Biochemical Pharmacology, Vol. 32, No. 2, pp. 275–280, 1983.
Printed in Great Britain.

0006-2952/83/020275-06 $03.00/0

MODULATION BY PROPRANOLOL OF THE LYSYL CROSS-LINKS IN AORTIC ELASTIN AND COLLAGEN OF THE ANEURYSM-PRONE TURKEY

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(*Received* 8 *March* 1982; *accepted* 10 *June* 1982)

Abstract—*dl*-Propranolol (propranolol) fed to immature and mature aneurysm-prone turkeys (Broad-Breasted White, BBW) for 6 weeks significantly raised the tensile strength of tissue rings from the abdominal aorta. The drug-mediated increase in tensile strength values was dose-related and independent of its heart rate- and arterial pressure-lowering effects. Propranolol acts, in part, by (a) stimulating lysyl oxidase to produce greater amounts of reactive aldehydes for intermolecular cross-links, (b) enhancing the progression of chemically unstable to stable forms of intermolecular elastin cross-links (lysinonorleucine and the desmosines), and (c) reducing the density of the age-related intermolecular cross-linking of collagen (pyridinoline). These propranolol effects on the lysyl cross-links were demonstrated in both the immature and mature animals and suggest a heretofore unrecognized potential for this widely used cardiovascular drug.

Arteries have unique mechanical versatility due to the complementary anatomic arrangements and functional properties of the constituent smooth muscle bundles, elastic membranes and collagen bundles of these tissues [1]. Functionally, the smooth muscle cells provide for active contraction and relaxation of the vessels while the elastic membranes and collagen bundles are complementary in supporting low and high pulse-pressure loads [2, 3].

The complementarity of the elastic membranes and collagen bundles in arterial tissues is due, in part, to a unique post-translational modification, the enzymatic deamination of the epsilon amino group of specific lysyl and hydroxylysyl residues of the proteins by lysyl oxidase to form the reactive aldehydes, allysine and hydroxyallysine [4, 5]. Following the alignment of the precursor elastin and collagen polypeptides, the reactive aldehydes interact either with adjacent aldehydes in an aldol condensation type of reaction [5] to form intra- and intermolecular cross-links or with the epsilon amino group of adjacent lysyl or hydroxylysyl residues to form Schiff-base intermolecular cross-links of the elastin and collagen polymers [6–10]. In their initial forms, the lysyl and hydroxylysyl cross-links are chemically unstable, but, with time, the unstable compounds are converted to stable compounds [8, 11, 12] or disappear [13, 14]. Then the elastin and collagen polymers acquire their distinctive and, in arterial tissues, their complementary mechanical properties.

Elastin and collagen concentrations may differ along the course of an artery. For example, in many species, the abdominal aorta has a lower elastin to collagen ratio than the thoracic aorta [15, 16] and the coronary arteries have a lower elastin to collagen ratio than the carotid arteries [17]. Furthermore, the arterial elastin and collagen concentrations may not be constant throughout life since they may change with alterations in intramural stresses [18] or with animal age [19, 20]. Whether the polymeric forms of elastin and collagen are regionally unique is uncertain, but limited information suggests different profiles of lysyl cross-links of elastin along the course of the aorta [21].

Reduction of the arterial pressure dynamics was considered to be the basis for the reserpine- and *dl*-propranolol (propranolol)-mediated prevention of beta-aminoproprionitrile (BAPN)-induced lethal aortic aneurysms in the aneursym-prone and hypertensive Broad-Breasted White (BBW) turkey [22, 23]. However, questions were raised as to a direct action for these drugs on the aortic tissue of the BAPN-fed animals when it was learned that an equally effective antihypertensive drug, hydralazine, increased the frequency of lethal aortic aneurysms in the BAPN-fed bird [23]. Additionally, reserpine and propranolol prevented, and hydralazine potentiated, the reduction in aortic tensile strength in the BAPN-fed BBW turkey [22, 23].

