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Increased Number of Cardiac Adrenergic Receptors following Local Heart Irradiation

S. LAUK,* M. BÖHM,† G. FEILER,† B. J. GEIST,* AND E. ERDMANN†

**Institut für Strahlenbiologie, GSF, Neuherberg, Federal Republic of Germany*
†Medizinische Klinik I, Universität München, Federal Republic of Germany

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The effect of local X irradiation on cardiac α and β receptors was studied in Wistar rats. Animals were given local heart irradiation with single doses of 15 or 20 Gy and were examined after a range of latency times of 7 to 400 days. Using the radioactive ligands [^{3}H]CGP-12177 and [^{3}H]prazosin, the maximal binding capacity was determined from saturation experiments. At 7 days after 20 Gy the maximal binding capacity of both α and β receptors was reduced to below the level of untreated control animals. Subsequently it rose continually to a maximum of 160% of the control level for β receptors and 130% for α receptors at 400 days postirradiation. The antagonist affinity as judged from the dissociation constant for [^{3}H]CGP 12177 and [^{3}H]-prazosin did not change significantly. A similar effect was observed after 15 Gy. An increase in adrenergic receptors may represent an important pathogenetic link between early morphological and late functional changes in the pathogenesis of radiation-induced heart disease.

INTRODUCTION

Local X irradiation of the rat heart with doses of 17.5 Gy or more causes cardiac failure after dose-dependent latency times (*1*). Sequential morphological examination showed that early radiation-induced capillary damage leads to myocardial degeneration and necrosis. These findings are supported by similar observations in locally irradiated dog hearts (*2*). Functional studies have revealed that congestive heart failure is preceded by an increased breathing rate at rest and an impaired recovery of heart rate after exercise challenge (*1*). However, functional changes could not be easily explained and could not be directly correlated to morphology. Alpha and beta adrenergic receptors, which both mediate positive inotropic effects in the human (*3*) and rat myocardium (*4*), are likely to play a major role in the regulation of cardiac function. Moreover, excessive stimulation of α adrenoceptors (*5*) or β adrenoceptors (*6*) produces cardiomyopathy. We therefore carried out sequential measurements of the concentrations of α and β adrenergic receptors after local heart irradiation.

MATERIALS AND METHODS

Animals and irradiation. Male Wistar rats aged 4–5 months were given local irradiation to the whole heart with 300 kV X rays at a dose rate of 2 Gy/min. Single doses of 15 or 20 Gy were given through a

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lateral field. To minimize radiation to other thoracic organs, the shape and position of the radiation field was determined individually for each animal using a chest X ray taken with the treatment beam on polaroid film. Details of the irradiation technique have been described (*1*). At 7, 49, 196, and 400 days following 20 Gy animals were sacrificed for receptor binding studies. After 15 Gy irradiation, animals were studied at intervals of 49 and 196 days. Six animals were irradiated for each time point and dose group, and an equal number of control animals was used.

Animals were kept two per cage throughout the observation period. One of the two animals was irradiated and the other served as control. If possible they were litter mates or at least age matched. Receptor binding studies were carried out simultaneously for each pair of animals.

