Effect of Chronic Digoxin on β -Adrenergic Receptors in Rabbits with Heart Failure

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SUMMARY

This study investigated the effect of chronic digitalis glycoside use on β -adrenergic sympathetic activities in heart failure. Twenty-two Japanese white rabbits were anesthetized by intravenous injection of chloral hydrate. Aortic regurgitation (AR) was produced by perforating aortic valves in 14 rabbits. Digoxin was given for 1 week to 7 rabbits with AR (AR + Dig) and saline to 7 rabbits with AR (AR + C). Sham operation was performed in the remaining 8 rabbits (S). The left ventricular end-diastolic pressure was higher in AR+C than S ($p < 0.05$). It was lower in AR + Dig than AR + C ($p < 0.05$). Cardiac output was lower in AR + C than S ($p < 0.05$). There was no difference between AR + Dig and S. Both the left ventricular end-diastolic and end-systolic diameters were larger in AR + C ($p < 0.05$) than S, but they were similar between AR + Dig and S. Plasma norepinephrine level was lower in AR + Dig than AR + C. Myocardial β -adrenergic receptors number determined by radioligand binding assay using 30–800 pM 125 I-iodocyanopindolol was lower in AR + C than S (28.8 ± 7.9 vs. 69.9 ± 12.3 fmol/mg protein, $p < 0.05$). It was higher in AR + Dig (39.9 ± 9.8) than AR + C ($p < 0.05$). Myocardial norepinephrine content was lower in both AR + C ($p < 0.05$) and AR + Dig than S ($p < 0.05$). Thus, digitalis glycosides exert favorable effects on β -adrenergic sympathetic activities in addition to the effects on hemodynamic variables in this animal model of heart failure. (Jpn Heart J 1997; 38: 263–272)

Key words: Digitalis glycosides, Heart failure, β -adrenergic receptor, Aortic regurgitation, Rabbits

THERE is still controversy regarding the optimal therapeutic strategy for long-term management of patients with congestive heart failure. Catecholamines or phosphodiesterase inhibitors possess a potent inotropic efficacy, but long-term use results in an attenuation of its efficacy and may even adversely

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affect survival in patients with heart failure.¹⁻³⁾ Digitalis glycosides have been used as a baseline drug for heart failure for more than 200 years. The inotropic response to digoxin is maintained in papillary muscle preparations derived from patients with endstage heart failure who underwent cardiac transplantation⁴⁾ which is quite different to that of catecholamines and phosphodiesterase inhibitors. Recent reports have shown that long-term use of the drug had some favorable effects despite the modest inotropic potency,^{5,6)} although the effect on overall survival was found to be neutral.

Myocardial β -adrenergic receptors are down-regulated in ventricular myocardium from patients with severe congestive heart failure.⁷⁾ Clinical observations show that digitalis glycosides attenuate excessive activation of the sympathetic nervous system⁸⁾ through sensitization of the carotid sinus baroreflex of parasympathetic activity which is profoundly disturbed in congestive heart failure.⁹⁾ A recent report has shown that ouabain binding sites are reduced in failing myocardium, and this change was prevented by β -blocker.¹⁰⁾ In addition, a reduction in ouabain binding sites is produced by long-term use of norepinephrine.¹¹⁾ It is hypothesized that ouabain-binding sites have some interactions with sympathetic nerve traffic. The present study was designed to examine the effects of chronic digitalis glycoside use on cardiac performance and β -adrenergic receptors in heart failure produced by aortic regurgitation in rabbits.

METHODS AND MATERIALS

All animal experiments were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" published by the US National Institutes of Health, and were approved by the Institutional Committee on Animal Experiment Ethics of Keio University School of Medicine.

Production of aortic regurgitation: Twenty-two Japanese white rabbits were anesthetized by intravenous administration of chloral hydrate (150 mg/kg). Aortic regurgitation was produced in 14 rabbits as described previously.^{12,13)} The right carotid artery was isolated from surrounding tissue. Aortic pressure was measured with a 5F micromanometer-tipped catheter. A 5F metal catheter was introduced from the right carotid artery and advanced toward the aortic root. The catheter was pushed toward the left ventricle where aortic valve motion was optimally detectable. Diastolic murmur was audible after the successful creation of aortic regurgitation which was confirmed by measuring the reduction in aortic diastolic pressure that was found to predict subsequent left ventricular overloading in our previous studies.^{12,13)} Sham operation was performed in another 8 rabbits in which aortic pressure was measured but aortic regurgitation not produced. After the procedure, the carotid artery was ligated, the wound sutured

and antibiotics injected.

