Steroids Produced in Vitro by Adrenal Glands of Normal and Arteriosclerotic Rats During and After Drug-Induced Myocardial Necrosis

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Acute infarctoid myocardial necrosis can be produced in virgin rats by injection of the potent catecholamine, isoproterenol. We have noted that these myocardial lesions are essentially identical, both grossly and microscopically, to acute infarcts which occur spontaneously in the hearts of breeder rats with arteriosclerosis. Rats with drug-induced myocardial necrosis show signs of shock which closely resemble those in the shock syndrome of myocardial infarction seen in man. The animals develop severe hypotension and congestive heart failure; with dyspnea, tachycardia and prostration. The serum levels of the transaminases (SGOT and SGPT) and lactic dehydrogenase rise sharply during the acute development of myocardial necrosis and gradually return to normal with repair of the myocardial damage.

The development of acute adrenal hyperplasia and involution of the thymus gland, coincident with the onset of myocardial damage, was of particular interest to us because these alterations indicated an intense activation of the adrenal cortex. Measurements of adrenal steroid production (in vitro) provided additional evidence that highly significant changes were taking place in the adrenal function of animals with isoproterenol-induced myocardial necrosis. Particular importance was attached to the observation that aldosterone production was elevated markedly during the acute stages of myocardial damage.

This paper deals with further investigations of the relationship between myocardial infarction and adrenal function. To amplify earlier results more detailed data are presented concerning the effects of myocardial necrosis on the steroids produced in vitro by the adrenal glands of virgin rats. The effects of myocardial necrosis were also investigated by using the adrenal glands of breeder rats. The breeder rats had varying degrees of pre-existing arteriosclerosis; the virgin rats were free of arterial disease. Highly significant differences were observed in the adrenal function of these virgin and breeder rats during the onset and repair phases of infarctoid myocardial necrosis. These differences may be related to the complications of pre-existing arteriosclerosis.

Methods

For this study virgin rats and breeder rats were obtained from the Sprague-Dawley Farms, Madison, Wisconsin. The virgin rats had ages comparable to those of breeder rats and were used as controls. The initial mean body weight of virgin males was 420 ± 30 g, and of the virgin females 260 ± 20 g. Two populations of breeder females were used: 1. breeders that had borne and nursed one or two litters, and had microscopic or early gross arteriosclerosis; and 2.
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breeders that had borne and nursed four or five litters, and had advanced gross arteriosclerosis. The initial mean body weights of these female breeders were 290 ± 20 g and 320 ± 20 g, respectively. The male breeders in this study had sired four to five litters, had developed microscopic arterial lesions only, and their initial body weight was 475 ± 30 g. Breeder rats tend to be obese and are 75 to 100 g heavier than virgin rats of the same age.

The grade of myocardial necrosis produced by isoproterenol can be regulated by adjusting the dose according to body weight. Isoproterenol was injected subcutaneously in two equal doses spaced 24 hours apart on the first and second day of the experiment. The virgin animals received 50 mg/100 g body wt/injection. The breeder animals were given 25 mg/100 g body wt. Each of these dosages insured the production of massive myocardial necrosis in virtually 100% of the animals treated. The reduced dose given to breeder rats was the highest dose compatible with an adequate number of survivors. Despite the lower dose given to breeder rats, the size and severity of their infarcts were equal to those induced in virgin rats by twice the dose. Each day for seven days animals from each of the categories described above were sacrificed by decapitation so that studies of adrenal function could be related in time to the onset and sequelae of myocardial necrosis. Comparable groups of untreated controls, e.g., male and female virgins, male breeders and female breeders having had one to two or four to five pregnancies, were sacrificed along with the injected animals each day during the experiment. The treated animals sacrificed on the first two days of the study had received the second injection of isoproterenol four hours prior to sacrifice. The severity of the myocardial necrosis was scored and the adrenal glands were prepared for histological studies by methods described previously. Conditions for preparation and incubation of adrenal tissue for in vitro steroid analyses have been published in detail.

The adrenal glands from two animals were pooled and used for each incubation. In all cases, the glands were stimulated maximally during the incubation by the periodic addition of ACTH. The gas chromatographic method of Kittinger was used for quantitative determinations of aldosterone, corticosterone, 18-hydroxy-deoxycorticosterone, and other steroids present in the incubation fluids.