The possibility of drug action at the level of the enzymatic deamination of the epsilon amino groups of lysyl and hydroxylysyl residues of aortic elastin and collagen was considered since BAPN is a rather specific inhibitor of aortic lysyl oxidase [4]. To investigate this possibility, we first identified an action of propranolol which, when fed alone, increased the tensile strength of the aorta from the BBW turkey. At these levels of propranolol feeding, tissue allysine levels were increased, and the profiles of intermolecular elastin and collagen lysyl-derived cross-links were altered in a manner which might predictably affect the mechanical properties of aortic tissue. These findings support a drug action on lysyl

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cross-linking reactions which, in a large measure, influence the elastic properties of aortic tissue.

MATERIALS AND METHODS

Groups of immature (4-week-old) and mature (8-month-old) BBW female turkey poults raised by conventional methods and fed a 20% protein diet were given propranolol as dietary supplements (at 0.03, 0.06, 0.09 or 0.12% of the diet weight) for 6 weeks. Arterial pressure, heart rate, maximum rate of arterial pressure rise (dp/dt_{max}) and response to an intravenous injection of *dl*-isoproterenol hydrochloride (isoproterenol) (1.25 μg/kg) were recorded on all animals through a carotid artery cannula 2 days prior to sacrifice. (The artery was cannulated following the injection of the operation site with 2% lidocaine hydrochloride.) Arterial pressure and heart rate were recorded using a linear-core P 1000 transducer, and dp/dt_{max} by a differentiator coupler-7301 (Narco Bio-System) as previously described [21]. After obtaining these hemodynamic measurements, the animals were killed, blood was drawn for plasma propranolol determinations [24], the aorta was removed, and a ring of aortic tissue was taken for tensile strength testing. The remaining tissue was stored at −20° for subsequent biochemical analyses.

Tensile strength testing was carried out on a ring of fresh aorta (standardized length of 2.9 mm) cut from a region immediately cephalad to the sciatic arteries (area of aortic rhexis in BAPN toxicity [21]) using the technique described by Kimball *et al.* [25], in which the tissue stress developed with the increasing tension delivered by a motor-driven device is recorded. The stress just prior to tissue rupture was related to the mean aortic thickness determined by measuring the width of the aortic ring at four 90° intervals around the circumference of the aorta and was expressed as per area (g/mm^2).

For studies of the lysyl-derived and chemically unstable cross-linking compounds, abdominal aortic tissue (40–120 mg) was homogenized and reduced with NaB^3H_4 (330 mCi/mole, 0.068 mg/mg estimated weight of collagen plus elastin) and hydrolyzed in 3 M toluene-*p*-sulfonic acid (105°, 24 hr). The borohydride-reducible allysine, lysinonorleucine, merodesmosine, dihydroxylysinonorleucine and hydroxylysinonorleucine were isolated by sequential cation-exchange chromatography of the tissue hydrolysate, and the radioactivity was quantitated using previously described techniques [26, 27].

To determine the ratio between borohydride-reducible dehydro-form and total lysinonorleucine, the lysinonorleucine-containing fraction isolated by the sequential cation-exchange chromatographic procedure was applied to a third cation-exchange column on the Beckman 120C Automatic Amino Acid Analyzer (AAA) to remove hydroxylysine and other contaminants. The lysinonorleucine was isolated using two citrate buffers [0.02 N, pH 4.25, 45 min; and 0.34 N, pH 5.25, plus methanol (100 ml 0.35 N citrate, 5 ml methanol), 100 min], and the counts per minute (cpm) and the leucine equivalents (÷1.8) in the effluent fraction were determined. These extensive isolation steps were required to assure the purity of the compound so that the ratio between the dehydro and the total forms of lysinonorleucine might be used as an index of the maturation of the lysyl cross-links.

Radioactivity in the cross-link-containing fractions was determined in the Packard 3003 Tri-carb liquid scintillation spectrometer and reported as cpm/μg of hydroxyproline (Hyp) or as cpm/nmole of lysinonorleucine (leucine equivalents).

