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Research Article

Experimental myocardial ischemia produced in dogs by proximal left anterior descending coronary artery ligation is accompanied by relatively rapid (1 h) increases in the number of (-) [3H]dihydroalprenolol binding sites without changing their dissociation constants in ischemic left ventricular tissue. The changes, persist for at least 8 h and are accompanied by marked decreases in myocardial tissue ischemic region norepinephrine content. In contrast, in the same canine model 1 h of proximal left anterior descending coronary artery ligation did not result in a significant change in the number of [3H]quinuclidynl benzilate binding sites of their dissociation constants. However, the number of [3H]quinuclidynl benzilate binding sites (muscarinic cholinergic receptors) are 50--70% greater than (-) [3H]dihydroalprenolol binding sites (beta adrenergic receptors) in canine left ventricular tissue. Thus, the data suggest that proximal left anterior descending coronary artery occlusion for 1 h significantly increases the number of beta adrenergic receptors in ischemic left ventricular tissue without changing the number of muscarinic cholinergic receptors. Whether the ischemia-produced increase in cardiac beta-receptor content is causally related to increased cyclic AMP levels that develop in ischemic tissue and/or an etiologic factor in arrhythmias originating from ischemic myocardial tissue will have to be determined in additional studies.

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Beta Adrenergic and Muscarinic Cholinergic Receptors in Canine Myocardium

EFFECTS OF ISCHEMIA

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ABSTRACT Experimental myocardial ischemia produced in dogs by proximal left anterior descending coronary artery ligation is accompanied by relatively rapid (1 h) increases in the number of (–) [³H]dihydroalprenolol binding sites without changing their dissociation constants in ischemic left ventricular tissue. The changes persist for at least 8 h and are accompanied by marked decreases in myocardial tissue ischemic region norepinephrine content. In contrast, in the same canine model 1 h of proximal left anterior descending coronary artery ligation did not result in a significant change in the number of [³H]quinuclidinyl benzilate binding sites or their dissociation constants. However, the number of [³H]quinuclidinyl benzilate binding sites (muscarinic cholinergic receptors) are 50–70% greater than (–) [³H]dihydroalprenolol binding sites (beta adrenergic receptors) in canine left ventricular tissue. Thus, the data suggest that proximal left anterior descending coronary artery occlusion for 1 h significantly increases the number of beta adrenergic receptors in ischemic left ventricular tissue without changing the number of muscarinic cholinergic receptors. Whether the ischemia-produced increase in cardiac β -receptor content is causally related to increased cyclic AMP levels that develop in ischemic tissue and/or an etiologic factor in arrhythmias originating from ischemic myocardial tissue will have to be determined in additional studies.

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INTRODUCTION

Previous studies have demonstrated that clamping or transection of the aorta in dogs (1, 2) and rats (3, 4), and coronary artery ligation in dogs, (5), baboons (6), and cats (7) increases cyclic AMP (cAMP)¹ levels in myocardium. This increase is prevented by beta receptor blockade (1–4).

It has been suggested that increases in cAMP levels in ischemic tissue occur in association with the development of ventricular fibrillation (6, 7). In dogs with distal coronary artery ligations that do not develop ventricular fibrillation, cAMP levels in ischemic tissue are reported not to rise (6).

Increases in cAMP levels in ischemic myocardial tissue might be caused by an alteration in the number or affinity of cardiac beta adrenergic receptors. However, it has also been reported that when myocardial ischemia is prolonged for more than an hour, depletion of myocardial norepinephrine content occurs (8, 9) and cAMP levels return toward normal.

