Basic Res Cardiol 94: 1 – 8 (1999)

ORIGINAL CONTRIBUTION

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The influence of maturation and gender on the anti-arrhythmic effect of ischaemic preconditioning in rats

Received: 8 April 1998
Returned for revision: 2 June 1998
Revision received: 9 July 1998
Accepted: 21 July 1998

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Abstract The aim of this study was to investigate the influence of maturation and gender on the anti-arrhythmic effect of myocardial ischaemic preconditioning in rats. Coronary artery occlusion was carried out in either rats anaesthetised with sodium pentobarbitone or in rat isolated hearts. Cardiac arrhythmias occurring in the 30 min post-occlusion period were assessed. In anaesthetised 3 month (m) old male rats ischaemic preconditioning, with a 3 min temporary coronary artery occlusion, significantly reduced the total number of ventricular ectopic beats (VEBs) from 2074 ± 206 to 490 ± 139 and the incidence of ventricular fibrillation (VF) from 40 to 0 % during a subsequent 30 min occlusion ($P < 0.05$). In middle-aged male rats (16 m) the anti-arrhythmic effect of preconditioning was unaltered (VEBs were reduced from 1958 ± 121 to 245 ± 66 and VF from 70 to 0 %). In 3 m old anaesthetised female rats the effect of ischaemic preconditioning was also evident (VEBs reduced from 961 ± 170 to 154 ± 48; $P < 0.05$). In non-preconditioned age-matched female animals the total number of VEBs (961 ± 170), VF (0%) and mortality (0 %) were significantly ($P < 0.05$) lower than in respective male animals. In female rats, attenuation of ischaemia-induced arrhythmic severity was most pronounced in the oestrus state. In hearts isolated from weight-matched male and female rats the incidence of ventricular tachycardia (81 vs 25 %) and the total number of VEBs (351 ± 73 vs 81 ± 50) were significantly ($P < 0.05$) different. It is concluded that in rats neither maturation nor gender influence the anti-arrhythmic effect of ischaemic preconditioning. However, female rats exhibit a lower level of arrhythmic activity during sustained coronary artery occlusion than male rats both *in vivo* and *in vitro*.

Key words Ischaemic preconditioning – rat – age – gender – arrhythmias – ischaemia

Introduction

Myocardial ischaemic preconditioning is the protective adaptive mechanism produced by exposing the myocardium to short periods of ischaemia. This protection increases the tolerance of the myocardium to subsequent longer periods of ischaemia, hence, reducing the severity of the resultant ischaemic changes. This endogenous protection is manifested three ways, limitation of infarct size (15), enhanced recovery of contractile function following a period of ischaemia and reperfusion (5) and suppression of ischaemia and reperfusion-induced ventricular arrhythmias (17).

The majority of previous studies investigating preconditioning have been carried out in young healthy male animals

Basic Research in Cardiology, Vol. 94, No. 1 (1999)

and the possible influence of factors such as age and hormonal status has been ignored, yet these factors are important because they profoundly influence cardiac function. In this respect, perhaps the most significant alteration with age is the increased susceptibility of the myocardium to arrhythmias. This has been demonstrated in the Framingham heart study on human subjects where the incidence of cardiac arrhythmias increased progressively with age (13). Various animal studies have also demonstrated increased susceptibility to arrhythmias with advancing age (4, 9, 16). It is also well established that cardiovascular mortality due to the development of fatal arrhythmias is lower among premenopausal women than among similarily aged men (10) and that after the loss of ovarian function the risk of sudden cardiac death rises markedly (8). This gender difference has frequently been attributed to the cardioprotective effects of ovarian hormones. Indeed, oestrogen therapy can reduce postmenopausal mortality resulting from cardiovascular disease by up to 50 % (24). In addition to its effects on lipids and lipoproteins (3) oestrogen has been shown to have a protective effect on the myocardium by reducing ST segment depression, a marker of ischaemia, in patients suffering from exercise induced angina (20). Interestingly, previous studies have demonstrated that the severity of ischaemia-induced arrhythmias resulting from coronary artery ligation in rats is significantly lower in females than in males (6, 28) but whether this gender difference is due to hormonal influences remains to be investigated.

Given these influences of age and gender it is possible that the anti-arrhythmic effect of preconditioning may be modified by these factors. Thus, the main aim of this study was to investigate, in an anaesthetised rat model of coronary artery occlusion, whether maturation or gender influence the ability of preconditioning to attenuate ischaemia-induced arrhythmias. The factors which influence the effect of gender on the severity of ischaemia-induced arrhythmias were also investigated both in anaesthetised rats and in rat isolated perfused hearts.