Elastin was isolated from the aortic tissue by a modification of the procedure of Lansing *et al.* [28]. The tissue was finely diced, autoclaved twice in water (3 hr, 20 lb pressure) and centrifuged, and the supernatant fraction was conserved for pyridinoline and Hyp [29] determinations. The residue was washed with water, extracted with 0.1 N NaOH at 98° for 25 min, washed with water until the wash was pH 7, and then lyophilized and weighed. A sample of the lyophilized elastin was hydrolyzed (6 N HCl, 105°, 58 hr), the acid was removed by flash evaporation, and the residue was dissolved in water and filtered (Millipore, 0.45) to remove inorganic crystalline material or other insoluble contaminants. The weight of the Millipore retentate was subtracted from the initial elastin weight to obtain the final elastin weight.

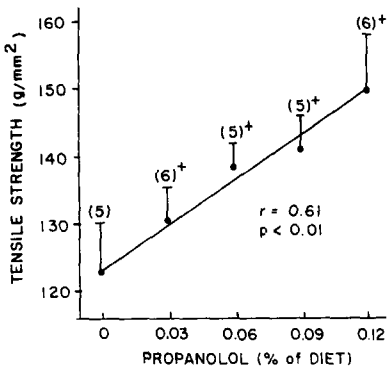
The amino acids and the desmosines (isodesmosine and desmosine) in the elastin hydrolysate were separated by cation-exchange chromatography with a 6-buffer system on the AAA as previously described [26]. Ratios among the principal non-polar amino acids were calculated as a measure of elastin purity, and the ninhydrin positive values for the effluent fractions containing the desmosines were converted from leucine equivalents (÷3.6) to nmoles of desmosines and related to the corrected elastin weight (mg).

The pyridinoline cross-linking compound was isolated from the supernatant fraction according to the method of Fujimoto and Moriguchi [30]. The supernatant fraction was lyophilized and hydrolyzed (6 N HCl, 110°, 24 hr), the HCl was removed, and the residue was dissolved in water, applied to a phosphocellulose column (H^+ form), separated on a cation-exchange column of the AAA, and expressed as nmoles leucine equivalents per mole of collagen.

Elastin concentration was derived from the gravimetric procedure, and collagen concentration was calculated from the Hyp content using the standard conversion factor (×7.46) [31]. Elastin and collagen values were expressed as μg/mg wet weight.

RESULTS

Propranolol significantly lowered the heart rate and the rate of arterial pressure rise (dp/dt_{max}) and raised the tensile strength index at plasma levels of 10 ng/ml (0.03% propranolol in the diet), as seen by a comparison of the tissues from the treated and untreated immature BBW turkeys (Fig. 1). Increasing the plasma propranolol levels produced no additional hemodynamic changes but caused a progressive and a significant increase in the tensile strength of the tissues. At both the low and high doses of propranolol, the beta-adrenergic receptor response to intravenous isoproterenol was blocked,



Propanolol (% of diet)	0%	0.03%	0.06%	0.09%	0.12%
Heart rate (beats/min) ± S.E.	285 ±3	242++ ±7	240++ ±7	235++ ±7	232++ +5
Arterial Pressure (dp/dt max. ± S.E.)	1777 ±69	1362++ ±83	1159++ ±43	1301++ ±140	1108++ ±99

Fig. 1. Propranolol and the tensile strength of aortic tissue from the BBW turkey (immature, female). Values are presented as means ± S.E. Tensile strength versus drug doses represented by the best-fit line determined by the method of least squares; r = slope and P = significance. Key: (+) Control vs propranolol-treated was tested by the Wilcoxon matched-pair sign test or (++) by the Student's t-test [32] using $P < 0.05$ as the level of confidence for significant differences. Refractoriness of heart rate and dp/dt_{max} changes were measured following isoproterenol challenges (five out of six animals at 0.03% and all animals at higher propranolol doses). Plasma propranolol, representing the mean value of three determinations, was 10 ng/ml at 0.03% and 100 ng/ml at 0.12% of the diet.

indicating adequate levels of propranolol to antagonize the beta-adrenergic receptor.

