.01 and 0.10 mU/mL) had no effect on collagen synthesis in human aortic smooth muscle cells **in vitro.** Insulin (0.025-25 mU/mL) was shown to enhance the synthesis of proteoglycans (a component of the SMC extracellular matrix) in porcine aortic smooth muscle cells cultured in serum-free media, although the maximum stimulation achieved with insulin was only 60-80% of that obtained with 10% fetal calf serum (Breton **et al.**, 1988). Supraphysiological concentrations of insulin (10-100 mU/mL) **in vitro** increase noncollagen protein synthesis in the bovine mesenteric artery (Arnquist, 1974) but not in the rat aorta (Arnquist and Dahlquist 1979). In general, treatment of diabetic animals with insulin **in vivo** has a much more

dramatic effect on vascular protein synthesis than incubation of vascular tissue with insulin **in vitro**. Two week insulin treatment **in vivo** restored aortic noncollagen protein synthesis to levels observed in the non-diabetic control rat (Arnquist and Dahlquist, 1979). Later, it was shown that once daily insulin treatment for 48 hours was necessary for the restoration of aortic protein synthesis in the diabetic rat, and that treatment for 12 or 24 hours did not increase noncollagen protein synthesis in the aorta (Arnquist and Norby, 1980). In this study insulin did not have a stimulatory effect on protein synthesis **in vitro** unless 5% fetal bovine serum was present in the incubation medium. These results led Arnquist and Norby (1980) to conclude that the increase in aortic protein synthesis observed with insulin treatment in the diabetic rat was the result of the normalization of the diabetic state rather than a direct action of the hormone itself. However, the inability of insulin to stimulate protein synthesis **in vitro** does not rule out a trophic effect of insulin on vascular smooth muscle. Miller **et al.** (1984) have shown that triiodothyronine (T_3) and cortisol are both required for insulin to completely restore the activity and the proper regulation of glycogen synthase in diabetic rat hepatocytes **in vitro**. Thus, the equivocal results obtained with insulin and VSM collagen and noncollagen protein synthesis **in vitro** may reflect the absence of proper cofactors needed for an insulin-induced activation of protein synthesis.

Insulin also has a stimulatory effect on cell growth (quantified by mean cell count and/or ^{3}H-thymidine incorporation into DNA) in vascular smooth muscle cells derived from a variety of species. It is possible that insulin may have a similar effect on VSM collagen synthesis as well. The addition of insulin (0.01-10 mU/mL) to cultured human (Pfeifle **et al.**, 1981), non-human primate (Stout **et al.**, 1975), and bovine (King **et al.**, 1985) aortic SMCs under non-growing conditions was found to dose-dependently stimulate cell proliferation. A dose-dependent increase in DNA synthesis was obtained with insulin (0.01-10 mU/mL) in rat (Pfeifle **et al.**, 1980; Taggart and Stout, 1980) and bovine (King **et al.**, 1983), but not rabbit (Ledet, 1976;1977), aortic SMCs in culture. A common feature of these studies is that insulin was approximately 3-5 times less effective in stimulating SMC growth than a high concentration of serum (5-10%) containing comparitively low levels of insulin (0.003-0.005 mU/mL). However, Stout **et al.** (1975) observed that removal of insulin from the serum prior to addition significantly attenuated the mitogenic effect of the high concentration. These results indicate that while insulin has a proliferative effect on cultured arterial smooth muscle cells, other serum factors are also involved in the overall regulation of SMC replication. Regardless, the stimulatory effect of insulin on SMC proliferation may have an attendant effect on the rate of collagen synthesis in this cell type. However, this is not likely as there

seems to be an inverse relationship between the mitotic activity of the arterial SMC and the synthesis of collagen. The rate of collagen synthesis in bovine aortic SMCs is very low during the time period after initial seeding (when the cells are rapidly dividing), while the rate of collagen synthesis markedly increases as the cells approach and reach confluence (Stepp **et al.**, 1986).

Another approach to examining the effect of diabetes on vascular collagen synthesis is incubating tissue or cultured cells with serum from diabetic animals and assessing the effect on protein synthesis. The effect of diabetic rat serum (DRS) on collagen and noncollagen protein synthesis has been well-characterized in non-vascular tissue such as dermal fibroblasts and costal cartilage of the rat. Spanheimer **et al.** (1988b) demonstrated that diabetic serum dose-dependently reduced collagen synthesis in non-diabetic costal cartilage compared to buffer-incubated control tissue. Diabetic serum also reduced noncollagen protein synthesis in costal cartilage, but the concentration required for maximal inhibition was 2-fold greater than that required for maximal inhibition of collagen synthesis. This indicates that collagen synthesis in cartilage is more sensitive to the inhibitory effect of DRS than noncollagen protein synthesis. Using molecular sieve chromatography, it was determined that the inhibitory activity of DRS was present in the high molecular weight (> 5000 daltons)

fraction. Spanheimer **et al.** (1987) obtained similar results using diabetic rat serum and rat dermal fibroblasts. They found that DRS reduced collagen synthesis to a greater degree than noncollagen protein synthesis at every concentration studied, and that again the high molecular weight fraction (> 5000 daltons) of the serum was responsible for the observed changes in fibroblast collagen and noncollagen protein synthesis. These findings are supported in part by those of Phillips **et. al** (1979) who showed that serum from diabetic rats contained a factor that inhibited both ^{3}H-uridine incorporation into RNA and ^{35}S-sulfate incorporation into mucopolysaccharide (proteoglycan) in rat costal cartilage. Furthermore, the ability of normal rat serum (NRS) to stimulate RNA and mucopolysaccharide synthesis was reduced or abolished by the addition of DRS, indicating that DRS contained an inhibitory factor instead of lacking a stimulatory one. Phillips termed this factor the "somatomedin-inhibitor" because RNA and mucopolysaccharide synthesis in rat costal cartilage is considered to be an index of somatomedin bioactivity. It is possible that the respective collagen and proteoglycan synthesis inhibitors described by Spanheimer **et al.** and Phillips **et al.** are one in the same, as the putative "somatomedin-inhibitor" of Phillips was also present in the high molecular weight fraction of DRS. However, there is no direct evidence either supporting or refuting this possibility. More recently, Spanheimer **et al.** (1988c) have

better-characterized the inhibitory factor(s) present in DRS using rat dermal fibroblasts. They discovered that the inhibitory component of DRS was in the 20-40,000 dalton molecular weight range, and that it could be inactivated by either heat (60^{o}C x 1 hr.) or trypsin treatment, indicating a proteinacious substance. To further study the mechanism by which DRS reduced collagen synthesis, Spanheimer **et al.** (1988c) isolated RNA from fibroblasts incubated for 24 hours in either NRS or DRS and measured its translation in a cell-free reticulocyte lysate system. The translation of procollagen mRNA obtained from fibroblasts incubated in DRS was significantly lower than the translation of procollagen mRNA isolated from cells incubated in NRS. Furthermore, the ratio of procollagen to noncollagen protein mRNA translation was 84% lower in RNA isolated from DRS- versus NRS-incubated fibroblasts, indicating a specific reduction in the translation of procollagen mRNA upon exposure to DRS. This suggests that the inhibitory component of DRS affects collagen synthesis in rat dermal fibroblasts at a pre-translational step, as evidenced by the selective reduction in the translation of procollagen mRNA in a standard RNA translation assay system.