Administration of digitalis glycosides: 0.25 mg/kg of digoxin was injected intravenously in 8 rabbits with aortic regurgitation (AR + Dig). Thereafter, digoxin was injected continuously at the rate of 2.0 $\mu\text{g/kg/hr}$ using an osmotic minipump (Alzet 2ML1, Alza Corp., Palo Alto, CA, USA) implanted subcutaneously for one week. Physiological saline was injected as a control in the same manner in the other 7 rabbits with aortic regurgitation (AR + C).

Measurement of hemodynamic data: Hemodynamic data were collected under an open-chest anesthetized condition one week after the procedure. Ventilation was controlled via the intubation tube (4.0 or 4.5 mm in internal diameter), introduced from the tracheal incision. Tidal volume was adjusted to 50 ml and frequency was 20/min. Aortic root pressure was measured by a 5F micromanometer-tipped catheter (PC-350, Millar Instruments, Houston, TX, USA), introduced from the left carotid artery. The left ventricular pressure was measured by a 3F micromanometer catheter (PC-330, Millar Instruments), introduced from an apical incision. The left ventricular diameter was measured by a pair of piezoelectric crystals attached to the anterior and posterior epicardial surface. Aortic flow was measured by an electromagnetic flow probe (MFV120, Nihon-Kohden, Tokyo) placed around the aortic root. After the collection of hemodynamic data, heart beat was stopped by rapid injection of potassium chloride, and coronary arteries were flushed with ice-cold physiological saline to completely eliminate residual blood. The left ventricular free wall was isolated, weighed and its thickness determined.

Membrane preparation: Approximately 0.5 g of the left ventricular myocardium was rapidly frozen by liquid nitrogen for determination of catecholamine content.¹⁴⁾ The remaining myocardium was processed for membrane preparation for β -adrenergic receptor assay as described previously.^{15,16)} The left ventricular myocardium was soaked in ice-cold isotonic sucrose buffer (250 mM sucrose, 1 mM KHCO_3 , 1 mM MgCl_2). Connective tissues, pericardium and endocardium were removed as much as possible and minced with scissors. They were homogenized with a tissue disrupter (Physoctron, Niti-on, Chiba, Japan) at maximal speed for 5 seconds and filtered through two layers of Japanese silk screen. The homogenates were centrifuged at 700 g for 10 minutes to remove nuclei and remaining connective tissues. The supernatant was further centrifuged at 17,000 g for 15 minutes. Pellets were resuspended in Tris buffer (100 mM Tris, 1 mM MgCl_2 , 5 mM EGTA, pH 7.4). Membrane samples were frozen by liquid nitrogen and kept under -70°C until assay. Membrane protein was determined by a modified Lowry method¹⁷⁾ and adjusted to 0.3 mg/ml when β -adrenergic receptor was assayed.

β -Adrenergic receptor assay: Density of myocardial β -adrenergic receptors

was determined by saturation isotherm in triplicate as described previously.^{15,16)} Briefly, 30–800 pM of ¹²⁵I-iodocyanopindolol (Amersham Japan, Tokyo) with 1.7×10^{-6} M propranolol or Tris buffer was added to 100 μ l of membrane sample and the mixture incubated at 37°C for 60 minutes. Reaction was terminated by adding 750 μ l of ice-cold Tris buffer. The preparations were filtrated through Whatman GF/C filters using a filtration apparatus (Millipore Corp., Bedford, MA, USA) and washed twice with ice-cold Tris buffer. Radioactivity was counted using an ARC-600 gamma counter (Aloka Corp., Tokyo) with a 76.5% counting efficiency. Specific activity was determined by subtracting nonspecific binding in the presence of propranolol from the total binding. Maximal binding sites and dissociation constant were determined using Scatchard analysis.¹⁸⁾

Blood sampling for plasma norepinephrine: A polyethylene catheter was placed in the right jugular vein before production of aortic regurgitation in 5 rabbits each with AR + C and AR + Dig for determination of plasma norepinephrine concentration. Five ml of blood were drawn in unanesthetized condition on the following day and 1 week after production of aortic regurgitation.