The specificity of the gas chromatographic method for aldosterone is based on the retention time and other properties of the 17-β-carboxyl-11-β-ol-andros-4-ene-3-one-18-ol-18, 11-hemiacetal-α-ketol derivative of aldosterone. Rat adrenal α-ketolic steroids, when treated with periodate, give rise to a mixture of the corresponding etiocholenic acid derivatives. Gas chromatography of these gives a peak with a retention time identical to that given by the authentic aldosterone derivative. Prior treatment of this mixture of derivatives with acetic anhydride and pyridine causes no diminution of the peak corresponding to the aldosterone derivative or change in its retention time (the authentic derivative of aldosterone cannot form an acetate). The peak corresponding in retention time to the aldosterone derivative could not be removed or diminished by prior extraction of a CH₂Cl₂ solution of the periodate reaction mixture with sodium bicarbonate or 0.1 N NaOH (the authentic derivative of aldosterone forms an internal ester which cannot be extracted from organic solvents by aqueous alkali). Finally, none of the cortical steroids known to be present in rat adrenal incubates give peaks with retention times which correspond to the retention time of the aldosterone derivative. Additional evidence for the specificity, recovery, and reproducibility of the gas chromatographic method has been published.

Results

A. GENERAL OBSERVATIONS

Signs of Shock

The signs of shock due to myocardial damage were not as pronounced in breeder rats with pre-existing arteriosclerosis as in virgin rats free of arterial disease. For example, breeder rats did not display the same degree of dyspnea, prostration or tachycardia, as did virgin rats.

Adrenal Hypertrophy and Thymic Involution

At autopsy, the thymus glands of the arteriosclerotic breeder rats with infarctoid necrosis were severely involuted and the adrenal glands greatly hypertrophied. The adrenal hypertrophy and thymic involution in all animals were most pronounced on the third day when myocardial necrosis became fully established (figs. 1 and 2). During the repair phase (4th-7th day) the adrenal glands decreased in size (figs. 1 and 2) but the thymus remained severely involuted.

*1-(3,4-dihydroxy-phenyl)-2 isopropyl amino ethanol hydrochloride, made available to us by Dr. A. Scribner, Winthrop Laboratories.

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Changes in adrenal weight of male Sprague-Dawley rats during the onset and repair of myocardial infarctoid necrosis (mean and se).

Changes in adrenal weight of female Sprague-Dawley rats during the onset and repair of myocardial infarctoid necrosis (mean and se).

Pulmonary Congestion

One of the striking findings in these animals was the accumulation of great quantities of clear fluid in the thorax during the acute stages of infarctoid necrosis. This hydrothorax increased each day, reaching a peak on the third day in virgin rats and, if the animal survived, receded steadily each day thereafter. The arteriosclerotic breeder rats showed distinctly less accumulation of thoracic fluid than virgin rats. The peak of fluid accumulation in breeder rats occurred on the second day. Concomitant with the rise and fall in volume of thoracic fluid, the blood became first more viscous and then less so, and at corresponding times the intestines seemed to be severely dehydrated and then rehydrated. There appeared to be a massive shift of fluid leading to pulmonary congestion and hydrothorax during the acute onset of myocardial necrosis. During the repair of the myocardium, the hydrothorax receded and normal distribution of fluid appeared to be restored.

B. ADRENAL STEROID PRODUCTION

The data presented in figures 3 and 4 demonstrate that the adrenals of isoproterenol-treated rats undergo profound changes during the development and repair of myocardial damage. The consequences of these changes are reflected in the total quantity, and in the relative proportions, of the several steroids produced by the glands. The adrenals of virgin, as well as breeder rats showed a marked
loss of functional reserve when stimulated by ACTH in vitro.

**Total Steroids**

Total steroid production decreased during the stage of acute myocardial necrosis. In all three groups of female rats the level of the in vitro total adrenal steroid production (µg/hr/100 mg adrenal tissue) reached its lowest level on the second day of the experiment (fig. 4). It should be noted that these animals had been sacrificed four hours after the second and final injection of isoproterenol. It can also be seen that, on the second day, the decrease (from control levels) of total steroid production by the adrenals of the two groups of female rats with gross arteriosclerosis was less than that of the virgin females. It should be emphasized, however, that the control values for arteriosclerotic animals were less at the outset of the experiment. Furthermore, comparison of the absolute levels of total steroid production shows no significant differences between virgin and breeder females on the second day. By the seventh day total steroid production returned to normal at a rate which paralleled the extent of repair of the myocardial lesions.

A two-way analysis of variance of the daily steroid production and extent of arterial pathology revealed highly significant differences (P<0.0005) between the pattern of adrenal response of females with and without gross arteriosclerosis. The changes in total steroid production by virgin male rats and male breeders with microscopic arteriosclerosis followed a time course which was nearly identical to that observed in the virgin females. An analysis of variance showed no significant differences in total steroid production by the adrenal glands of male rats with or without microscopic arteriosclerosis.