The idea that propranolol acts on the aortic lysyl oxidase reaction was supported by the higher tissue levels of hydroxynorleucine (allysines) in aorta of treated as compared to untreated animals (Table 1). The propranolol-mediated increase in the tissue allysine levels was accompanied by a parallel rise in tissue levels of dehydromerodesmosine, suggesting a drug-mediated increase in the condensation reaction of two allysine residues and one lysyl residue [7] to form this intermolecular elastin cross-link.

Propranolol treatment affected the aortic tissue lysyl cross-linking reactions at steps beyond the formation of allysines and dehydromerodesmosine. Propranolol treatment was accompanied by a significant lowering of the dehydrolysinonorleucine to the lysinonorleucine ratio (the reduced ratio resulting from the presence of larger amounts of lysinonorleucine, since levels of dehydrolysinonorleucine were similar in tissues from treated and untreated animals) and by significant increases in the density of the desmosines in the elastin isolated from the aortic tissue (Table 1). These propranolol-mediated effects on the densities of lysinonorleucine and the desmosines occurred without altering the elastin concentration or the relative proportions among the non-polar amino acids of elastin and suggested a qualitative modification of the elastin polymer by the drug.

Table 1. Propranolol and lysyl cross-linking compounds of elastin in the abdominal aorta of the immature, female BBW turkey*

	Elastin (μg/mg wet wt)	Hydroxynorleucine (allysine) (cpm/μg Hyp)	Merodesmosine (cpm/μg Hyp)	Lysinonorleucine (cpm/μg Hyp)	(cpm/nmole)	Desmosines (nmoles/mg elastin)
Control	13 ± 2	440 ± 32	122 ± 7	86 ± 15	6624 ± 749	21 ± 2
N	6	6	6	12	12	6
Propranolol (0.12%)	11 ± 3	701 ± 112	178 ± 17	88 ± 7	4742 ± 309	32 ± 7
N	6	8	9	12	12	6
P	NS†	<0.05‡	<0.02§	NS	<0.05§	<0.02§

* Values are means ± S.E.; N = number of animals. The relative proportions of principal amino acids in the isolated elastins (valine:alanine:glycine:proline) were 1:0.9:0.5:1.1 for control and 1:0.9:0.5:1.0 for propranolol-treated animals. Hyp = hydroxyproline.

† No significant difference.

‡ Statistical probability as tested by the Wilcoxon two-sample rank test [32].

§ Statistical probability as tested by Student's t-test [32].

Table 2. Propranolol and hydroxylysyl cross-linking compounds in abdominal aortic collagen of the immature, female BBW turkey*

	Collagen (μg/mg wet wt)	Borohydride-reducible Hydroxylysinonorleucine (cpm/μg collagen)	Borohydride-reducible Dihydroxylysinonorleucine (cpm/μg collagen)	Pyridinoline (nmoles Leu equiv./mole collagen)
Control	9 ± 2	25.7 ± 3.2	44.9 ± 2.7	0.71 ± 0.03
Propranolol (0.12%)	8 ± 2	20.7 ± 1.4	44.7 ± 3.1	0.41 ± 0.03
P	NS†	NS	NS	0.02

* Values are means ± S.E. for at least five tissues in each group; differences between the two groups were tested by Student's *t*-test [32].

† No significant difference.

Propranolol treatment modified the intermolecular cross-links of aortic collagen (Table 2), but the effect was more circumscribed than the crosslinking changes observed in elastin. Without affecting the collagen concentration or the tissue levels of the borohydride-reducible intermolecular cross-links, hydroxylysinonorleucine or dihydroxylysinonorleucine, propranolol mediated a significant lowering of the pyridinoline density from 0.71 to 0.41 nmoles/collagen molecule.

The question of a propranolol effect on the lysyl-derived cross-links in aortic elastin and collagen of the fully developed mature animals was then explored. As shown by the data in Table 3, amounts of propranolol (0.03% of diet), which blocked the beta-adrenergic receptor response to isoproterenol in adult animals and significantly lowered heart rate, systolic and diastolic arterial pressures and the dp/dt_{max} of the arterial pressure, increased the density of the desmosines in the isolated elastin. The pyridinoline density in the collagen appeared to be lower in the propranolol-treated animals. And as in the immature animals, propranolol treatment did not modify the principal amino acid composition of the elastin isolated from the aortic tissue of the adult animal.