Radioligand binding studies. Left ventricles were dissected from the hearts of irradiated and control rats. The ventricles were chilled in 30 ml ice-cold homogenization buffer (10 mmol/liter Tris–HCl, 1 mmol/ EDTA, pH 7.4). The hearts were minced with scissors and homogenized with a motor-driven glass–Teflon homogenizer for 1 min. Afterward the preparation was homogenized by hand for 1 min with a glass–glass Potter Elvehjem homogenizer. This homogenate was diluted with an equal volume of ice-cold 1 mol/liter KCl and stored on ice for 10 min. The solution was centrifuged for 45 min at 100,000*g*. The pellet was resuspended in 50 vol of incubation buffer (50 mmol/liter Tris–HCl, 10 mmol/liter $CaCl_2$, pH 7.4) and homogenized for 1 min with a Potter homogenizer. This suspension was again centrifuged with 100,000*g* for 45 min. The pellet was resuspended in incubation buffer (50 vol). Only fresh tissue was used in these experiments. Postsynaptic α adrenoceptors in the myocardium are of the α-1 subtype, which mediates positive inotropy (*4*). Therefore the radiolabeled α-1-selective antagonist [^{3}H]prazosin was used to investigate α adrenoceptors. Since both β-1 and β-2 adrenoceptors are located on myocardial cells and mediate positive intropism (*7*), the nonselective β adrenoceptor antagonist (−)-4(3-*t*-butylamino-2-hydroxypropoxy)-5(5,7-[^{3}H]benzimidazol-2-one) [^{3}H]CGP-12177) is suitable for detection of the total amount of cardiac β receptors. In brief, homogenates were incubated with different concentrations of [^{3}H]prazosin (0.01–3.0 nmol/l) and [^{3}H]CGP-12177 (0.05–10.0 nmol/liter). The assay was performed in a total volume of 250 μl incubation buffer. The incubation was carried out at 37°C for 60 min for both [^{3}H]prazosin and [^{3}H]CGP-12177 binding. Binding of [^{3}H]prazosin and [^{3}H]CGP-12177 to cardiac membranes rose linearly with increasing protein concentrations. These conditions allowed complete equilibration of the receptor with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters. The filters were washed immediately three times with 6 ml of ice-cold incubation buffer. All experiments were performed in triplicate. Homogenates of irradiated and control rats were studied in parallel. Filters were dried at 90°C and placed in 10 ml scintillation fluid (Quickszint 501, Zinser Analytics Frankfurt, FRG) and radioactivity was determined in a liquid scintillation counter. Specific binding was defined as the difference in binding in the absence and presence of phentolamine (α adrenoceptors) or (−)-propanolol (β adrenoceptors), respectively. A concentration of 10 μmol/liter propanolol was used for [^{3}H]prazosin. Different propanolol concentrations of 1, 3, 10, and 30 μmol/liter propanolol did not alter nonspecific binding which amounted to about 30% of total binding at K_D and about 50% at 10 nmol/liter [^{3}H]CGP-12177. Therefore, 10 μmol/liter (−)-propanolol was used for experiments with [^{3}H]CGP-12177. There was no difference in nonspecific binding using the β adrenoceptor agonist isoprenaline (300 μmol/liter). The use of different antagonists is mandatory, since identical labeled and unlabeled substances could interfere at nonspecific binding sites and hence lead to an overestimation of specific binding. The density (B_{max}) and apparent affinity (K_D) of binding sites were obtained in individual experiments from Scatchard plots determined by linear regression analysis. Protein concentration was determined according to Lowry *et al.* (*8*), using bovine serum as standard.

RESULTS

Body and heart weights of irradiated animals were not different from those of their age-matched controls. The relative heart weight was 0.33 ± 0.06% and 0.34 ± 0.06% of total body weight in controls and irradiated animals, respectively. However, animals treated with 20 Gy did show mild clinical signs of heart failure at 196 days and pronounced symptoms at 400 days. They were not as active as their age-matched controls, they showed forced abdominal breathing which was particularly obvious when the animals were resting following exertion, and they had a rough coat. The

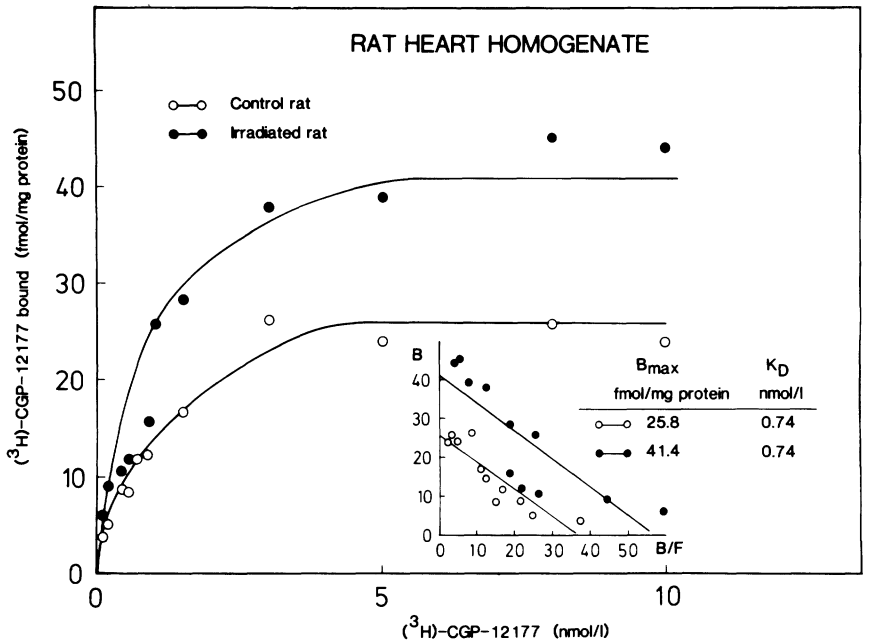

FIG. 1. Specific binding of [^{3}H]CGP-12177 to cardiac membranes of an irradiated rat heart (closed symbols) and a control heart (open symbols). Specific binding was defined as total binding minus nonspecific binding as measured in the absence and presence of 10 μmol/liter (−)-propanolol, and is expressed as fmol/mg protein. The inset shows a Scatchard analysis where [^{3}H]CGP-12177 binding is plotted as a function of the ratio of bound to free radioligand. The intercept with the ordinate is the maximal number of binding sites (B_{max}), the slope is the apparent affinity (K_D). The irradiated animal had been treated with 20 Gy, 400 days before receptor binding study.

median survival time after 20 Gy was 390 days in a parallel experiment.[1] Fifteen Gy did not lead to any clinical symptoms within the observation period of this study.