**Aldosterone**

The data obtained in these experiments on aldosterone production confirmed the results previously reported for male and female virgin rats. The absolute in vitro production of aldosterone by ACTH-stimulated adrenal glands was significantly elevated in virgin

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Changes of the in vitro production of steroids by adrenal glands of male Sprague-Dawley rats during the onset and repair of myocardial infarctoid necrosis (control values: µg/hr/100 mg).

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animals of both sexes during the second to the sixth day of the experiment (figs. 3 and 4). In male virgins aldosterone production increased to a maximum of approximately four times the control values. This peak of aldosterone production coincided with the time of maximum destruction of myocardial tissue. In the female virgins the corresponding peak increase of aldosterone was two to three times control values. It is noteworthy that the aldosterone production by the adrenals of virgin animals of both sexes had begun to rise sharply by the second day even though total steroid production was considerably below control levels. In arteriosclerotic females, however, aldosterone production lagged and did not rise above control values until the fourth day. Male rats, with microscopic arteriosclerosis only, showed also a delayed rise in aldosterone production. The differences between the maximum values in males are statistically significant (P < 0.01).

Corticosterone and 18-hydroxy-deoxycorticosterone

The in vitro production of corticosterone (compound B) and 18-hydroxy-deoxycorticosterone (18-OH-DOC) followed a pattern of change similar to that seen in the production of total steroids. In general, the production of these substances remained below normal during the periods when total steroid production was also depressed. No significant differences were noted within the groups of male or female rats with respect to corticosterone production (figs. 3 and 4). However, the production of 18-OH-DOC was significantly less in both groups of arteriosclerotic females (P < 0.0005) than in virgin females. Males with microscopic arteriosclerosis also produced less 18-OH-DOC (P < 0.025) than did the virgin males.

C. ADRENAL HISTOLOGY

Virgin Rats Without Arterial Disease

The histological pattern of distribution of lipid in the adrenal glands of male virgin rats was uniform during the acute onset of myocardial necrosis. The zona glomerulosa became depleted of its normally rich complement of lipid and showed only a patchy distribution of lipid during the first five days (fig. 5). At the same time the zona reticularis and inner portions of the zona fasciculata were completely stripped of lipid (fig. 5). By the close of the experiment (sixth to seventh day) all of these zones had become replete with lipid.

In the virgin females the histological
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FIGURE 5

Adrenal cortex of a male virgin rat after two injections of isoproterenol. Myocardial necrosis, signs of congestive heart failure and increasing levels of aldosterone had reached a peak in this animal. The zona glomerulosa (arrows) is depleted of lipid (lipid material appears black: see fig. 6). The z. reticularis and inner portions of the zona fasciculata are also depleted of lipid, indicating active glucocorticoid release. Sudan black B.

changes in the adrenal glands did not develop as promptly as in the male virgins. Four hours after the injection of isoproterenol (day one) all zones of the cortex were filled with lipid. By the second and third day the zona glomerulosa became progressively depleted of lipid and by the fifth day this zone was empty of lipid in practically all of the virgin females. As with male virgins, the adrenal glands of female virgins were refilled with lipid by the sixth and seventh day.

Male Breeder Rats With Microscopic Arteriosclerosis

In male breeders with pre-existing microscopic arteriosclerosis the histophysiological changes in the adrenal glands were different from those observed in virgin rats. On the first day of the myocardial necrosis, the zona glomerulosa was found to be quite narrow and contained only scattered patches of lipid. On the second day, the cortex showed some evidence of hyperplasia but, unlike the findings in virgin rats, all zones began to show restoration of lipid stores. At the height of the development of infarctoid necrosis (third to fourth day) most adrenal glands from this group showed multiple hemorrhages in the zona reticularis with dilatation of the medul-
many foci of cortical hemorrhage, thrombosis and necrosis (fig. 7). By the third day the adrenal medullary sinusoids and central vein of all animals were greatly dilated and remained this way throughout the seven days of observation. In addition, the breeder rats with advanced arteriosclerosis showed intense lipid depletion from the zona glomerulosa on the fourth day coincident with the rise of aldosterone on this same day (fig. 4). In some cases, large nodules of regenerating cortical cells were found just beneath the capsule (fig. 8). By the seventh day the adrenal glands of female breeders with early and advanced arteriosclerosis appeared normal in size and all zones were re-filled with lipid. By this time, female breeders showed little or no gross evidence of their earlier myocardial damage.