Perhaps similar studies using vascular smooth muscle cells will reveal a mechanistically analagous effect of diabetes or diabetic serum on collagen synthesis in this cell type. However, there is only one report in the

literature, to my knowledge, that has examined the effect of diabetic serum on VSM protein synthesis **in vitro.** Jarvelainen **et al.** (1987) reported that serum from human diabetics in "poor" metabolic control increased collagen synthesis in human aortic smooth muscle cells compared to serum from non-diabetic controls, whereas serum derived from diabetics with "good" metabolic control had no effect on collagen synthesis in these cells. The significance of this finding is unclear at this time, especially in light of the fact that diabetes reduces collagen and noncollagen biosynthesis in virtually every tissue studied thus far. More studies are needed, then, to better-characterize the effect of diabetic serum on VSM collagen biosynthesis and to assess its relevance to the effect of diabetes on collagen synthesis in this tissue **in vivo.**

The abnormal metabolic environment, such as the hyperglycemia and hyperketonemia of diabetes, could contribute to the altered vascular connective tissue synthesis observed in animal models of this disease. There are very few reports in which the direct effect (i.e. without the presence of serum) of ketones or excess glucose on protein synthesis have been studied **in vitro.** Arnquist and Dahlquist (1979) found no effects of glucose (400 mg/dL) on the incorporation of ^{14}C-leucine into noncollagen protein in the rat aorta **in vitro.** Glucose (150 mg/dL) slightly blunted the production of type I collagen in rabbit aortic

medial cell cultures, while it had no effect on type III collagen or fibronectin production in these cells (Ledet and Vuust,1980). Ketones (0.20 mM acetoacetate) had no effect on the synthesis of either collagen type or fibronectin in this study. Glucose (700 mg/dL) attenuates the stimulatory effect of insulin on collagen and noncollagen protein synthesis in human skin fibroblasts **in vitro** (Kjellstrom and Malmquist, 1984). Spanheimer **et al.** (1988b) observed that normal rat serum (NRS) stimulated collagen and noncollagen protein production in rat costal cartilage **in vitro**, and they examined the ability of glucose and ketones to affect this phenomenon. Glucose in a concentration of 300 mg/dL or less did not affect NRS-stimulated collagen or noncollagen protein synthesis in rat cartilage, while concentrations of 600 and 900 mg/dL attenuated the stimulatory effect of NRS on collagen, but not noncollagen, protein synthesis. Conversely, ketones (20 mM beta-hydroxybutyrate) slightly reduced the effect of NRS on noncollagen protein synthesis without affecting NRS-stimulated collagen synthesis. This study did not, however, clearly examine the direct effects of glucose and ketones on collagen and noncollagen protein synthesis, but it did demonstrate that these molecules can affect protein synthesis under certain conditions. Excess glucose and ketones seem to reduce the responsiveness of certain tissues to stimulatory influences (such as normal serum or insulin) **in vitro** without directly affecting tissue function itself. These studies suggest, then, that the

abnormal metabolic environment may contribute to the impaired arterial collagen synthesis characteristic of diabetes, but that it does not appear to play a direct role in this phenomenon.

Hypothyroidism attends both clinical (Pittman **et al.**, 1979) and experimental (Rodgers, 1986; Table 1) diabetes, and may therefore be involved in the reduction of vascular collagen biosynthesis observed in diabetic animals. Unfortunately, there is a paucity of studies examining the direct effect of thyroid hormones (T_3 and T_4) on vascular smooth muscle collagen synthesis **in vitro**. Mikkonen **et al.** (1966) found that T_4 treatment **in vivo** slightly reduced the incorporation of ^{14}C-proline into collagen and noncollagen protein in rat granulation tissue **in vitro**, while T_3 (10^{-4}-10^{-3}M) added to tissue **in vitro** slightly increased both collagen and noncollagen protein synthesis. Brosnan **et al.** (1973) reported that thyroxine (T_4) treatment (3 weeks) reduced aortic collagen content in the hypophysectomized dog (hypophysectomy itself did not affect aortic collagen content). Thyroid hormones have an entirely different effect on protein synthesis in the heart. Thyroxine treatment **in vivo** increases total cardiac protein synthesis measured **in vitro** in the rabbit (Parmacek **et al.**, 1986), dog (Bonnin **et al.**, 1983), and rat (Siehl **et al.**, 1985), while T_3 directly stimulates total protein synthesis in fetal mouse heart cells in culture (Crie **et al.**, 1983). Thyroxine treatment **in**

vivo also causes the development of cardiac hypertrophy in the rat and rabbit (Tanaka **et al.**, 1985; Parmacek **et al.**, 1986). The stimulatory effect of T_3 and T_4 on cardiac protein synthesis, however, may be unique to that tissue, because thyroid hormones were found to reduce proteoglycan, collagen, and noncollagen protein synthesis in skin fibrobalsts (de Rycker **et al.**, 1984; Shibasha **et al.**, 1988) and osteoblasts (Ernst and Froesch, 1987) in culture. The thyroid hormones are well established anabolic regulators of the myocardium, while their role in the regulation of vascular tissue still remains obscure. Recently, it was shown that T_3 replacement therapy reverses the impaired cardiac performance of STZ-diabetic rats nearly as effectively as does insulin replacement (Davidoff and Rodgers, submitted). There is insufficient evidence in the literature, however, to support a causal relationship between the hypothyroidism and the decrease in vascular collagen synthesis of diabetes. The little indirect evidence that does exist, in fact, suggests that T_3 and T_4 may reduce, rather than stimulate, collagen synthesis in the vasculature. The studies of Miller **et al.** (1984; 1986) using diabetic rat hepatocytes have demonstrated that T_3 and T_4 have an obligatory role in modulating tissue response to other circulating hormones such as insulin, and it is possible that this may be true in vascular tissue as well.