Data analysis: Hemodynamic data were recorded using a thermal array recorder (Nihon-Kohden, Tokyo) at 200 mm paper speed. Total forward stroke volume (TSV) and regurgitant volume (RV) were determined by digitization of aortic flow using MYPAD-A3 Logitec DIGITIZER (MODEL K-510, Tokyo). Stroke volume was defined by subtracting the regurgitant volume from the total forward stroke volume, and regurgitant fraction by dividing regurgitant volume by total forward stroke volume. Least squares method was used for Scatchard analysis. Data are expressed as a mean $\pm$ SD. Differences between the three groups were determined by analysis of variance followed by Student t-test. Changes in each group were assessed by paired t-test. Statistical significance was considered to be $p < 0.05$.

RESULTS

No deaths were observed during the observation period. Plasma digoxin concentration was 1.6 ± 1.1 ng/ml in AR + Dig. The Table shows the hemodynamic variables for the three groups. The decrease in aortic diastolic pressure immediately after production of aortic regurgitation was similar between AR + C and AR + Dig. Aortic diastolic pressure and mean pressure were lower in both AR + C and AR + Dig than sham-operated rabbits, although there was no difference between the two groups for aortic regurgitation. Regurgitant fraction was also similar between the two groups. Left ventricular end-diastolic pressure was higher in AR + C than sham-operated rabbits, although it was lower in AR + Dig than AR + C. Cardiac output was lower in AR + C than sham-operated rabbits.

Table. Hemodynamic Variables

	Sham	AR + C	AR + Dig
dAODP (mmHg)	/	27 ± 11	23 ± 10
Heart rate (bpm)	205 ± 18	224 ± 21*	217 ± 24
LV end-diastolic pressure (mmHg)	5.7 ± 2.0	18.0 ± 6.5*	11.9 ± 4.6*+
AoP (mmHg)			
systolic	101 ± 22	89 ± 10	84 ± 8*
diastolic	76 ± 21	52 ± 13*	59 ± 4*
mean	84 ± 21	67 ± 13*	67 ± 5*
Cardiac output (ml/min/kg)	164 ± 29	128 ± 33*	145 ± 30
Regurgitant fraction (%)	/	48 ± 10	48 ± 16
LV diameter (mm)			
end-diastole	22.4 ± 1.3	24.5 ± 2.6*	22.7 ± 2.2
end-systole	20.7 ± 1.0	22.7 ± 2.3*	20.9 ± 1.9

dAODP = decrease in aortic diastolic pressure immediately after production of aortic regurgitation (AR); LV = left ventricle; AoP = aortic pressure; AR + C = rabbits with AR given saline; AR + Dig = rabbits with AR given digoxin. * $p < 0.05$ compared with sham; + $p < 0.05$ compared with AR + C.

Figure 1. Myocardial β -adrenergic receptors.

Bmax = maximal binding sites; Kd = dissociation constant; AR + C = rabbits with aortic regurgitation (AR) given saline; AR + Dig = rabbits with AR given digoxin.

* $p < 0.05$ compared with sham; + $p < 0.05$ compared with AR + C.

Both end-diastolic and end-systolic dimensions were larger in AR + C than sham-operated rabbits. There was no difference in cardiac output or the dimensions between AR + Dig and sham-operated rabbits.

The left ventricular free wall weight was higher in AR + C (0.95 ± 0.11 g/kg) than sham-operated rabbits (0.83 ± 0.05 g/kg, $p < 0.05$). There was no difference between AR + Dig (0.94 ± 0.12 g/kg) and sham-operated rabbits. The left ventricular free wall thickness was similar in the three groups (sham-operated rabbits, 3.7 ± 0.5 mm; AR + C, 3.8 ± 0.2 mm; AR + Dig, 3.9 ± 0.1 mm). Figure 1 shows maximal binding sites and the dissociation constant of myocardial β -

Figure 2. Myocardial norepinephrine content. Abbreviations are described in Figure 1.