To determine whether experimental canine myocardial ischemia is associated with an alteration in either the number or affinity of beta adrenergic receptors in left ventricular ischemic tissue, we have performed the following experiments. (–) [³H]Dihydroalprenolol (DHA), a potent beta adrenergic antagonist, has been used to label beta adrenergic receptors to allow direct measurement of their number and affinity (10–13). [³H]Quinuclidinyl benzilate (QNB), a muscarinic cho-

¹Abbreviations used in this paper: cAMP, cyclic AMP; DHA, (–) [³H]dihydroalprenolol; LAD, left anterior descending coronary artery; LV, left ventricle; QNB, [³H]-quinuclidinyl benzilate.

linergic antagonist, was used to estimate the number and affinity of muscarinic cholinergic receptors (14). In the present experiments, various periods of proximal left anterior descending coronary artery occlusion were evaluated to study the influence of experimental canine myocardial ischemia on left ventricular ischemic and nonischemic regions DHA and QNB binding and norepinephrine content.

METHODS

73 mongrel dogs (25–35 kg) of either sex (30 sham-operated control and 43 experimental animals) were used. The dogs were anesthetized with pentobarbital sodium (30 mg/kg i.v.), intubated, and ventilated with oxygen on a Harvard respirator (Harvard Apparatus Co., Millis, Mass.). A left thoracotomy was performed, the pericardium opened, and the proximal left anterior descending coronary artery (LAD) isolated. The proximal LAD and several branch vessels of the mid and lower portion of the LAD were ligated so as to create an anterior left ventricle (LV) region with intense cyanosis for 15 min in 12 dogs, for 1 h in 19 dogs, for 3 h in 7 dogs, and for 8 h in 5 dogs. Sham-operated control dogs were treated similarly except the proximal LAD and branch vessels of the mid level LAD were never ligated. After the designated time period of LAD occlusion, the heart was removed, and LV quickly divided into ischemic and control zones, and tissue frozen in liquid nitrogen and stored at -76°C until further analyzed. Ischemic myocardial tissue was recognized by: (a) a region of intense cyanosis developing in an anterior left ventricular myocardial region occurring immediately with, and continuing for the duration of LAD occlusion and (b) epicardial electrocardiographic mapping to document that this region contained sites of ST segment elevation (15). Control left ventricular tissue was taken from the lateral left ventricular myocardium in a region that did not develop cyanosis after the LAD occlusion and one that did not demonstrate epicardial ST segment elevation.

Crude membranes from cardiac tissue were prepared for DHA binding according to the procedure of Williams et al. (10) and for QNB binding by the method of Fields et al. (14). In brief, 7–10 g of heart tissue was minced in 70–100 ml of cold buffer (25% sucrose, 5 mM Tris HCl, pH 7.4, and 1 mM MgCl_2). The mince was homogenized in a polytron PT10 for three 5-s bursts with cooling in between. The homogenate was centrifuged at 1,000 g (max) for 10 min at 4°C . The pellet was discarded and the supernatant centrifuged at 39,000 g (max) for 10 min. For the DHA binding studies the pellet was washed twice in cold incubation buffer (50 mM Tris HCl, pH 7.5, and 10 mM MgCl_2) and finally suspended in buffer containing 75 mM Tris HCl, pH 7.5, and 25 mM MgCl_2 at 3–6 mg protein/ml. For the QNB binding studies the pellet was washed twice with 50 mM potassium phosphate buffer, pH 7.4, and finally suspended in the same buffer at 3–6 mg protein/ml.

The membrane preparations were assayed for DHA binding as described (12, 13). 0.1 ml membrane suspensions were incubated for 10 min at 37°C with varied concentration of DHA (0.3–15 nM) in a total vol of 150 μl . Final concentrations of Tris and MgCl_2 were 50 and 17 mM, respectively. Incubations were terminated by diluting the incubation mixture with 2 ml of ice-cold incubation buffer and rapid vacuum filtration through Whatman GF/C filters (Whatman Inc., Clifton, N. J.). The filters were immediately washed with 10 ml of ice-cold incubation buffer. Filters were air dried overnight, and placed in a scintillation cocktail (AquaSol 2; New England Nuclear, Boston, Mass.) and radioactivity measured in a Packard

liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.) at an efficiency of 40%. In each experiment nonspecific binding to the membranes was determined by measuring the amount of radioactivity retained on GF/C glass fiber filters when incubations were performed in the presence of 10 μM (\pm) propranolol. QNB binding was measured as described by Fields et al. (14). 0.1-ml membrane suspensions were incubated for 40 min at 37°C with varied concentrations of QNB (10–200 pM) in a total vol of 2 ml. Incubations were terminated by rapid vacuum filtration through Whatman GF/C filters. The filters were immediately washed with 10 ml of ice-cold 50 mM potassium phosphate buffer pH 7.4 and the subsequent procedures were followed as stated above. Nonspecific binding was determined in separate experiments by measuring the radioactivity retained on GF/C filters in the presence of 1 μM atropine.