Finally, it is possible that the autonomic neuropathy characteristic of diabetes may be involved in the diabetes-induced reduction in vascular collagen synthesis. A possible link between diabetic autonomic neuropathy and vascular collagen synthesis comes from indirect evidence supporting a stimulatory effect of catecholamine and adrenergic innervation on vascular smooth muscle cell protein synthesis. Diabetic autonomic neuropathy is characterized by morphological alterations of both sympathetic and parasympathetic nerve fibers including neuroaxonal dystrophy (accumulation of abnormal subcellular structures), axonal swelling, and general axonopathy (Tomlinson and Mayer, 1984; Yagihashi and Sima, 1986; Schmidt and Plurad, 1986). Diabetic nerves are also more susceptible to ischemic damage and have an impaired ability to regenerate upon injury (Nukada, 1986; Longo **et al.**, 1986). Nerve conduction velocity as well as axonal transport of neurotransmitters and their biosynthetic enzymes are reduced or impaired in diabetes (Troni **et al.**, 1984; Tomlinson and Mayer, 1984). These latter defects have been linked to endoneurial sorbitol accumulation and myoinositol depletion in diabetic nerve (Greene **et al.**, 1988). Tomlinson and Yusof (1983) indirectly demonstrated a reduced norepinephrine release from adrenergic neurons with electrical stimulation in the alloxan-diabetic rat. Some studies provide indirect evidence suggesting that catecholamines have a regulatory influence on VSM collagen

synthesis and vascular geometry. Chronic adrenergic denervation significantly reduced both medial thickness and cross-sectional area in the central ear artery of the rabbit (Bevan and Tsuru, 1981; Bevan, 1984). The sympatholytic antihypertensive agents (e.g. reserpine, clonidine) reduce vascular collagen and noncollagen protein synthesis and content to a greater degree than do non-sympatholytic agents causing equivalent reductions in blood pressure (Ooshima **et al.**, 1974; Spector **et al.**, 1978; Carlier and Rorive, 1985). In addition, non-sympatholyic antihypertensive drugs (hydralazine, captopril) were found to prevent or reverse elevations in systolic arterial pressure in the SHR and Goldblatt RVH rat without affecting the vascular hypertrophy characteristic of clinical and experimental hypertension (Rorive **et al.**, 1980; Smeda **et al.**, 1988). Chichester and Rodgers (1987) showed that chronic low dose doxazosin (an alpha adrenoceptor blocking agent) treatment reduced aortic collagen synthesis without affecting arterial pressure in the SHR. There is circumstantial evidence supporting an involvement of the alpha adrenoceptor in the regulation of collagen synthesis in vascular smooth muscle. Alpha adrenoceptor activation in vascular smooth muscle cells causes the release of 1,2 diacylglycerol (DAG) from membrane phospholipids which then activates protein kinase C (Michell, 1983). Alpha adrenoceptor blocking agents can antagonize this effect (Nishizuka, 1984). Phorbol esters, which are direct activators of protein kinase C, increase

collagen biosynthesis in chondrocytes (Finer **et al.**, 1985) and skin fibroblasts (Marian and Mazzucco, 1985). If, in fact, alpha adrenoceptor activation increases collagen biosynthesis in vascular smooth muscle, then a reduction of catecholamine-mediated alpha adrenoceptor activation caused by sympathetic autonomic neuropathy could be responsible, in part, for the reduction in vascular collagen biosynthesis observed with diabetes. Studies examining the effect of alpha adrenoceptor agonists on VSM cell collagen synthesis **in vitro** would provide useful information regarding the role of catecholamines in the regulation of collagen synthesis in VSM. Consequently, the effect of norepinephrine (NE; 10^{-6}M) on rat aortic collagen synthesis **in vitro** was examined in this laboratory. Briefly, NE actually reduced collagen synthesis in the WKY rat aorta, possibly due to a direct toxic effect on the VSM cell. The results are presented and discussed in Appendix 4.

Summary and Conclusions

It was shown in this study that renovascular hypertension increased aortic collagen synthesis and PH activity in the rat. This is in agreement with the current literature which suggests that the elevations in vascular collagen synthesis in experimental hypertension are primarily the result of the distensional stress imposed on the arterial wall by the rising blood pressure. Chronic STZ-induced diabetes did not reduce SAP in the RVH or WKY rat, suggesting that the pressure-lowering effect of diabetes observed in the SHR is unique to that genetic strain. Finally, diabetes reduced vascular collagen synthesis in both the RVH and normotensive WKY rats, without affecting SAP at any time during the eight-week period of diabetes. This indicates that diabetes has an inhibitory effect on VSM collagen biosynthesis regardless of the model of hypertension or the strain of rat, and that these changes in vascular collagen synthesis are independent of any associated changes in arterial pressure. The mechanism for the inhibition of collagen synthesis by diabetes is still unclear, but the endocrine imbalances of diabetes, such as hypoinsulinemia and hypothyroidism, are likely to be responsible for this effect. However, **in vivo** and **in vitro** insulin and thyroid hormone replacement studies will be needed to clarify the role of these hormones in the reduction of VSM collagen biosynthesis caused by diabetes.

Appendix I: Effect of unlabelled proline on aortic collagen synthesis.

A positive correlation between the extracellular total proline concentration and the uptake of ^{14}C-proline has been demonstrated in mouse Ki-3T3 cells in culture (Peterkofsky **et al.**, 1982). If this is true of vascular smooth muscle, then this has interesting implications for the study of collagen synthesis using the incorporation of ^{14}C-proline into collagen **in vitro.** Consequently, the effect of cold proline addition to the pulsing medium was tested in the present assay system for the determination of collagen synthetic rates in the rat aorta. The experiment was performed using two 14 week-old WKY rats. Each animal was killed by cervical dislocation, and the aorta was excised, cut in half lengthwise, and minced. One half of each aorta was pulsed with ^{14}C-proline in the presence of unlabelled proline, while the remaining half of the aorta served as control and did not receive cold proline addition to the medium. The total concentration of proline in the medium was 0.0375 mM without cold proline addition and 0.2375 mM with cold proline addition, while the amount of radioactivity was held constant at 10 uCi/mL.

The results of the experiment are presented in table 5. Supplelmentation of the Krebs-Henseleit pulsing buffer with cold proline resulted in a 6.1-fold reduction in the

incorporation of radiolabeled proline into aortic collagen. This treatment also caused a 5.9-fold reduction in the incorporation of ^{14}C-proline into total protein, expressed as dpm proline/umole proline (1167 $\pm$ 78 without cold proline addition versus 197 $\pm$ 10 with proline addition, x10^{-3}). Similar changes in the synthetic rates of vascular collagen and total proline-containing protein (a 6.4- and 7.0-fold decrease, respectively) were also obtained when the data was expressed as dpm imino acid per mg. protein (data not shown). There was, however, approximately a 6.3-fold difference in the specific activity of proline in the medium between the two treatments.

The apparent reduction in collagen synthetic activity observed with cold proline treatment thus appears to be the result of the effective dilution of the radiolabel by the unlabeled proline in the medium. Since the acid-soluble radioactivity was not measured, it could not be directly determined whether or not the dilution of the radiolabel in the surrounding medium also resulted in a decrease in the specific activity of the intracellular aortic proline pool. However, it has been shown that changes in the specific activity of proline in the pulsing medium are reflected in the intracellular pool of this imino acid. Experiments using chick embryo skeletal muscle cells (Paterson and Stiohman, 1972) indicate that ^{3}H-leucine in the extracellular medium equilibrates with the intracellular free pool of this amino

Table 5. Effect of unlabelled proline (pro) addition to the pulsing medium on **in vitro** incorporation of 14-C-proline into 14-C-hydroxyproline in the rat aorta. Values are means ± S.D.