Methods

Study design

Four groups of sprague-Dawley rats were studied. Three month old males (300–350 g, n = 68); sixteen month old male rats (800–1300 g, n = 20), three month old females (200–270 g, n = 22) and females weight-matched (300–350 g, n = 28) to the three month old male rats. Each group under investigation was randomised with the appropriate control group. Throughout the study time matched controls (i.e., 3 month old males) were performed. The experiments were performed in accordance with the Animal Scientific Procedures Act, 1986.

Surgical procedures for the in vivo studies

Rats were anaesthetised with sodium pentobarbitone 60 mg/kg administered intraperitoneally. The left jugular vein was cannulated for the administration of any additional anaesthetic required and the trachea for artificial respiration. Systemic arterial blood pressure was monitored from the left carotid artery with a Statham P231D transducer. A standard lead 1 electrocardiogram was recorded, together with arterial blood pressure, on a Linton Graphtec Linearcorder WR 3310. A precalibrated steel thermistor probe was inserted into the rectum to measure core temperature which was maintained at 37–38 °C with the aid of a heating lamp.

A longitudinal skin incision was made to the left of the midline and the muscle layers covering the chest opened by blunt dissection. The chest was opened using a left thoracotomy performed between the fourth and fifth ribs approximately 3 mm from the sternum. Artificial respiration with room air was immediately initiated using a respirator (C.F. Palmer, UK, 48 strokes/min, 1.5 ml/100 g animal weight). The two ribs above the thoracotomy were sectioned and the pericardium incised to allow access to the heart. A 6–0 braided silk suture attached to a 10 mm micropoint reverse cutting needle was then placed under the left main coronary artery as previously described (6). The ends of the ligature were threaded through a length of polyethylene tubing. Any animal in which this procedure produced arrhythmias or a sustained fall in blood pressure to less than 70 mmHg was discarded.

Rat isolated perfused hearts

Male and female weight-matched Sprague Dawley rats were anaesthetised as described above then given 500 U heparin intravenously into the tail vein. A thoracotomy was performed to enable complete exposure of the heart. A suture was loosely placed under the left main coronary artery as described above. Hearts were then rapidly removed and transferred to a Langendorff apparatus where retrograde perfusion at a constant flow 10 ml min^{-1} was initiated. The perfusate was a modified Krebs-Henseleit solution containing (in mmol l^{-1}): NaCl 118, KCl 3.2, $CaCl_2$ 2.52, $MgSO_4$ 1.66, $NaHCO_3$ 26.88, KH_2PO_4 1.18, glucose 5.55 and sodium pyruvate 2.0. The solution was gased with 95 % O_2/5 % CO_2, pH maintained at 7.4 and filtered and heated to 37 °C. Fine platinum subdermal electrodes (Grass type E2) were placed on the right atrium and apex of the left ventricle to enable the ECG to be recorded. The ends of the suture were threaded loosley through a segment of plastic tubing (15 mm) forming a snare.

Measurements

In the *in vivo* studies an on-line data analysis system (John Dempster, University of Strathclyde) recorded systolic, diastolic, mean arterial blood pressure and heart rate. In the isolated hearts, coronary perfusion presence and heart rate were recorded continuously on a Grass model 79 pen recorder. Ventricular arrhythmias were analysed under the guidelines of the Lambeth Conventions for the analysis of experimental arrhythmias (28). The total number of ventricular ectopic beats (VEBs) was calculated as the sum of the individual arrhythmias over the 30 min period of permanent occlusion only in animals (or isolated hearts) that did not experience irreversible VF. The incidence of ventricular tachycardia, defined as a run of four or more ventricular ectopic beats at a rate faster than resting sinus rhythmn, and the incidence of reversible and irreversible VF and mortality where appropriate was recorded.

The area at risk was measured by the administration of Evans blue dye (1 % and 0.1 % *in vivo* and *in vitro*, respectively) intravenously or to the perfusate at the end of the experimental protocol. The stained hearts were then removed and frozen. Once frozen the hearts were weighed then sliced enabling the unstained (area at risk) to be separated from the stained tissue. The area at risk was then calculated as a percentage of the whole heart weight.

To determine if left ventricular hypertrophy had developed in middle-aged animals, the hearts from 3 and 16 month old male rats were removed at the end of the experimental protocol. The right ventricular free wall and the left ventricle plus septum were separated, dried and weighed individually.

Experimental protocols

Animals or hearts were allowed to stabilise for 15 min after placing the ligature. In control (non-preconditioned) animals the ligature around the left coronary artery was tightened to produce myocardial ischaemia for a 30 min period. In preconditioned animals a temporary occlusion of the coronary artery was achieved by placing the snare firmly against the wall of the heart and securing the threads of the ligature. This temporary preconditioning occlusion was held for 3 min. The artery was then reperfused for 10 min before the 30 min permanent occlusion.