Specific binding was determined by subtracting nonspecific from total binding. Using these direct binding methods, the number and affinity of DHA and QNB binding sites in membranes were analyzed according to Scatchard (16) fitting the regression lines by the method of least squares. Five to six data points were obtained for the Scatchard analysis. Each determination was done in duplicate (and often in triplicate) and the r value of the line drawn was always within 0.80–0.95. Specific binding for DHA was 40–60% of total binding depending upon the concentration of DHA used. The specific binding of QNB was 70–90% of the total binding. The exact amount of DHA or QNB specifically bound was relatively less at the highest concentrations of the ligand used.

Protein concentration in the membrane fractions was measured by the Lowry method (17). Total protein concentrations in the crude membrane preparations were measured in all experiments. In four experiments, in which LV ischemic and nonischemic tissue were obtained from canine hearts with 1 h of proximal LAD occlusion, mitochondrial protein concentration was also measured.

To test if the binding of DHA to membrane was activated by detergent treatment, a nonionic detergent, Lubrol-PX (Sigma Chemical Co., St. Louis, Mo.) was incubated with the crude homogenates at different ratios of protein to detergent (1:0.1–1:4) for 10 min at 37°C . Specific DHA binding was then determined as mentioned above.

Norepinephrine content in heart tissue was measured spectrofluorometrically according to the method of Chang (18). In brief, tissue protein was precipitated with perchloric acid and catecholamine was adsorbed on alumina. Catecholamine was eluted with acetic acid and oxidized with iodine. Norepinephrine content was determined (activation at 385 nm and emission at 485 nm) using the Aminco Bowman spectrofluorometer (American Instrument Co., Travenol Laboratories Inc., Silver Spring, Md.) The minimum amount of norepinephrine in tissue that gives a fluorescence reading twice that of tissue blank is ≈ 10 –20 ng.

RESULTS

Proximal LAD ligation for 1, 3, and 8 h resulted in significant increases in the number of DHA binding sites in LV ischemic myocardial tissue as compared to nonischemic regions (Table I). After 15 min of coronary occlusion, there was no significant increase in DHA binding sites although 9 of 12 animals studied did demonstrate increases. After 1, 3, and 8 h of coronary occlusion, the number of DHA binding sites in ischemic LV tissue were significantly increased and were 163 ± 18 SE, 141 ± 12 , and 154 ± 20 fmol/mg

TABLE I
Specific Binding and Dissociation Constants (K_D) of DHA in Crude Particulate Fractions from Ischemic and Nonischemic Zones of Sham and Proximal LAD-Occluded Dogs

Time	Sham					LAD-occluded				
	Number of dogs	Ischemic		Nonischemic		Number of dogs	Ischemic		Nonischemic	
		B max*	K_D	B max	K_D		B max	K_D	B max	K_D
		fmol DHA bound/ mg protein	nM	fmol DHA bound/ mg protein	nM		fmol DHA bound/ mg protein	nM	fmol DHA bound/ mg protein	nM
15 min	6	93±14	7.35±1.34	81±10	6.10±0.87	12	110±8 NS† NS§	7.77±0.86	97±10	7.41±1.03
1 h	6	86±7	8.42±1.55	94±10	9.21±1.05	12	163±18 $P < 0.01$ † $P < 0.02$ §	11.77±1.06	101±18	9.86±1.44
3 h	6	68±7	7.93±0.81	85±10	8.31±1.09	7	141±12 $P < 0.001$ † $P < 0.005$ §	10.28±1.09	101±18	7.79±1.74
8 h	6	93±10	6.15±0.43	89±8	7.58±0.48	5	154±20 $P < 0.05$ † $P < 0.05$ §	8.33±1.53	124±13 $P < 0.1$ †	9.20±1.00

* B Max, total number of specific DHA binding sites extrapolated from the Scatchard plots±SEM.