Treatment	Proline specific activity in the media (uCi/umole)	**Collagen synthesis ($x10^{-3}$)** DPM hypro/ umole hypro
- cold pro	268.1	510 ± 73
+ cold pro	42.1	84 ± 7[1]

The experiment was performed using 14 week-old WKY rats, n=2 for each treatment. The amount of radioactivity in the pulsing media was identical in both treatments (10uCi/mL) and the concentration of proline was 0.0375mM without unlabelled proline supplementation and 0.2375mM with unlabelled proline supplementation.

[1]Significantly different from the minus cold pro incubation (unpaired Student's t test, $p < 0.0001$).

acid within a relatively short time span (approximately 15 minutes). Additional studies have demonstrated that there is a rapid equilibration between amino acids in the extracellular medium and their intracellular pool (Loftfield **et al.**, 1956; Fern and Garlick, 1974). It is likely that the specific activity of the intracellular proline pool was reduced by the addition of cold proline to the incubation medium in this experiment. Therefore, the similar differences in both the collagen synthetic rate and the extracelleulr proline specific activity between the two treatments (approximately 6-fold) suggests that there was no real decrease in collagen synthesis **per se**. It suggests, rather, that there was merely a decrease in the amount of radioactive proline incorporated into collagen due to a decrease in the specific activity of the intracellular precursor pool.

Peterkofsky **et al.** (1982) showed that the rate of uptake of radiolabelled proline into mouse Ki-3T3 cells was dependent on the total extracellular proline concentration. There was no decrease in the amount of radioactivity taken up into Ki-3T3 cells when the radioactive proline in the medium was diluted up to 200-fold with cold proline, supposedly due to the stimulation of the imino acid uptake system by the increasing extracellular proline levels. Because the K_m for proline transport (6.5 mM; Ornder **et al.**, 1977) is relatively high, Peterkofsky **et al.** (1982)

suggested that collagen synthesis would proceed at a suboptimal rate at the concentration of proline provided by the addition of isotope alone. In the present experiment, however, a 6-fold increase in the concentration of proline in the pulsing medium (with a concomitant 6-fold decrease in its specific activity) resulted in a nearly identical decrease in the amount of radioactive proline incorporated into collagen and other proteins. It is apparent, then, that in the present system no advantage is gained by supplementing the pulsing medium with unlabeled proline regardless of any effects it may have on the transport of the imino acid into the tissue.

Appendix II: Influence of noncollagenous protein on the index of collagen synthesis.

A frequently reported expression of the rate vascular collagen synthesis is DPM hydroxyproline per milligram total protein per incubation period (Ooshima **et al.**, 1977; Iwatsuki **et al.**, 1977a; Foidart **et al.**, 1978). This expression of collagen synthesis has potential limitations, however, because diabetes also reduces the synthesis and content of noncollagen protein in different tissues (Pain and Garlick, 1974; Dice **et al.**, 1978; Arnquist and Dahlquist, 1979). This could lead to an underestimation of the effect of diabetes on aortic collagen synthesis. A reduction in the denominator (total Lowry protein) would artificially inflate the calculated value for collagen synthesis in the diabetic animals, which could partially or entirely mask a true effect of diabetes. Despite this concern, diabetes was found to reduce aortic collagen biosynthesis in both the RVH and WKY rat (Table 6). However, in the RVH group this effect was not as pronounced as when the data was expressed as DPM hypro per umole hypro (Table 2). Overall, expressing collagen synthesis as DPM hypro per mg. protein did not change the qualitative effect of diabetes on this measurement in the hypertensive or normotensive rat aorta.

Using the alternative index of collagen synthesis did, however, mask elevations in aortic collagen biosynthesis

Table 6. Aortic collagen synthesis in renovascular hypertensive (RVH) and Wistar-Kyoto (WKY) rats 6 and 12 weeks following surgery. The 12 week animals were sacrificed 8 weeks after STZ injection. Values are means ± S.D.

			Collagen Synthesis ($x10^{-3}$) (DPM hypro/ mg. protein)		
Group	Treatment	n	6 weeks	n	12 weeks
RVH	control	8	14.9 ± 7.6	7	31.3 ± 14.1
	diabetic	8	———	7	19.3 ± 9.2[2]
WKY	control	7	11.4 ± 9.6	5	33.7 ± 12.7
	diabetic	–	———	7	9.2 ± 2.4[2]

[2]significantly different from control of the same group.

(Table 6) that were observed when synthesis was expressed as the specific activity of hydroxyproline in collagen. This apparent lack of a hypertension-induced enhancement of vascular collagen synthesis may be explained by the findings of Wolinsky (1971; 1972). He showed that there was a disproportionate increase in the accumulation of noncollagenous versus collagenous protein in the RVH rat aorta after 10 weeks of hypertension. However, the accumulation of collagenous protein continued to increase beyond this time point, whereas there was no further accumulation of noncollagenous protein with long-term hypertension (64 weeks). The absence of elevated vascular collagen synthesis in the RVH rats (Table 6) probably reflects concurrent increases in both collagen and noncollagen protein deposition. If the duration of hypertension was extended beyond the 12 week period used in this study, then perhaps an increase in the synthesis of collagen per mg. protein would be observed in the RVH aorta.

Appendix III: Serum glucose, insulin, thyroid hormones and aortic collagen synthesis of WKY and RVH rats 3 weeks after STZ injection.

Collagen synthesis was measured in RVH diabetic, RVH non-diabetic, and WKY non-diabetic rats 3 weeks following STZ injection (Table 7) in order to gain a better understanding of the temporal relationship between the onset of diabetes and the reduction in vascular collagen synthesis. Surprisingly, the severity of diabetes in these animals was in question, and therefore the collagen synthesis values for this study were not presented in the main body of the thesis. However, there is some conflict among the several indexes used to verify the presence of diabetes (Table 8). The two most direct measures of diabetes, serum glucose and serum insulin levels, indicate that the STZ-injected animals were not diabetic. The serum insulin levels of the non-diabetic RVH and WKY rats were well below normal values, rendering the validity of these measurements suspect. It is interesting to note that the diabetic RVH rats lost body weight and were hypothyroid compared to the non-diabetic groups. These indexes were within the expected ranges for diabetic rats, suggesting that at least some degree of diabetes was present. Although the measurements used to verify the presence of diabetes were not in complete agreement, it appears that the RVH rats injected with STZ were at least somewhat diabetic, as there

is no other apparent explanation for the co-occurance of weight loss and hypothyroidism in these animals.

Table 7. The effects of STZ diabetes of 3 weeks duration (6 weeks after renal artery clipping) on aortic collagen concentration and synthesis in the RVH and WKY rat. Values are means ± S.D. and collagen synthesis and concentration are expressed as dpm hypro per umole hypro ($x10^{-3}$) and nmole hypro per mg. protein, respectively.

Group	n	Collagen Concentration	Collagen Synthesis
RVH-C	8	385 ± 68	40.1 ± 23.5
RVH-D	8	431 ± 56	24.6 ± 8.4[2]
WKY-C	7	444 ± 86	24.1 ± 18.0

[2]Significanly different from the control of the same group ($p < 0.05$, unpaired Student's t test)

Table 8. Final body weight and serum values 3 weeks after STZ injection (6 weeks following surgery) in RVH and WKY rats. Value are means ± S.D.