Binding of [^{3}H]CGP-12177 and [^{3}H]prazosin to cardiac membranes is monophasic; Fig. 1 presents a typical example of a β receptor binding study. Incubation with increasing radioligand concentrations yields a higher level of ligand binding in the irradiated heart compared to that in the control heart. However, the concentrations at which half-maximal binding is achieved (K_D value) are similar in both animals. This is derived from Scatchard analysis, shown in the inset of Fig. 1. When the bound [^{3}H]CGP-12177 is plotted against the ratio of bound to free [^{3}H]CGP-12177, a straight line can be fitted through the experimental data points by linear regression analysis. The intercept with the ordinate gives B_{max}, and the negative slope is an estimate of the apparent equilibrium dissociation constant K_D for the interaction of [^{3}H]CGP-12177 with the binding sites. Since the two fitted lines are parallel in this case, K_D and thus the antagonist affinity of β receptors were similar in both animals,

[1] B. G. Geist, K.-R. Trott, and S. Lauk, Physiological consequences of local heart irradiation in the rat. Manuscript submitted for publication.

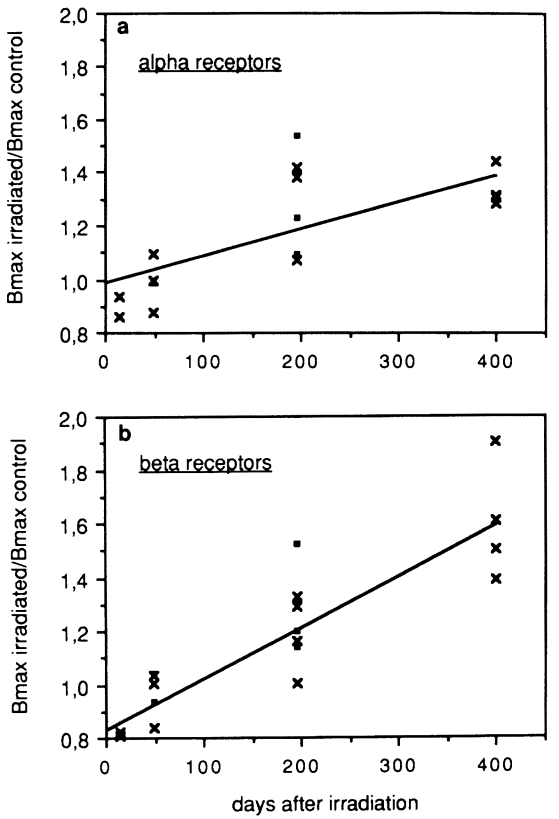

FIG. 2. Variation of B_{max} of α (a) and β (b) with postirradiation time. Each point represents the ratio of B_{max} in one irradiated and one control animal. Lines are derived by linear regression analysis. (x = 20 Gy, ■ = 15 Gy.)

whereas the number of β receptors was higher in the irradiated heart. The ratio of B_{max} irradiated to B_{max} control was derived from this analysis for each pair of animals. Similar alterations were obtained for α adrenoceptors, i.e., an increased number but an unchanged antagonist affinity of [^{3}H]prazosin.

The time course of changes with postirradiation time in B_{max} for binding to α receptors is represented in Fig. 2a. The number of α receptors increases continuously to an average of 133% at 196 days and remains approximately constant thereafter. The number of β receptors (Fig. 2b) showed a similar time course. B_{max} in irradiated animals was 80% of control levels at 14 days, regained control levels at 49 days, and rose to 160% by 400 days. The increase in B_{max} with postirradiation time can be described by a linear regression analysis. The P values for the intercept and slope were <0.0002 for both α and β adrenergic receptors (Figs. 2a and 2b). Following 15 Gy only two times, 49 and 196 days postirradiation, were studied, and the effect was not different from that at 20 Gy for either time. In control animals the average B_{max} and standard deviation of α and β adrenergic receptors was 60.5 ± 7.2 and 26.2 ± 4.5 fmol/mg protein, respectively. Over the period studied, these control values did not change