† P values refer to group t test comparisons between LAD occluded and sham occluded animals.

§ P values refer to paired t test comparisons between ischemic and nonischemic LV tissue in dogs with proximal LAD occlusion.

protein, respectively. These values were also significantly greater in the LV ischemic area as compared to those obtained from the same general region in the sham-operated control animals, which showed values for the same time periods of 86±7, 68±7, and 93±10 fmol DHA bound/mg protein, respectively. After 8 h of proximal LAD occlusion, the number of DHA binding sites in LV nonischemic tissue (124±13 fmol DHA bound/mg protein) appeared to be greater than in the same tissue obtained from sham-operated animals (89±8 fmol DHA bound/mg protein) ($P < 0.1$). No significant alterations in the affinity of DHA binding were demonstrated (Table I).

Proximal LAD ligation for 1, 3, and 8 h also resulted in a significant decrease in the norepinephrine content in the LV ischemic region (Fig. 1). However, there was not a clear relationship between the magnitude of decrease in tissue norepinephrine content and the increase in DHA binding sites in animals with experimental coronary artery occlusions for 15 min or 1 h (Fig. 1).

In seven additional canine hearts with proximal LAD occlusions for 1 h both DHA and QNB binding were determined. DHA binding in the LV ischemic region increased in each of these hearts (63% increase, $P < 0.001$ in LV ischemic as compared to nonischemic tissue), but there was no significant difference in QNB

binding between LV ischemic and nonischemic regions in these same hearts (154±13.5 in LV ischemic region and 147±8.0 fmol QNB bound/mg protein in the nonischemic region). When the number of QNB binding sites obtained in these seven dogs were compared with similar values from comparable LV regions in six sham-operated animals there was still no significant change in QNB binding sites or dissociation constants (K_D) in the various LV regions. It was of interest to note in the sham-operated animals and in the animals with proximal LAD ligations that the number of nonischemic LV QNB binding sites were 50–75% greater than DHA (140 fmol QNB bound/mg protein vs. 86 fmol DHA bound/mg protein in sham-operated animals and 147 fmol QNB bound/mg protein as compared to 101 fmol DHA bound/mg protein in nonischemic LV in animals with LAD ligations).

The yields of final crude membrane protein per gram of heart wt in the LV ischemic and nonischemic regions were 1.5–3 mg/g of wet wt. The specific protein contents of the membrane preparations in LV ischemic and nonischemic tissue after the various periods of proximal LAD occlusion are as listed below. Protein concentrations in the membrane fractions were 1.6±0.19 in ischemic and 1.9±0.14 mg/g tissue wet wt in LV nonischemic tissue after 1 h (NS), 1.9±0.18 in ischemic and 2.4±0.12 mg/g tissue wet wt in non-