Group*	Glucose (mg/dL)	Insulin (uU/mL)	T_3 (ug/mL)	T_4 (ug/mL)
RVH-control	91 ± 12	12.8 ± 6.0	83.0 ± 5.2	5.21 ± 0.69
RVH-diabetic	137 ± 32	8.8 ± 2.5	55.7[1] ± 17.1	3.70[1] ± 1.47
WKY-control	121 ± 34	24.4 ± 11.6	82.1 ± 4.0	5.03 ± 0.64

*The body weights (BW) for the groups are 322 ± 19, 266 ± 34, and 322 ± 16 for the RVH-control, RVH-diabetic, and WKY-control, respectively. The BW of the RVH diabetic group is significantly different from its control (unpaired Student's t test, $p < 0.05$)

[1]Significantly different from the control of the same group.

The collagen synthetic rates in the RVH and WKY non-diabetic control groups of this study were lower than those measured in the animals sacrificed 12 weeks following surgery. This may not be a valid comparison, however, because aortic collagen synthesis in these two studies was measured in separate assays performed on different days. Therefore the difference in synthetic rates between these two studies may represent inter-assay variability. This is difficult to explain at the present time because the same methodology was used in both of the studies. Aortic collagen synthesis in RVH rats 3 weeks after STZ injection was significantly lower than that in the corresponding vehicle-injected controls. If the assumption that the STZ-injected RVH rats were at least mildly diabetic is valid, then it appears that vascular collagen synthesis is reduced 3 weeks after the onset of diabetes. This is in line with the findings of Arnquist and Dahlquist (1979), who showed that diabetes reduced aortic noncollagen protein synthesis in the rat as early as 2 weeks after the induction of experimental diabetes. There were no significant differences in aortic collagen concentration among any of the groups, and no trends were apparent. Perhaps it was too early in the course of either hypertension or diabetes for any changes in the composition of the arterial wall to be observed. The results of Arnquist and Dahlquist and of this study suggest that the reductions in vascular protein synthesis occur relatively early in the course of diabetes.

Appendix IV: Reduction of aortic collagen synthesis by norepinephrine **in vitro.**

The possible stimulatory influence of alpha adrenoceptor activation on VSM collagen synthesis led us to test the effect of the catecholamine norepinephrine (NE) on aortic collagen biosynthesis in the rat. Fourteen week-old WKY rats were killed by cervical dislocation, and the aortas were excised, cut in half longitudinally, and minced. Collagen synthesis was measured using the protocol outlined in Methods. Norepinephrine significantly reduced the incorporation of ^{14}C-proline into collagen in the WKY rat aorta (Table 9). Roughly equivalent reductions in collagen synthesis were observed when the data was expressed as either DPM hypro per mg. protein or DPM hypro per umole hypro. Although these results do not directly support the above hypothesis, definitive conclusions can not be drawn from these data. The VSM cell is rarely exposed to the concentration of NE used in this study (10^{-6}M), so it is possible that the hormone had a toxic effect on the tissue. A complete dose-effect relationship must be determined before the involvement of catecholamines in the regulation of VSM collagen synthesis can be critically evaluated.

Table 9. The effect of norepinephrine (NE; 10^{-6}M) on aortic collagen synthesis in the WKY rat. Values are means ± S.D.

		Collagen Synthesis (x 10^{-3})[a]	
Treatment	n	dpm/ mg. protein	dpm/ umole hypro
Control	5	422.3 ± 109.0	781.1 ± 108.7
NE	5	116.9 ± 33.8[1]	284.5 ± 71.9[1]

[a]Data is expressed as DPM hydroxyproline per mg. protein and dpm hydroxyproline per micromole hydroxyproline (specific activity) [1]Significantly different from control ($p < .001$)

BIBLIOGRAPHY

Ahnoff, M., Grundevik, I., Arfwidsson, A., Fonselius, J. and Persson, B.A. Derivatization with 4-chloro-7-nitrobenzofurazan for liquid chromatographic determination of hydroxyproline in collagen hydrolysate. Analytical Chem, 53:485, 1981.

Andreassen, T.T. and Oxlund, H. Changes in collagen and elastin of the rat aorta induced by experimental diabetes and food restriction. Acta Endocrinol, 115: 338, 1987.

Arnquist, H.J. Action of insulin on vascular and intestinal smooth muscle: Effects on amino acid transport, protein synthesis and accumulation of glucose carbon. Acta Physiol Scand, 90:132, 1974.

Arnquist, H.J. and Dahlquist, H.H. Effect of experimental diabetes on the incorporation of amino acids into protein in rat aorta. Horm Metab Res, 11: 384, 1979.

Arnquist, H.J. and Norrby, K. Effect of insulin in vivo and in vitro on incorporation of amino acid into protein in normal and diabetic rat aorta. Horm Metab Res, 12:15, 1980.

Berg, R.A. Determination of 3- and 4-hydroxyproline. Meth Enzymol, 82:372, 1982.

Bevan, R.D. Trophic effects of peripheral adrenergic nerves on vascular structure. Hypertension, 6:III-19, 1984.

Bevan, R.D. and Tsuru, H. Functional and structural changes in the rabbit ear artery after sympathetic denervation. Circ Res, 49:478, 1981.

Bonnin, C., Sparrow, M.P. and Taylor, R.T. Increased protein synthesis and degradation in the dog heart during thyroxine administration. J Moll Cell Cardiol, 15:245, 1983.

Bowersox, J.C. and Sorgente, N. Altered growth kinetics of dermal fibroblasts and arterial smooth muscle cells from spontaneously diabetic BB rats. Diabetes, 34:628, 1985.

Breton, M., Berrou, E., Deudon, E. and Brahimi-Horn, M.C. Effect of insulin on sulfated proteoglycan synthesis in cultured smooth muscle cells from pig aorta. Exp Cell Res, 177:212, 1988.

Brosnan, M.E., Sirek, O.V., Sirek, A. and Przybylska, K. Action of growth hormone and thyroxine on aortas of hypophysectomized dogs. Diabetes, 22:243, 1973.

Brownlee, M., Vlassara, H., Kooney, A., Ulrich, P. and Cerami, A. Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. Science, 232: 1629, 1986.

Cardinale, G.J. and Udenfriend, S. Prolyl hydroxylase. Adv Enzymol, 41:245, 1974.

Carlier, P.G. and Rorive, G.L. Pathogenesis and reversibility of the aortic changes in experimental hypertension. J Cardiovasc Pharmacol, 7:S46, 1985.

Cavaliere, T.A., Taylor, D.G., Kerwin, L.J. and Antonaccio, M.J. Cardiovascular effects of alloxan diabetes in normotensive and spontaneously hypertensive rats. Pharmacology, 20:211, 1980.

Chichester, C.O. and Rodgers, R.L. Effects of doxazosin on vascular collagen synthesis, aterial pressure and serum lipids in the spontaneously hypertensive rat. J Cardiovasc Pharmacol, 10:S21, 1987.