adrenergic receptor of the left ventricular free wall. Maximal binding sites were lower in AR + C (28.8 ± 7.9 fmol/mg protein) than sham-operated rabbits (69.9 ± 12.3 fmol/mg protein, $p < 0.05$). The number was higher in AR+Dig (39.9 ± 9.8 fmol/mg protein) than AR + C ($p < 0.05$). There was no difference in dissociation constant except it was higher in AR + Dig (167 ± 83 pM) than AR + C (114 ± 47 pM, $p < 0.05$). Figure 2 presents the myocardial norepinephrine content of the left ventricular free wall. Norepinephrine content was lower in both AR + C (1.30 ± 0.30 μg/g tissue) and AR + Dig (1.16 ± 0.49 μg/g tissue) than sham-operated rabbits (1.83 ± 0.38 μg/g tissue, $p < 0.05$ respectively). There was no difference between AR + C and AR + Dig.

Plasma norepinephrine concentration increased from 305 ± 133 pg/ml to 589 ± 132 pg/ml in AR + C ($p < 0.05$), but not in AR + Dig (from 339 ± 60 pg/ml to 396 ± 134 pg/ml). Plasma norepinephrine level 1 week after production of aortic regurgitation was lower in AR + Dig than AR + C ($p < 0.05$).

DISCUSSION

Aortic regurgitation as an animal model for heart failure: Hemodynamic findings showed that 1 week after induction of aortic regurgitation, left ventricular end-diastolic pressure was elevated and cardiac output reduced despite ventricular enlargement present, suggesting that left ventricular contractile performance was impaired. Down-regulation of myocardial β -adrenergic receptors and depletion of catecholamines were also observed. In a previous study we found that left ventricular end-diastolic pressure was elevated and β -adrenergic receptor number reduced as early as 1 day after induction of aortic regurgitation.¹⁵⁾ However, neither cardiac output nor myocardial catecholamine level was reduced at that time. Left ventricular function and sympatho-neuronal regulation progres-

sively deteriorated during the week following induction of aortic regurgitation. Myocardial β -adrenergic receptor density and norepinephrine content were both reduced in the failing left ventricle although not in the non-failing right ventricle, findings in accordance with the hemodynamic data showing left ventricular failure.¹⁹⁾ Gilson et al.²⁰⁾ reported that the myocardial β -adrenergic receptor number was reduced in rabbits with pressure and volume overload. In contrast, Yamazaki et al.²¹⁾ reported that the number of myocardial β -adrenergic receptors was increased in rats with aortic regurgitation. Florenzano et al.²²⁾ reported that left ventricular contractility was increased following induction of aortic regurgitation in chronically instrumented conscious dogs. However, aortic regurgitation induced in our study was severe enough to explain this discrepancy in the findings. Aortic regurgitation can result if severe enough in left ventricular failure in animal models. Magid et al.²³⁾ produced aortic regurgitation in rabbits, and observed pathological evidence for heart failure. Mean regurgitant fraction was 52%, which is comparable to our study.

Effect of digitalis glycosides on acute left ventricular failure: The left ventricular end-diastolic pressure was higher and cardiac output lower in rabbits with aortic regurgitation than sham-operated rabbits. Left ventricular end-diastolic pressure was lower in rabbits with aortic regurgitation given digoxin than those given saline. There was no difference in cardiac output between rabbits with aortic regurgitation given digoxin and sham-operated rabbits. Likewise, myocardial β -adrenergic receptor density was lower in rabbits with aortic regurgitation, while the density was higher in rabbits with aortic regurgitation given digoxin than those given saline. Two explanations are possible for the mechanisms of the attenuation of myocardial β -adrenergic receptor down-regulation. It is conceivable that digitalis glycoside improved hemodynamic status through its positive inotropic action, which in turn resulted in the attenuation of myocardial β -adrenergic receptor down-regulation.