Evaluation of vaginal smears

In all female animals a vaginal smear was taken during the 15 min stabilisation period. A cotton swab was dipped into sterile saline before being carefully inserted into the vagina using gentle rotation. The vaginal smear slides were then evaluated under a microscope using x10 eyepiece and x40 objective. Cells present were identified as cornified (flat and keratinized) or non-cornified and the presence of leucocytes and stringy mucous, often described as mucous ferning, noted. The prescence of many cornified cells and the absence of leucocytes indicated the animal to be in oestrous. The presence of leucocytes and mucous ferning indicated non-oestrus (2).

Statistical analysis

Values are expressed as means ± SEM. Differences between means were compared by applying a two-tailed unpaired Students's t-test. Incidences of ventricular tachycardia and ventricular fibrillation were compared using Fisher-Irwin test. $P < 0.05$ was considered to be statistically significant; n = number of animals or hearts used.

Results

Effect of preconditioning in 3 and 16 month old male rats in vivo

Figs. 1 and 2 compare the number of ectopic beats and the incidence of VF respectively in 3 and 16 month old male rats. Preconditioning in 3 month old male rats resulted in a marked reduction in arrhythmia severity compared to controls; the total number of VEBs was significantly reduced (Fig. 1), VT

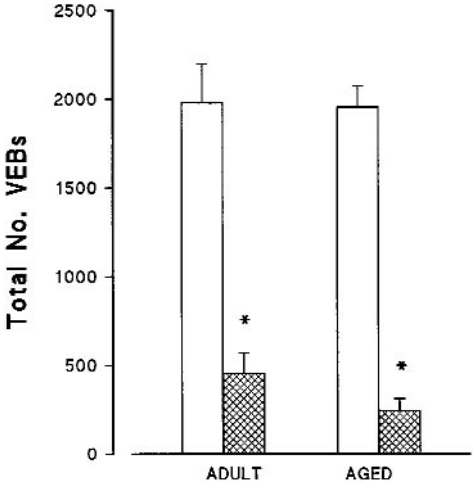

Fig. 1 The number of ventricular ectopic beats (VEBs) over a 30 min period of coronary artery occlusion in anaesthetised control (open column) and preconditioned (shaded column) male rats aged 3 (adult) or 16 month (aged). * $P < 0.05$ indicates significantly different from appropriate non-preconditioned control.

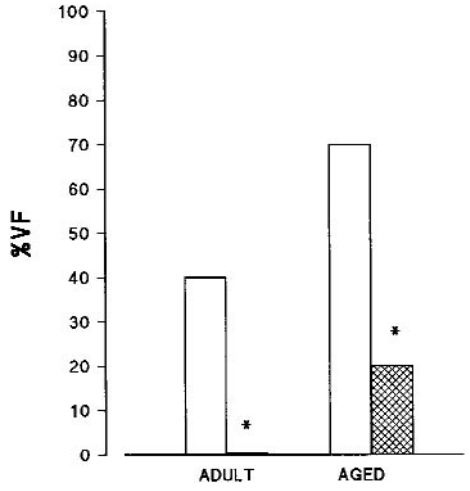

Fig. 2 The incidence of VF which occurred during a 30 min coronary artery occlusion in anaesthetised control (open column) and preconditioned (shaded column) male rats aged 3 (adult) or 16 month (aged). * $P < 0.05$ indicates significantly different from appropriate non-preconditioned control; n = 10 in all groups.

number was reduced from 1743 "250 in control to 351" 98 in preconditioned animals and VF was abolished (Fig. 2). Preconditioning also significantly ($P < 0.05$) delayed the onset of arrhythmias from 4.9 ± 1 to 7.5 ± 1 min post occlusion. Preconditioning in aged (16 month) male rats also resulted in a significant reduction in the occurrence of ectopic beats (Fig. 1), VT number (reduced from 1727 ± 155 in control to 134 ± 51 in preconditioned rats) and the incidence of VF (Fig. 2). The onset of arrhythmic activity was signifigantly delayed in 16 month rats from 2 ± 1 min in control to 5 ± 1 min in preconditioned animals. The protective effect of preconditioning on these arrhythmic indices was thus similar in middle-aged and 3 month old animals. Although the incidence of VF tended to be greater in 16 month compared with 3 month old rats, this difference did not reach statistical significance. Mortality in middle-aged controls was 60 % compared to 40 % in 3 month old males and preconditioning reduced this to 20 % and 0 % ($P < 0.05$), respectively.

Haemodynamic measurements in preconditioned 3 and 16 month old male rats

Prior to coronary artery occlusion mean arterial blood pressures were similar in 3 (118 ± 10 mmHg) and 16 (112 ± 10 mmHg) month old rats and did not alter significantly between groups throughout the experimental protocol. Heart rate before coronary artery occlusion was 312 ± 6 and 339 ± 6 beats min^{-1} in 3 and 16 month old rats respectively. Neither the temporary nor the permanent coronary occlusion significantly modified heart rate in either experimental group.