Crie, J.S., Wakelend, J.R., Mayhew, B.A. and Wildenthal, K. Direct anabolic effects of thyroid hormone on isolated mouse heart. Am J Physiol, 245:C328, 1983.

deRycker, C., Vandelam, J.L. and Hennen, G. Effects of 3, 5,3'-triiodothyronine on collagen synthesis by cultured human skin fibroblasts. FEBS Lett, 174:34, 1984.

Deyl, Z., Jelinek, J., Macek, K., Chaldakov, G. and Vankov, V.N. Collagen and elastin synthesis in the aorta of spontaneously hypertensive rats. Blood Vessels, 24:313, 1987.

Dice, J.F., Walker, C.D., Byrne, B. and Cardiel, A. General characteristics of protein degradation in diabetes and starvation. Proc Natl Acad Sci, 75:2093, 1978.

Ernst, M. and Froesch, E.R. Triiodothyronine stimulates proliferation of osteoblast-like cells in serum-free culture. FEBS Lett, 220:163, 1987.

Factor, S.M., Bhan, R., Minase, Y. and Sonnenblick, E.H. Hypertensive-diabetic cardiomyopathy in the rat. Am J Pathol, 102:228, 1981.

Falcy, C., Farjanel, J., Rattner, A., Grochulski, A., Gilson, N., Borsos, A.M., Christef, N. and Sternberg, M. Aorta collagen metabolism in spontaneously hypertensive and aorta-constricted rats: Variations in enzyme activities concerned with disaccharide unit synthesis and degradation according to blood pressure and age. Coll Rel Res, 5:519, 1985.

Fein, F.S., Capasso, J.M., Aronson, R.S., Cho, S., Nordin, C., Miller-Green, B., Sonnenblick, E.H. and Factor, S.M. Combined renovascular hypertension and diabetes in rats: A new preparation of congestive cardiomyopathy. Circulation, 70:318, 1984.

Fern, E.B. and Garlick, P.J. The specific radioactivity of the tissue free amino acid pool as a basis for measuring the rate of protein synthesis in the rat in vivo. Biochem J, 142:413, 1974.

Finer, M.H., Gerstenfeld, L.C., Young, D., Doty, P. and Boedtker, H. Collagen expression in embryonic chicken chondrocytes treated with phorbol myristate acetate. Moll Cell Biol, 5:1415, 1985.

Foidart, J.M., Rorive, G.L., Nusgens, B.V. and Lapiere, M. The relationship between blood pressure and aortic collagen metabolism in renal hypertensive rats. Clin Sci Mol Med, 55:27s, 1978.

Franklin, T.J., Morris, W.P. and Loveday, B.E. Effects of inhibitors of collagen maturation on hypertension in rats. Blood Vessels, 19:217, 1982.

Fukumoto, M., Himeno, N., Asai, M. and Midorikawa, O. Effects of prolyl hydroxylase inhibition on arterial collagen synthesis and blood pressure in hypertensive rats. Pharmacology, 29:255, 1984.

Ganguly, P.K., Beamish, R.E., Dhalla, K.S., Innes, I.R. and Dhalla, N.S. Norepinephrine storage, distribution, and release in diabetic cardiomyopathy. Am J Physiol, 252:E734, 1987.

Gilligan, J., Hempstead, J. and Spector, S. Arterial wall protein synthesis in chronically hypertensive rats: The effect of cloniodine treatment. Fed Proc, 41:1092, 1982.

Gilligan, J.P. and Spector, S. Synthesis of collagen in cardiac and vascular walls. Hypertension, 6:III44, 1984.

Goldblatt, H., Lynch, J., Hanzel, R. and Summerville, W. Studies on experimental hypertension. J Exp Med, 59: 347, 1934.

Greene, D.A., Lattimer, S.A. and Sima, A.A. Pathogenesis and prevention of diabetic neuropathy. Diabete Metab Rev, 4:201, 1988.

Hume, W.R. and Bevan, J.A. Amino acid uptake in rabbit blood vessels two weeks after induction of hypertension by coarctation of the abdominal aorta. Cardiovasc Res, 12:106, 1978.

Hutton, J.J., Tapel, A.L. and Udenfriend, S. A rapid assay for collagen proline hydroxylase. Anal Biochem, 16:384, 1966.

Iwatsuki, K., Cardinale, G.J., Spector, S. and Udenfriend, S. Hypertension: Increase of collagen biosynthesis in arteries but not in veins. Science, 198:403, 1977a.

Iwatsuki, K., Cardinale, G.J., Spector, S. and Udenfriend, S. Reduction of blood pressure and vascular collagen in hypertensive rats by bets-aminoproprionitrile. Proc Natl Acad Sci, 74:360, 1977b.

Jarvelainen, H., Ronnema, T. and Lehtonen, A. Effect of sera from male type 1 (insulin-dependent) diabetics on human aortic smooth muscle cells in culture. Acta Endocrinologica, 114:362, 1987.

King, G.L., Buzney, S., Kahn, C.R. and Hetu, N. Differential responsiveness to insulin of endothelial and support cells from micro- and macrovessels. J Clin Invest, 71:974, 1983.

King, G.L., Goodman, A.D., Buzney, S., Moses, A. and Kahn, C.R. Receptors and growth-promoting effects of insulin and insulin-like growth factors on cells from bovine retinal capillaries and aorta. J Clin Invest, 75:1028, 1985.

Kjellstrom, T. and Malmquist, J. Insulin effects on collagen and protein production in cultured human skin fibroblasts from diabetic and non-diabetic subjects. Horm Metab Res, 16:168, 1984.

Ledet, T. Growth hormone stimulating the growth of arterial medial cells in vitro: Absence of effect of insulin. Diabetes, 25:1011, 1976.

Ledet, T. Growth hormone antiserum suppresses the growth effect of diabetic serum: Studies on rabbit aortic medial cell cultures. Diabetes, 26:798, 1977.

Ledet, T. and Vuust, J. Arterial procollagen type 1, type 2, type3, and fibronectin: Effects of diabetic serum, glucose, insulin, ketone, and growth hormone studied on rabbit aortic myomedial cell cultures. Diabetes, 29:964, 1980.

Leung, D.Y.M., Glagov, S. and Mathews, M.B. Cyclic stretching stimulates synthesis of matrix components by arterial smooth muscle cells in vitro. Science, : 475, 1976.

Leung, M.K., Folkes, G.A., Ramamurthy, N.S., Schneir, M. and Golub, L.M. Diabetes stimulates procollagen degradation in rat tendon in vitro. Biochim Biophys Acta, 880:147, 1986.

Loftfield, R.B. and Harris, A. Participation of free amino acids in protein synthesis. J Biol Chem, 219: 151, 1956.

Longo, F.M., Powell, H.C., Lebeau, J., Gerrero, M.R., Heckman, H. and Myers, R.R. Delayed nerve regeneration in streptozotocin diabetic rats. Muscle & Nerve, 9:385, 1986.