Alternatively, a growing body of evidence suggests that there are some interactions between digitalis glycosides and sympathetic nervous system activity. First, clinical trials on digoxin show that digitalis glycosides lower plasma norepinephrine levels in patients with heart failure.²⁴⁻²⁶⁾ In these studies, however, an indirect effect of digoxin through stabilization of hemodynamic variables may explain the decrease in plasma norepinephrine level. Ferguson et al.⁸⁾ examined the effects of short-term digitalis administration on sympathetic nervous activity in patients with moderate-to-severe heart failure using direct microneurographic recordings of efferent sympathetic nerve activity, and demonstrated that muscle sympathetic nerve activities were attenuated by digitalis glycosides before any detectable improvement in hemodynamic parameters. By contrast, they found that dobutamine which similarly improved hemodynamic parameters did not

attenuate muscle sympathetic nerve activities. In addition, microneurographic recordings showed that digitalis but not dobutamine or placebo selectively potentiated sympathetic neural responses to baroreflex perturbation induced by lower body negative pressure in normal humans.²⁷⁾ Digitalis glycoside did not alter sympathetic nerve responses to the cold pressure test, suggesting that cardiopulmonary baroreflex function was selectively potentiated by the agent. Leenen et al.²⁸⁾ found that brain ouabain-like activity existed especially in the hypothalamus, using two animal models of heart failure, and reported that intracerebroventricular administration of Fab fragments significantly decreased renal sympathetic nerve activity and plasma norepinephrine level.

Conversely, there are several lines of evidence suggesting that overactivity of the sympathetic nervous system can lead to a decrease in Na^+ , K^+ pump activity, which reflects digitalis-binding site. Fan et al.¹⁰⁾ reported that Na^+ , K^+ -ATPase activity and ^3H -ouabain binding sites were decreased in myocardium with right ventricular failure induced by progressive pulmonary constriction and tricuspid avulsion, and these alterations were reversed by the β -blocker nadolol. The same group further showed that Na^+ , K^+ -ATPase $\alpha 3$ isoform protein along with ouabain-binding sites was reduced in failing myocardium induced by rapid ventricular pacing.¹¹⁾ Similar findings were noted in intact myocardium administered norepinephrine. They concluded that the decrease in Na^+ , K^+ -ATPase activity seen in congestive heart failure was mediated by excessive activation of the sympathetic nervous system. This finding is consistent with the report by Wang et al.⁹⁾ who showed that perfusion of the carotid sinus with ouabain caused a significant decrease in threshold pressure and a significant increase in peak discharge frequency, as well as an increase in the slope of the carotid sinus pressure-discharge curve in dogs with pacing-induced heart failure. They speculated that carotid sinus Na^+ , K^+ -ATPase activity was stimulated by prolonged activation of the sympathetic nervous system in congestive heart failure,²⁹⁾ resulting in a subsensitivity of carotid sinus baroreceptor function, which was restored by the sodium-potassium pump inhibitors, digitalis glycosides.

In this study, it is possible that digitalis glycosides attenuated excessive activation of the sympathetic nervous system after production of aortic regurgitation resulting in a partial prevention of down-regulation in myocardial β -adrenergic receptors, although myocardial norepinephrine content in rabbits with aortic regurgitation given saline and those given digoxin was similar. Plasma norepinephrine level was also lower in rabbits given digoxin than those given saline one week after production of aortic regurgitation. Differences in the time course of the regulation may be responsible for the persistent decrease in myocardial norepinephrine content in the presence of the improved β -adrenergic receptor and plasma norepinephrine. We cannot explain the exact mechanisms of the

antiadrenergic effects of digoxin in the present study. A recent study has shown that acute administration of digoxin reduces the β -adrenergic contractile response in intact rabbit hearts.³⁰⁾ The authors speculated that Ca^{2+} mobilized via $\text{Na}^+/\text{Ca}^{2+}$ exchange inhibited the catalytic unit of the adenylate cyclase system without affecting the β -adrenergic receptor-G protein system or contractile proteins.

Limitations of this study: We examined different groups of rabbits to compare the results, which potentially leads to misinterpretation of the results. The degree of aortic regurgitation was checked immediately after the procedure and again just before obtaining a membrane preparation 1 week after the procedure. Parameters reflecting the volume overloading were essentially the same between the two groups with aortic regurgitation including the decrease in aortic diastolic pressure immediately after production of aortic regurgitation, aortic diastolic pressure and regurgitant fraction 1 week after the procedure. Therefore, we believe the difference between the two groups with aortic regurgitation was not caused by a difference in severity, but rather by a difference in the treatments themselves. Digoxin is not a common therapeutic option for chronic compensated aortic regurgitation. We showed that digoxin was effective in restoring the hemodynamics and β -adrenergic receptor regulation in acute left ventricular failure induced by aortic regurgitation.

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