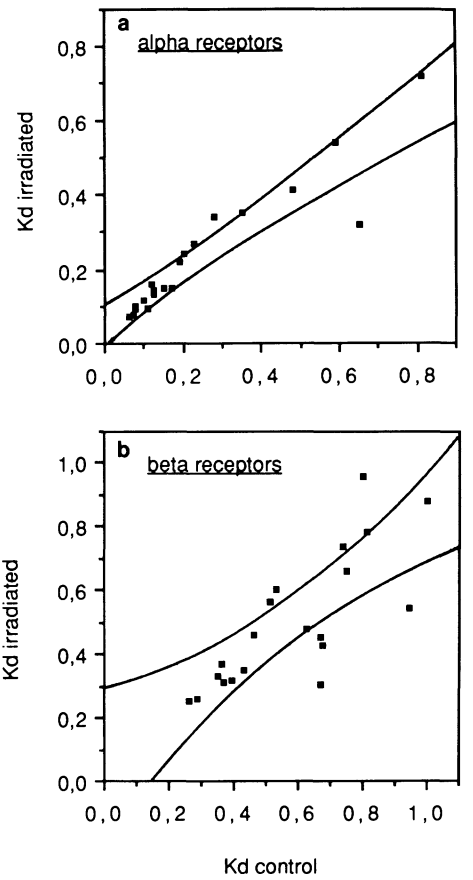

FIG. 3. K_D in irradiated animals as function of K_D in control animals for α (a) and β (b) receptors. Each point represents one pair of animals. Lines represent the 95% confidence intervals.

systematically with age of the animals, and their variability partly reflects variability between measurement procedures. Therefore postirradiation changes are expressed as percentage of the respective controls.

The K_D of irradiated animals was compared to the respective control values for α and β receptors (Figs. 3a and 3b). The 95% confidence limits for the data points are plotted, indicating some scatter of data. However, a separate analysis for dose groups and time points did not reveal any systematic pattern of values deviating from 1. The few data points where K_D of irradiated and control animal appeared to be different from each other were derived from both dose groups and from both short and long observation periods. This suggests that variations of K_D within one measurement procedure are within the range of the method rather than an effect of radiation on receptor affinity. Absolute values and standard deviations of K_D for α and β adrenergic receptors in control animals were 0.11 ± 0.05 and 0.57 ± 0.17 nmol/liter, respectively.

DISCUSSION

During the observation period of 14 to 400 days following local heart irradiation, the number of β receptors rose continually. However, the starting point of this rise at 14 days was below control levels. Measurement of the concentration of α receptors gave similar results during the first 200 days, but no further increase was seen between 200 and 400 days. The early decrease in β receptor concentration agrees with the observations reported by Timmermans and Gerber (*9*), who reported that concentrations of β receptors in rabbits decreased to 80% of control values at 3 days after local heart irradiation with 10 Gy. One might hypothesize that norepinephrine is acutely released from nerve endings during the acute phase, resulting in receptor down-regulation.

In previous experiments, local X irradiation of the heart has been shown to cause cardiac failure after dose-dependent latency times of 110 days after 40 Gy to 408 days after 17.5 Gy (*1*). Animals treated with 15 Gy developed mild clinical symptoms as well as pathological signs of congestive heart failure, but these were not life threatening. The sequence of histopathological changes leading to clinically manifest heart failure was studied following 15 or 20 Gy (*10*). At around 28 days after 20 Gy, the myocardial capillary network started to decrease rapidly in volume density and length density to less than 50%. This was accompanied by a focal loss of the capillary endothelial alkaline phosphatase activity. At 70 days after 20 Gy focal myocardial degeneration started to develop as a result of capillary rarefication. The maximum extent as well as the latency until onset of these changes was dose dependent; that is, they occurred later and to a lesser extent after 15 than after 20 Gy.

Hearts examined at 196 days after 20 Gy or later therefore contained a certain amount of degenerated, necrotic myocardial tissue. This may have influenced the protein content of the cardiac membrane preparation. If receptors are more susceptible to degenerative processes than other tissue proteins, the analysis would underestimate the concentration of receptors in the remaining viable myocardium. If the protein content decreases while binding capacity of existing adrenergic receptors remains unimpaired, the receptor density increases. However, the maximum extent of myocardial degeneration is 30% after 20 Gy and only 5% after 15 Gy (*10*). If increased receptor concentrations were only reflecting protein loss due to myocardial degeneration, a similarly clear dose dependence would be expected. Since receptor concentrations were not different following 15 or 20 Gy, we conclude that there is a real increase in the number of adrenergic receptors following heart irradiation in Wistar rats.