Lowry, O.H., Rosenbrough, A.L. and Randall, R.J. Protein measurement with the Folin-phenol reagent. J Biol Chem, 193:265, 1951.

Marian, B. and Mazzuco, K. Dermal collagen metabolism during tumor promotion with 12-O-tetradecanoylphorbol-13-acetate in mouse skin. Carcinogenesis, 6:501, 1985.

McClellan, S., Yue, D.K., Fisher, E., Capogreco, C., Heffernan, S., Ross, G.R. and Turtle, J.R. Deficiency of ascorbic acid in experimental diabetes: Relationship with collagen and polyol pathway abnormalities. Diabetes, 37:359, 1988.

Michell, B. Ca and protein kinase C: Two synergistic cellular signals. TIBS, 8:263, 1983.

Mikkonen, L., Lampiaho, K. and Kulonen, E. Effect of thyroid hormones, somatotropin, insulin and corticosteroids on synthesis of collagen in granulation tissue both in vivo and in vitro. Acta Endocrinologica, 51:23, 1966.

Miller, T.B., Garnache, A. and Cruz, J. Insulin regulation of glycogen synthase phosphatase in primary cultures of hepatocytes. J Biol Chem, 259: 12470, 1984.

Miller, T.B., Garnache, A., Cruz, J., McPherson, K. and Wolleban, C. Regulation of glycogen metabolism in primary cultures of rat hepatocytes: Restoration of acute effects of insulin and glucose in cells from diabetic rats. J Biol Chem, 261:785, 1986.

Monnier, V.M., Sell, D.R., Abdul-Karim, F.W. and Emancipator, S.N. Collagen browning and cross-linking are increased in chronic experimental hyperglycemia: Relevance to diabetes and aging. Diabetes, 37:867, 1988.

Murphy, W.R., Coleman, T.G., Smith, T.L. and Stanek, K.A. Effects of graded renal artery constriction on blood pressure, renal artey pressure, and plasma renin activity in Goldblatt hypertension. Hypertension, 6: 68, 1984.

Nishizuka, Y. Turnover of inosotol phospholipids and signal transduction. Science, 225:1365, 1984.

Nukada, H. Increased susceptibility to ischemic damage in streptozotocin-diabetic nerve. Diabetes, 35:1058, 1986.

Ooshima, A., Fuller, G.C., Cardinale, G.J., Spector, S. and Udenfriend, S. Increased collagen synthesis in blood vessels of hypertensive rats and its reversal by antihypertensive agents. Proc Natl Acad Sci, 74: 777, 1974.

Ooshima, A., Fuller, G.C., Cardinale, G.J., Spector, S. and Udenfriend, S. Reduction of collagen biosynthesis in blood vessels and other tissues by reserpine and hypophysectomy. Proc Natl Acad Sci, 74:777, 1977.

Ornder, D.L., Lee, M., Moore, P.A. and Cecchini, G. Neutral amino acid transport systems of tissue culture cells. J Biol Chem, 252:2675, 1977.

Oshima, T., Matsushita, Y., Miyamoto, M. and Koike, H. Effects of long term blockade of angiotensin converting enzyme with captopril on blood pressure and aortic prolyl hydroxylase activity in spontaneously hypertensive rats. Eur J Pharmacol, 91: 283, 1983.

Pain, V.M. Influence of streptozotocin diabetes on the ability of muscle cell sap tp support protein synthesis by ribosomes in cell-free systems. Biochim Biophys Acta, 308:180, 1973.

Pain, V.M. and Garlick, P.J. Effect of streptozotocin diabetes and insulin treatment on the rate of protein synthesis in tissues of the rat in vivo. J Biol Chem, 249:4510, 1974.

Parmacek, M.S., Magid, N.M., Lesch, M., Decker, R.S. and Samarel, A.M. Cardiac protein synthesis and degradation during thyroxine-induced left ventricular hypertrophy. Am J Physiol, 251:C727, 1986.

Paterson, B. and Stiohman, R.C. Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. Devel Biol, 29:113, 1972.

Peterkofsky, B., Chojkier, M. and Bateman, J. Determination of collagen synthesis in tissue and cell culture systems. In H Furthmayr (ed.) Immunochemistry of the Extracellular Matrix, CRC Press, Inc., Boca Raton, 1982, p. 19.

Pfaffman The effects of streptozotocin-induced diabetes and insulin treatment on the cardiovascular system of the rat. Res Commun Chem Pathol Pharmacol, 28:27,

1980.

Pfeifle, B. and Ditschuneit, H. Effect of insulin on growth of cultured human arterial smooth muscle cells. Diabetologia, 20:155, 1981.

Pfeifle, B., Ditschuneit, H.H. and Ditschuneit, H. Insulin as a cellular growth regulator of rat arterial smooth muscle cells in vitro. Horm Metab Res, 12:381, 1980.

Phillips, L.S., Vassilopoulou-Sellin, R. and Reichard, L. A. Nutrition and somatomedin. VIII.The "somatomedin inhibitor" in diabetic rat serum is a general inhibitor of growing cartilage. Diabetes, 28:919, 1979.

Pittman, C.S., Suda, A.K., Chambers, J.B. and Ray, G.Y. Impaired 3,5,3' triiodothyronine (T3) production in diabetic patients. Metabolism, 28:333, 1979.

Reddi, A.S. Collagen metabolism in the myocardium of normal and diabetic rats. Exp Mol Pathol, 48:236, 1988.

Rodgers, R.L. Depressor effect of diabetes in the spontaneously hypertensive rat: Associated changes in heart performance. Can J Physiol Pharmacol, 64:1177, 1986.

Rodgers, R.L., Breault, P.H., Sessa, W.C. and Chichester, C.O. Depressor effect of diabetes in the spontaneously hypertensive rat: Role of vascular reactivity and prolyl hydroxylase and lysyl oxidase activities. Life Sci, 37:2237, 1985.

Rodrigues, B. and McNeill, J.H. Cardiac function in spontaneously hypertensive diabetic rats. Am J Physiol, 251:H571, 1986.

Rorive, G.L., Carlier, P.J. and Foidart, J.M. Hyperplasia of rat arteries smooth muscle cells associated with development and reversal of renal hypertension. Clin Sci, 59:335s, 1980.

Rowe, D.W., Starman, B.J., Fujimoto, W.Y. and Williams, R.H. Abnormalities in proliferation and protein synthesis in skin fibroblast cultures from patients with diabetes mellitus. Diabetes, 26:284, 1977.

Sasaki, S. and Bunag, R. Hypothalamic responsiveness in DOCA hypertensive rats augmented by streptozotocin-induced diabetes. J Cardiovasc Pharmacol, 4:1042, 1982.

Schmidt, R.E. and Plurad, S.B. Ultrastructural and biochemical characterization of autonomic neuropathy in rats with chronic streptozotocin diabetes. J Neuropathol Exp Neurol, 45:525, 1986.