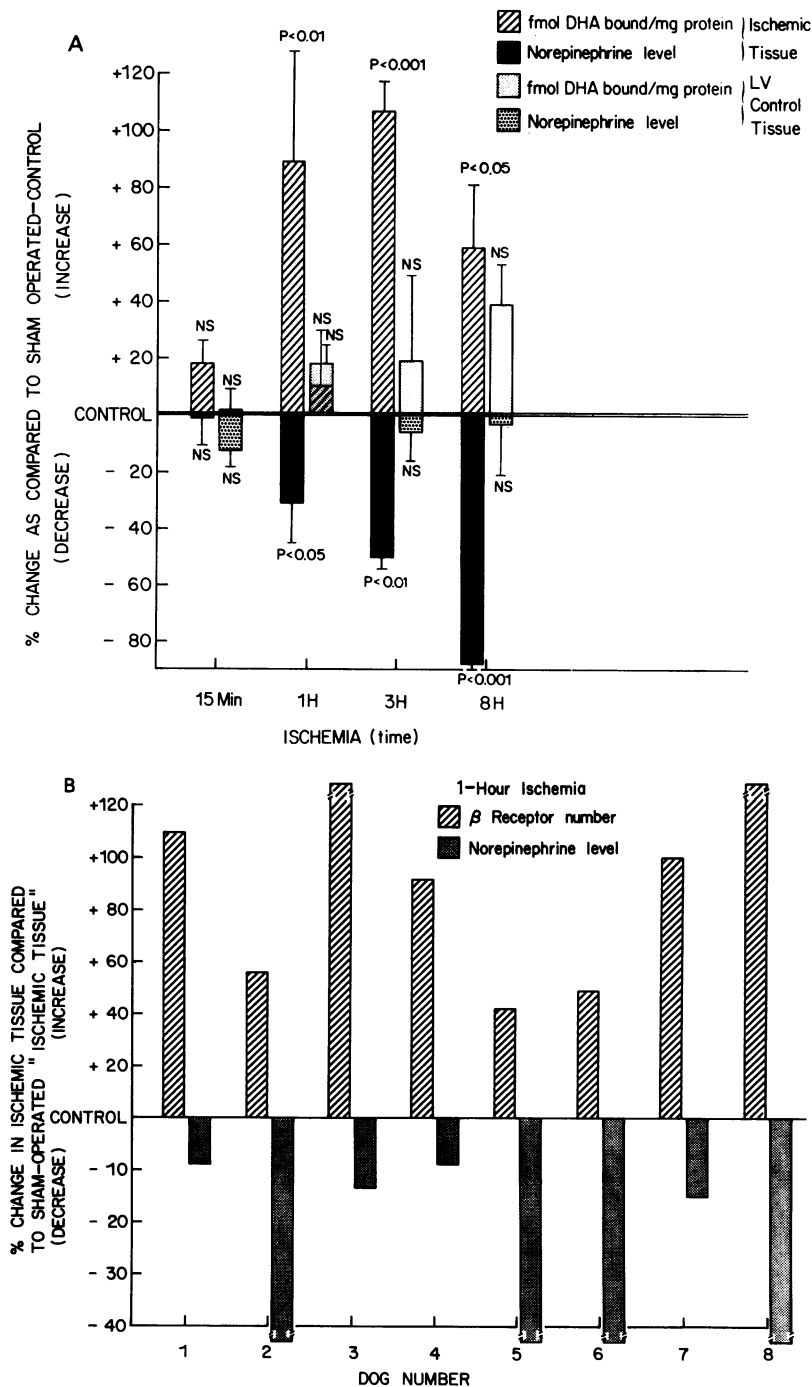


FIGURE 1 (A) Percent changes in DHA binding sites (beta adrenergic receptor numbers) and norepinephrine content in LV ischemic tissue as compared to the LV control tissue in sham-operated dogs. Ischemia was experimentally produced by occluding the proximal LAD for 15 min–8 h. Subsequently, crude membrane fractions were prepared and DHA binding assays carried out as described in the text. The results are the mean \pm SE of 6–12 experiments. (B) Percentage changes in eight separate dogs in DHA binding sites and norepinephrine content in LV ischemic tissue as compared to the control tissue in sham-operated dogs. The data demonstrate that there is not a clear relationship between the increase in the number of DHA binding sites and the magnitude of catecholamine depletion in the LV ischemic region in individual animals that were studied.

ischemic tissue after 3 h (NS), and 2.1 ± 0.09 in ischemic and 2.7 ± 0.05 mg/g tissue wet wt in nonischemic LV tissue ($P < 0.01$) after 8 h of proximal LAD occlusion. There was no difference in mitochondrial protein concentrations in these same preparations obtained after 1 h of LAD occlusion.