DISCUSSION

Propranolol, at plasma levels which block the beta-adrenergic receptors to reduce heart rate and arterial pressure, increased the tensile strength of aortic tissue from the aneurysm-prone BBW turkey (Fig. 1). The drug effect on the aortic tensile strength measurement appears to have been dose-related and, in part, independent of the hemodynamic effects since, without additional lowering of heart rate or arterial pressure, increasing amounts of propranolol raised the tensile strength of the aortic tissue in a linear fashion. A dose-related mediation of increased tensile strength suggests a drug action on processes in the aortic tissues which influence the static mechanical properties of the tissue.

A key metabolic process in the development of the static mechanical properties of the aorta is the lysyl oxidase-catalyzed reactions leading to the formation of covalent intermolecular cross-links in the two structural proteins, elastin and collagen. The idea of a propranolol action at the level of the aortic lysyl oxidase reactions is supported by the relatively high levels of allysine, the immediate product of the enzyme, in the drug-treated animals (Table 1). The mechanism whereby propranolol enhanced aortic lysyl oxidase reactions may be biochemical since non-peptidyl amines are reported by Trackman and Kagan [33] to interact with this enzyme system. Or propranolol may indirectly stimulate the aortic lysyl oxidase reaction by reducing the heart rate, the systolic and diastolic pressures, and the dp/dt_{max} of the arterial pressure. The latter possibility appears unlikely since parallel rather than inverse relationships between arterial pressure and the levels of elastin and collagen metabolism, including lysyl oxidase activity, in aortic tissues are well-documented [34–36].

Propranolol treatment enhanced the formation and maturation of the lysyl cross-links in aortic elastin. Evidence supporting this conclusion includes: (1) the significantly elevated level of dehydromerodesmosine, a chemically unstable cross-link which may form during the dimerization of monomeric elastin [37]; this drug-mediated enhancement of dehydromerodesmosine formation may have been secondary to an increase in available allysines (Table 1); (2) the significant lowering of the dehydrolysinonorleucine:total lysinonorleucine ratio (Table 1) since dehydrolysinonorleucine is probably the precursor of lysinonorleucine [8] and the desmosines [38]; and (3) the increased density of desmosines in the isolated elastin (Tables 1 and 3).

The mechanisms underlying the enhanced maturation of aortic elastin cross-links may be a direct action of propranolol as a secondary amine on the stabilization of the Schiff-base cross-link or an indirect action of propranolol of reducing the rate of elastin turnover, thus favoring the time-dependent [9, 11, 12] formation of chemically stable elastin cross-links. Additional studies are required to distinguish between these, or other, explanations.

Comparing the densities of the desmosines in the elastin of abdominal aortic tissue from immature and mature animals (Tables 1 and 3) indicates that in the BBW turkey, as in aortic tissue from other species including man [19, 39], the concentration of desmosines increases to adult levels during the early developmental period. An incomplete maturation of the lysyl cross-links in some fraction of aortic elastin in both age periods in the BBW turkey is

Table 3. Propranolol and the densities of desmosines and pyridinoline in the abdominal aorta of the mature, female BBW turkey*

	Wt (lb)	Heart rate (beats/min)	Arterial pressure			Desmosines (nmoles/mg elastin)	Pyridinoline (nmoles Leu equiv./mole collagen)
			Systolic (mm Hg)	Diastolic (mm Hg)	dp/dt_{max}		
Control	20.4 ± 1.1	274 ± 6	217 ± 8	169 ± 4	1404 ± 124	22.8 ± 0.9	0.94 ± 0.10
N	5	5	5	5	5	4	4
Propranolol (0.03%)	21.1 ± 1.1	192 ± 7	172 ± 15	129 ± 16	1040 ± 137	37.1 39.1 32.3	0.61 0.68 0.76
N	5	5	5	5	5	3	3
P	NS†	<0.001‡	<0.05§	<0.05§	<0.05§	<0.02‡	<0.02‡

* Values are means ± S.E.M.; N = number of animals or tissues. All propranolol-treated animals were refractory to isoproterenol.