Discussion

The marked adrenal hypertrophy and severe thymic involution in animals undergoing myocardial infarctoid necrosis have been consistent findings, indicating an intense activation of the adrenal cortex. Breeder rats characteristically have hyperplastic adrenal glands and involuted thymi in conjunction with arteriosclerosis. The adrenal cortices of the more severely arteriosclerotic animals are depleted of lipid, hemorrhagic, thrombosed and, in some cases, infarcted. It has also been shown by ACTH stimulation that the steroidogenic capacity of these glands is partially exhausted. Therefore, it was noteworthy that the adrenal glands of arteriosclerotic breeder rats were still capable of a dynamic increase in size, and the thymus glands of even further involution, when challenged by the stress of catecholamine-induced myocardial necrosis (figs. 1 and 2).

The development of pulmonary congestion and large quantities of fluid in the thorax is similar to that observed in congestive heart failure which develops in man, particularly after severe myocardial infarction. It is not clear whether the hyperaldosteronism observed in congestive heart failure is operative during all stages of heart failure or whether it contributes to the edema and electrolyte changes late in the syndrome. In the experimental model of infarctoid necrosis and pulmonary edema, as described here, the loss of vascular fluid preceded the in vitro increase of aldosterone production. Further, the amount of vascular fluid loss and the associat-
ed aldosterone production appeared to be related. The in vitro adrenal studies show that there was a fall in aldosterone production on the first day when transudate appeared in the thorax, followed by a sharp rise in aldosterone thereafter (figs. 3 and 4). Thus the loss of vascular fluid preceded the changes of aldosterone production in the virgin rats. Further, the male breeders with microscopic arteriosclerosis showed a prompt appearance of fluid transudate into the thorax on the first day accompanied by a decrease in aldosterone on the same day. This was followed by a less pronounced rise in aldosterone (fig. 3) compared to virgin rats. In female breeders with gross arteriosclerosis there was no increase in aldosterone production until the fourth day despite marked fluid accumulation in the thorax. When the extent of myocardial necrosis had reached a peak (third day in virgins, fourth day in breeders) and myocardial repair commenced, the extravasated fluid in the thorax began to recede. As rehydration proceeded in the surviving animals, the aldosterone levels returned to normal. Garagnani et al.\textsuperscript{13} have shown a similar pattern of aldosterone activity in human patients following myocardial infarction, i.e., a drop in aldosteronuria 24 hours after onset of infarction, an increase on the fourth day and a return to normal by the twentieth day.

The drastic decrease in total adrenal steroid production during the acute onset of myocardial necrosis (figs. 3 and 4) is interpreted as an acute loss of functional adrenal reserve. This is accompanied by an apparent shunting of biosynthetic pathways toward the production of more vital adrenal steroids such as aldosterone. It should be noted that the depression of total steroid production is most prolonged (four days) in male breeders (fig. 3) and it is the male breeder which shows the most prolonged duration of the myocardial necrosis process. The lower initial levels of total steroids in breeder rats is in accord with our reported observations that arteriosclerosis and decreased adrenal function are correlated.\textsuperscript{7,8} The prompt restoration of total steroid production to normal levels in the latter portion of the experiment indicates that once myocardial necrosis has reached a zenith and repair has begun, the acute need for adrenal steroids is relieved.

Despite the reduction in total steroids, the adrenal glands of virgin rats (which are free of arterial disease) and male breeders (with microscopic arteriosclerosis only) showed a promptly increased production of aldosterone after the first day's lag. The prolonged delay in aldosterone production observed in female breeders with gross arteriosclerosis indicates that there is probably a rate limiting process in the steps involved in aldosterone synthesis in their adrenal glands. This impairment must be temporary since aldosterone production eventually increases fourfold. It is interesting that maximal aldosterone production does not occur until the sixth day in animals with advanced arteriosclerosis. It is noteworthy that independent in vitro experiments have shown that equivalent increases in aldosterone production occur during active necrosis even in the absence of ACTH stimulation. Aldosterone production usually appears very promptly in response to certain alterations in fluid and electrolyte imbalance.\textsuperscript{12} In a related sense, Davis et al.\textsuperscript{14} have shown that significant increases in aldosterone appeared in dogs within 30 minutes after elevations of venous pressure that led to extravasation of fluid and electrolyte. In view of the vital electrolyte-regulating effects of aldosterone and the finding that aldosterone rises so strikingly during the acute stages of the infarctoid necrosis, and particularly because it does so in male rats which show the highest mortality (20\%) and poorest capacity for myocardial repair, we wonder whether the amount of aldosterone produced in these animals was helpful or deleterious to survival. Sanders and Melby\textsuperscript{15} found that hyperaldosteronism may precipitate or intensify edema and pulmonary congestion in patients with congestive heart failure.