DISCUSSION

The mechanism(s) responsible for the increased number of DHA binding sites and presumably beta adrenergic receptor numbers in ischemic left ventricular tissue as early as 1 h after proximal LAD occlusion are presently uncertain. However, several explanations may be considered. Increased protein synthesis might be the explanation although the 1-h time period seems relatively short for increased membrane protein turnover. The increase in DHA binding sites in LV ischemic tissue could reflect either translocation of a cytosolic receptor pool to the membrane and/or unmasking of latent receptors on the membrane. Recently, Kempson et al. (19) have shown that when heart slices are incubated in the presence of triiodothyronine there is an increase in beta receptor numbers, but this increase was not noted when the incubation was performed with crude membrane preparations. Thus, they postulated that there is a cytosolic pool of beta adrenergic receptors that is translocated to the membrane during triiodothyronine treatment. Also, Shutt et al. (20) have shown that when sarcolemmal membrane vesicles are treated with Lubrol-PX, a detergent, or alamethicin, an ionophore, a two- to three-fold increase in receptor numbers occurs indicating that some of the receptors are latent ones. However, our experiments done with crude membrane preparations did not show any increase in receptor numbers when treated with Lubrol-PX. In fact, receptors were either solubilized or denatured when the protein to Lubrol ratio exceeded 1:0.2.

Another possibility is that the increase in receptor numbers is an example of "up-regulation" of receptor numbers in response to decreased tissue catecholamine levels. Such increases in β -receptor numbers in brain and heart have been described when tissue catecholamines are depleted by such agents as 6-hydroxydopamine or guanethidine (21, 22). Somewhat against this possibility, however, is that the increases in receptor number did not clearly correlate with the extent of catecholamine depletion at the various time periods that were studied.

It should also be emphasized that in the present study beta adrenergic and muscarinic cholinergic receptor numbers have been expressed as DHA and QNB bound per milligram of protein, respectively. Therefore, another possible explanation for the increase in DHA binding sites in LV ischemic tissue

is that the change is an artifactual one based upon alterations in membrane protein content such that LV ischemic tissue contains relatively more receptor protein than nonischemic areas and thus only apparently more beta adrenergic receptors. If this were the correct explanation for the increase in DHA binding sites, there should logically have also been an increase in the number of QNB binding sites because both beta adrenergic and muscarinic cholinergic receptors exist in the sarcolemmal membrane (23). Since there was no change in QNB binding sites in the same animals in whom DHA binding sites were increased, this possibility does not appear to be a valid explanation for the alteration in beta adrenergic receptor numbers that was observed. Furthermore, we found essentially the same crude homogenate and mitochondrial protein contents in ischemic and nonischemic left ventricular regions after 1 h of proximal LAD ligation in this experimental model.

The physiological significance of the increase in DHA binding sites and apparently in beta adrenergic numbers in LV ischemic tissue after experimental proximal LAD occlusion is presently uncertain, but it does appear that the increase in receptor numbers after one hour of experimental myocardial ischemia is specific for beta adrenergic as opposed to muscarinic cholinergic receptors. Depending on the occupancy-response relationship, an increase in beta adrenergic receptor numbers could have at least two possible implications for physiological responses. One implication is that such an increase might result in a greater maximally obtainable physiological response as regards metabolic, inotropic, and other physiological aspects of catecholamine stimulation. The response to agonist stimulation would be expected to parallel the degree of receptor occupancy and the increase in the maximally obtainable physiological response would occur in circumstances in which there are either none or few "spare" receptors. The second possible implication of an increase in beta adrenergic receptor numbers would be to increase only the sensitivity of the physiological response, i.e., an increase in the apparent affinity of the agonist without a change in the maximal response. This would be expected to occur when there are spare receptors available. Alternatively, the increased number of beta adrenergic receptors might be "uncoupled" from intracellular physiological responses by the regional ischemic influence.

The present studies appear to identify an increase in the number of beta adrenergic receptors in the ischemic region of the canine LV after 1 h of proximal LAD ligation, but additional studies will be necessary to identify the mechanism(s) responsible for the increased beta adrenergic receptor numbers and the pathophysiological consequences and implications of this phenomenon.

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