† NS, no significant difference.

‡ Statistical probability for significant difference between mean values of the control and propranolol-treated groups was determined using Student's *t*-test [32].

§ Statistical probability for significant difference between mean values of the control and propranolol-treated groups was determined by the Wilcoxon two-sample rank test [32].

suggested by the propranolol-mediated enhancement in the density of the desmosines in both immature and mature animals (Tables 1 and 3). Perhaps the BBW turkey aortic elastin is unique in this respect, but the drug-mediated increase in the density of the desmosines in the isolated elastin without a parallel change in the concentration of elastin (Table 1) points up the potential for error in converting tissue desmosine values into elastin equivalents [40].

In addition to modifying the elastin lysyl cross-link profile, propranolol treatment reduced the aortic tissue levels of the numerically dominant hydroxylysyl-derived cross-link, pyridinoline [41], in the BBW turkey (Table 2). Without changing the collagen concentration or the levels of the borohydride-reducible hydroxylysyl-derived intermolecular cross-links, dihydroxylysinonorleucine and dehydrodihydroxylysinonorleucine, propranolol significantly lowered the density of pyridinoline in the collagen from the aorta of the immature and probably the mature animals (Tables 2 and 3). Pyridinoline is a chemically stable, polyfunctional cross-link found in collagens that are required to support large mechanical loads, e.g. cartilage, bone, Achilles tendon and aorta [30, 41–43] and is the only intermolecular collagen cross-link known to increase in density with animal age [44]. Age increased the density of pyridinoline in the BBW turkey aorta (Tables 2 and 3), and, most interestingly, propranolol treatment appears to have interfered with these increases.

The mechanism of the propranolol-mediated lowering of the pyridinoline may be biochemical through an inhibition of the post-translation enzymatic hydroxylation reaction leading to the formation of hydroxylysine. Propranolol reportedly acts as a competitive inhibitor in another hydroxylase reaction, the prolyl hydroxylase-catalyzed conversion of specific prolyl to hydroxyprolyl residues [45]. It is not clear whether propranolol reduces the density of hydroxyproline in the collagen molecule of aortic tissue [investigations are underway in our laboratories to answer this uncertainty since a propranolol-mediated reduction in hydroxyproline would increase the levels of borohydride-reducible cross-links and lower the density expression of pyridinoline (Table 2)]. A propranolol inhibition of lysyl hydroxylase may be more significant, however, since the number of hydroxylysine residues in collagen is considerably lower than the number of hydroxyproline residues, and hydroxylysine residues are requisite precursors of essentially all of the intermolecular cross-links including pyridinoline. Alternatively, propranolol by lowering arterial pressure (and aortic wall stress) may limit the age-related changes in the cross-linking of collagen and, thereby, indirectly reduce the density of pyridinoline.

Whatever the ultimate explanation(s) for the propranolol effects on the lysyl cross-links in elastin and collagen in the aneurysm-prone BBW turkey, the findings of this study suggest unique pharmacologic properties for propranolol in modifying the potential for elasticity in the biopolymers of elastin and collagen in aortic tissues. Should future studies show a propranolol-mediated effect on the elastic proper-

ties of aortic tissues of the BBW turkey and that this propranolol effect is not species related, then a heretofore unrecognized potential will have been identified for propranolol as a therapeutic choice for a variety of clinical situations associated with reduced tensile strength of elastin- and collagen-rich cardiovascular tissues.

Acknowledgements—The authors gratefully acknowledge the assistance of Mariko Ono, Nino Ravdin and Carla Thomasino in preparing the manuscript. The manuscript was supported in part by U.S. Public Health Service Research Grant, HL-17909, and the Sinclair Fund for Geriatric Research at the University of Miami. We acknowledge the generous supply of propranolol by the Ayerst Co.

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