Schneir, M., Bowersox, J., Ramamurthy, N., Yavelow, J., Murray, J., Edlin-Foliz, E. and Golub, L. Response of rat connective tissues to diabetes: Tissue-specific effects on collagen metabolism. Biochim Biophys Acta, 583:95, 1979.

Schneir, M., Ramamurthy.N. and Golub, L. Extensive degradation of recently synthesized collagen in gingiva of normal and streptozotocin-induced diabetic rats. J Dent Res, 63:23, 1984a.

Schneir, M., Ramamurthy, N. and Golub, L. Enhanced intracellular procollagen degradation in skins of streptozotocin-induced diabetic rats: A causal relationship to underhydroxylation. Prog Clin Biol Res, 180:667, 1985.

Seibold, J.R., Uitto, J., Dorwart, B.B. and Prockop, D.J. Collagen synthesis and collagenase activity in dermal fibroblasts from patients with diabetes and digital sclerosis. J Lab Clin Med, 105:664, 1985.

Sessa, W.C., Rodgers, R.L. and Chichester, C.O. Quantification of collagen synthesis by reverse phase high performance liquid chromatography utilizing 4-chloro-7-nitrobenzofuran derivitives. J Chromatog, 382:258, 1986.

Shibasha, Y., Yanagishita, M. and Hascall, V.C. Effect of thyroid hormone deficiency on proteoglycan synthesis by human skin fibrobalst cultures. Conn Tiss Res, 17:119, 1988.

Sidenius, P. and Jakobien, J. Reversibility and preventability of the decrease in slow axonal transport velocity in experimental diabetes. Diabetes, 31:689, 1982.

Siehl, D., Chua, B.H.L. and Lautensack-Belser, N. Faster protein and ribosome synthesis in thyroxine-induced hypertrophy of rat heart. Am J Physiol, 248:C309, 1985.

Smeda, J.S., Lee, R.M.K.W. and Forrest, J.B. Prenatal and postnatal hydralazine treatment does not prevent renal vessel wall thickening in SHR despite the absence of hypertension. Circ Res, 63:534, 1988.

Smith, B.D. and Silbert, C.K. Fibronectin and collagen of cultured skin fibroblasts in diabetes mellitus. Biochem Biophys Res Commun, 100:275, 1981.

Somani, P., Singh, H.P., Saini, R.K. and Rabinovitch, A. Streptozotocin-induced diabetes in the spontaneously hypertensive rat. Metabolism, 28:1075, 1979.

Spanheimer, R.G. Direct inhibition of collagen production in vitro by diabetic rat serum. Metabolism, 37:479, 1988b.

Spanheimer, R.G. and Stumpf, V. Decreased collagen production induced in vitro by a high molecular weight fraction of diabetic serum. Clin Res, 35:25A, 1987.

Spanheimer, R.G., Umpierrez, G.E. and Stumpf, V. Decresed collagen production in diabetic rats. Diabetes, 37:371, 1988a.

Spanheimer, R.G., Zlatev, T. and Umpierrez, G.E. Pre-translational regulation of collagen synthesis after exposure to diabetic serum. Clin Res, 36:24A, 1988c.

Spector, S., Ooshima, A., Iwatsuki, K., Fuller, G., Cardinale, G. and Udenfriend, S. Increased vascular collagen biosynthesis by hypertension and its reversal by antihypertensive drugs. Blood Vessels, 15:176, 1978.

Stepp, M.A., Kindy, M.S., Franzblau, C. and Sonenshein, G.E. Complex regulation of collagen gene expression in cultured bovine aortic smooth muscle cells. J Biol Chem, 261:6542, 1986.

Stout, R.W., Bierman, E.L. and Ross, R. Effect of insulin on the proliferation of cultured primate arterial smooth muscle cells. Circ Res, 36:319, 1975.

Taggart, H. and Stout, R.W. Control of DNA synthesis in cultured vascular endothelial and smooth muscle cells: Response to serum, platlet-deficient serum, lipid-free serum, insulin, and oestrogens. Atherosclerosis, 37:549, 1980.

Tanaka, T., Morita, H., Koide, H., Kawamura, K. and Takatsu, T. Biochemical and morphological study of cardiac hypertrophy: Effects of thyroxine on enzyme activities in the rat. Basic Res Cardiol, 80:165, 1985.

Tomlinson, D.R. and Mayer, J.H. Defects of axonal transport in diabetes mellitus: A possible contribution to the aetiology of diabetic neuropathy. J Autonom Pharmacol, 4:59, 1984.

Tomlinson, D.R. and Yusof, A.P.M. Autonomic neuropathy in the alloxan-diabetic rat. J Autonom Pharmacol, 3: 257, 1983.

Troni, W., Carta, Q., Cantello, R., Casselle, M.T. and Rainero, I. Peripheral nerve function and metabolic control in diabetes mellitus. Ann Neurol, 16:178, 1984.

Umpierrez, G.E., Goldstein, S., Phillips, L.S. and Spanheimer, R.G. The effects of altered metabolic and nutritional factors on collagen production in diabetes. Clin Res, 36:24A, 1988.

Umpierrez, G.E. and Spanheimer, R.G. The effects of weight loss and insulin therapy on collagen production in streptozotocin-induced diabetes. Clin Res, 35:25A, 1987.

Vater, C.A., Harris, E.D. and Siegal, R.C. Native cross-links in collagen fibrils induce resistance to human synovial collagenase. Biochem J, 181:639, 1979.

Williams, I.H., Chua, B.H.L., Sahms, R.H., Siehl, D. and Morgan, H.E. Effects of diabetes on protein turnover in cardiac muscle. Am J Physiol, 239:E178, 1980.

Wolinsky, H. Response of the rat aortic media to hypertension: Morphological and chemical studies. Circ Res, 26:507, 1970.

Wolinsky, H. Effects of hypertension and its reversal on the thoracic aorta of male and female rats: Morphological and chemical studies. Circ Res, 28:622, 1971.

Wolinsky, H. Long-term effects of hypertension on the rat aortic wall and their relation to concurrent aging changes. Circ Res, 30:301, 1972.

Wool, I.G., Stirewalt, W.S., Kurihara, K., Low, R.B., Bailey, P. and Oyer, D. Mode of action of insulin in the regulation of protein biosynthesis in muscle. Rec Prog Horm Res, 24:139, 1968.

Yagihashi, S. and Sima, A.A. Neuroaxonal and dendritic dystrophy in diabetic autonomic neuropathy: Classification and topographic distribution in the BB rat. J Neuropathol Exp Neurol, 45:545, 1986.

Yamabe, H. and Lovenberg, W. Increased incorporation of 14-C-lysine into vascular proteins of the spontaneously hypertensive rat. Eur J Pharmacol, 29: 109, 1974.

Yamamoto, J. and Masatsugu, N. Effects of moderate diabetes on cardiac performance in spontaneously hypertensive and Wistar-Kyoto rats. Hypertension, 11: 344, 1988.

Yue, D.K., McClellan, S.V., Dunwoodie, S.L. and Turtle, J.R. Prolyl hydroxylase deficiency in diabetic collagen. Diabetes, 36:101A, 1987.