Sequential radionuclide studies in Wistar rats revealed that ventricular perfusion is maintained following heart irradiation in spite of a rarefication of the capillary network by an increase in cardiac output. This was significant by 100 days after irradiation with 17.5 Gy (*11*). This important change in cardiac function could be mediated by the observed increase of adrenergic receptors. However, radiation-induced changes in cardiac function show much more interspecies and even interstrain variation than the underlying morphology. Sprague–Dawley rats maintain ventricular perfusion following heart irradiation by increasing the percentage of ventricular output distributed to the heart, while cardiac output is decreasing (*11*). No receptor binding studies have been carried out in Sprague–Dawley rats, but one might expect different results than in Wistar rats. In dogs the left ventricular ejection fraction was reduced

at 1 to 3 months after fractionated heart irradiation with 4 × 11 or 4 × 13 Gy but returned to normal by 6 months postirradiation (*2*). At this time heart rate was still increased; therefore, assuming an unchanged left ventricular volume, cardiac output of the dogs may have been increased. In groups of patients who had received thoracic radiotherapy including the heart, left ventricular ejection fractions at rest or during exercise challenge were found to be reduced in 17–33% of irradiated patients (*12–15*) without any consistent changes in heart rate. These changes were observed at long latency times of 2.5 to 20 years after radiation. Comparative investigation of these different types of functional expression of morphologically similar radiation-induced cardiac lesions is important for interpretation of experimental results and may allow conclusions on underlying mechanisms.

We can only speculate on the mechanism leading to an increase in the concentration of receptors. In patients with heart failure the plasma catecholamines are increased (*16, 17*) and β adrenergic receptors are down-regulated in response, while the concentration of α receptors remains constant (*3, 18*).

Following local heart irradiation, congestive heart failure in Wistar rats develops progressively. The earliest clinical sign of heart disease is that the time for return to a normal heart rate is prolonged after standardized exercise challenge (*1*). Only many weeks later do animals gradually develop signs of heart failure at rest. When animals were sacrificed because of heart failure, autopsy revealed pleural effusions, liver congestions, and subcutaneous edema. The longest observation period in the present experiment (400 days after 20 Gy) was beyond the median survival time for this dose group, and congestive heart failure is fully expressed by this time. Since an increase in the concentration of β receptors is observed, this finding suggests a different pathogenesis for radiation-induced heart disease in these animals in comparison to cardiac failure from other causes.

However, there are some conditions under which an increase of adrenergic receptors has been observed. In dogs Mukherjee *et al.* (*19*) found an increase of β receptors persisting for at least 8 h after ischemia had been induced by coronary artery ligation. This can be attributed to efferent sympathetic denervation induced by hypoxia (*20, 21*). However, chronic hypoxia investigated in rats at high altitude (*22*) had the opposite effect, causing a decrease in β receptors. The severe rarefication of the capillary network occurring after local heart irradiation may cause focal or general hypoxia in the myocardium. But since the reduction of the capillary network happens gradually, it should be compared to chronic hypoxia rather than to acute coronary artery ligation.

In various animal models for cardiac hypertrophy, different observations regarding adrenergic receptors have been made. In Syrian hamsters with dystrophic hereditary cardiomyopathy (*23*) and young spontaneously hypertensive rats (*4*), increased responsiveness to α adrenergic stimulation has been reported, while β adrenergic receptors were unchanged. However, in adult rats which are naturally hypertensive, the number of both α and β adrenergic receptors was found to be reduced (*24, 25*). In guinea pigs an increase in α and β receptors was reported following aortic constriction (*26, 27*) and was attributed to a depletion of endogenous catecholamine stores (*27*). Following X irradiation with 15 or 20 Gy the ratio of heart weight to body weight is

slightly but not significantly increased when animals develop symptoms of heart failure. This excludes at least gross cardiac hypertrophy.

Therefore, the pathophysiological changes in radiation-induced heart disease appear to be different from the alterations found in the above-mentioned experimental models for cardiomyopathy. No other experimental situation where adrenergic receptors have been studied is directly comparable to radiation-induced heart disease.

In summary, the initial decrease of α and β receptors could reflect an augmented release of norepinephrine as an acute event following X irradiation, leading to receptor down-regulation. In later stages α and β adrenoceptors are suggested to be up-regulated. It is not unreasonable to assume that up-regulation is initiated by a decreased sympathetic output of cardiac nerves. This could be due to the direct effect of radiation on terminal nerves containing catecholamine or could be mediated by radiation damage to the capillary network and local hypoxia.

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