The changes in adrenal histology correlate well with our in vitro estimations of adrenal function. For example, in keeping with the prompt increased production of aldosterone

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in virgin males (fig. 3) the zona glomerulosa in their adrenal cortices was strikingly depleted of lipid (fig. 5) four hours after beginning myocardial necrosis. The zona glomerulosa is generally considered to be the site of mineralocorticoid production. Lipid depletion from this zone is interpreted to indicate active secretion of mineralocorticoids.16 The lipid depletion from the zona reticularis and inner portions of the zona fasciculata in male and female virgin rats during active myocardial necrosis indicates active glucocorticoid release. The inner zones of the adrenal cortex are the sites for synthesis of the glucocorticoid adrenal steroids.16 These latter adrenal histological changes correlate well with the changes of total steroid production and of compound B.

The male breeders developed the most severe myocardial necrosis and displayed evidence of poor myocardial repair. Histological changes in their adrenal glands showed that on the first day the zona glomerulosa was narrow and contained patches of lipid, indicating less active release of mineralocorticoids as was observed in the case of male virgin rats (fig. 5). With recovery from infarctoid necrosis the zona glomerulosa of male breeders contained an unusually rich concentration of lipid (fig. 6), indicating active storage of steroid precursors in this zone. These histological changes correlate with the less active and different pattern of aldosterone production by male breeders in the acute stages of infarctoid necrosis as compared to male virgins. The hemorrhage in both the inner cortex and medulla, the dilatation of medullary sinusoids, and the distention of the main central vein draining the adrenal, occurring at the height of myocardial necrosis (fourth day) all indicate the severe stimulatory demands made on the adrenal glands at this time.

It is interesting that arteriosclerotic female breeder rats consistently show lipid depletion from the zona glomerulosa8 and, concomitantly, show decreased ability to produce additional aldosterone in vitro after stimulation by ACTH.5 During the active stages of myocardial necrosis the in vitro chemical analyses indicated very little or no increased production of aldosterone by arteriosclerotic female breeders (fig. 4). The zona glomerulosa, although narrow, contained demonstrable quantities of lipid, indicating availability of substrate but absence of production of aldosterone. However, coincident with the eventual rise in aldosterone production during myocardial repair in arteriosclerotic females (fig. 4), the zona glomerulosa showed active lipid depletion, i.e., active aldosterone production. At the same time, as the chemical analyses demonstrated restoration of normal total steroid production during myocardial repair (fig. 4), histological evidence indicated that the inner zones of the cortex recovered their normal lipid complement.

Summary

The subcutaneous injection of the potent catecholamine, isoproterenol, on two consecutive days causes active and massive myocardial necrosis in rats. Infarctoid myocardial necrosis was produced in male and female virgin rats free of arterial disease, in male breeder rats which have microscopic arteriosclerosis only, and in female breeder rats having early or advanced gross arteriosclerosis. During the active development of myocardial necrosis and heart failure, an apparent shift in distribution of body fluid produced systemic dehydration, pulmonary edema, and hydrothorax. Animals which survived the myocardial destruction showed gradual reduction of the congestive heart failure and beginning myocardial repair. Virgin animals with no pre-existing arterial disease developed more severe signs of shock and congestive heart failure than breeder rats with pre-existing arteriosclerosis.

In vitro estimation of the adrenal steroids produced by the animals with myocardial necrosis indicated intense in vivo stimulation of adrenocorticoid production coincident with the increasing severity of the myocardial necrosis. Steroid production returned to normal during progressive myocardial repair. Virgin rats showed a very prompt adrenal response.
to the stress of myocardial destruction but breeder rats, which also have pre-existing reduced adrenal function, showed a lag in the response of their adrenal glands. Chemical and histological analyses of the adrenal glands indicated a pronounced increase of aldosterone production during the experiment, this increase being associated with loss of fluid and electrolytes from the vascular space. The production of aldosterone appears to be vital to the physiological adjustments needed by these animals during heart failure because the production of aldosterone seems to occur at the expense of the other adrenal steroids, e.g., corticosterone, 18-hydroxy-deoxycorticosterone and total steroid. These comparative studies suggest that adrenal responsiveness during the stress of myocardial infarctoid necrosis is quantitatively different in animals free of previous arterial disease from that found in animals with pre-existing microscopic or grossly visible arteriosclerosis. The specific kind and the amount of adrenal steroid produced during myocardial destruction may be of vital importance for survival.

References
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