Effect of age on heart weight/body weight ratios

The 3 and 16 month old rats weighed 328 ± 8 and 958 ± 36 g respectively. The right ventricular to body weight ratio was the same in 3 and 16 month old male rats being 0.12 ± 0.01 and 0.12 ± 0.02 mg g^{-1} respectively. However, the left ventricular to body weight ratio was less (0.60 ± 0.05 mg g^{-1}) in the middle-aged than the 3 month old rats (0.77 ± 0.04 mg g^{-1} $P < 0.05$).

Effect of hormonal status on the severity of ischaemia-induced arrhythmias in control and preconditioned age-matched female rats

In female animals preconditioning also significantly reduced the total number of VEBs (from 961 ± 170 to 154 ± 48; (Fig. 3) and the VT number from 796 ± 158 in control to 76 ± 35 in preconditioned animals. The onset of arrhythmias was later in preconditioned (8 ± 1 min) than in control females (6 ± 1 min) but this was not statistically significant. Interestingly, control females exhibited significantly fewer VEBs than age-matched control males. It was not possible to determine the effect of preconditioning on VF in the female rats as none of the control animals exhibited irreversible VF during the 30 min period of coronary artery occlusion. This is in contrast to the effects of coronary artery occlusion in male rats (Table 1 and Fig. 2).

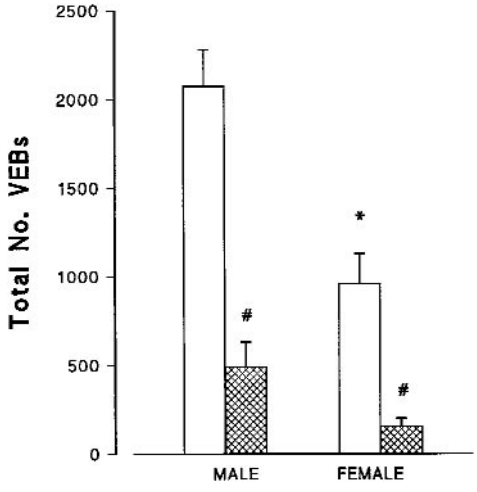

Fig. 3 The number of ventricular ectopic beats (VEBs) over a 30 min period of coronary artery occlusion in anaesthetised control (open column) and preconditioned (shaded column) male and female rats. # significantly different ($P < 0.05$) from non-preconditioned control. * P significantly different ($P < 0.05$) from male controls.

Table 1 The effect of hormonal status on the severity of arrhythmias during a 30 min coronary artery occlusion in anaesthetised male and female rats

	n	Total VEB	VT number	VT % incidence	% VF irreversible
Male 3 months old	24	1526 ± 229	1447 ± 231	100	46
Female age-matched oestrus	8	591 ± 44*	450 ± 46*	100	0*
Female age-matched non-oestrus	6	1455 ± 296	1256 ± 271	100	0*
Female weight-matched oestrus	7	895 ± 198*	777 ± 185*	100	0*
Female weight-matched non-oestrus	11	1007 ± 273	798 ± 252	100	27

n = number of rats. The number of ectopic beats was counted only in the animals that survived the 30 min period of occlusion. VT = ventricular tachycardia; VF = ventricular fibrillation; VEB = ventricular ectopic beats; * $P < 0.05$ indicates significant difference from male control

Haemodynamic changes in male and female age-matched rats subjected to coronary artery occlusion and to preconditioning

Fig. 4 shows the mean arterial blood pressure response of age-matched male and female animals to the preconditioning protocol. Prior to coronary artery occlusion mean arterial blood pressures were similar in male and female animals (116 ± 5 and 120 ± 6 mmHg, respectively). Both groups exhibited a statistically significant fall in mean arterial blood pressure during the temporary preconditioning occlusion which recovered slightly upon reperfusion (Fig. 4). Mean arterial blood pressure fell again following the prolonged occlusion and then recovered steadily to pre-ligation levels. Heart rate before coronary artery occlusion was 310 ± 8 and 314 ± 9 beats min^{-1} in male and female animals, respectively. Neither the temporary nor the permanent occlusion significantly modified heart rate in either group.

Arrhythmia severity in female rats during oestrus and non-oestrus

The severity of arrhythmias occurring during the coronary artery occlusion period in non-preconditioned 3 month old male rats was compared with that in both age and weight matched females in oestrus and non-oestrus (Table 1). It is clear that ectopic activity was significantly ($P < 0.05$) reduced in age-matched oestrus females (591 ± 44 beats), compared to female rats in non-oestrus (1455 ± 296 beats) or males (1526 ± 229 beats). When female animals were weight-matched with males then those animals in oestrus (895 ±198) still exhibited significantly lower levels of ectopic beat activity than males. The number of ectopic beats which occurred as VT was also significantly reduced in female age-matched (450 ± 46) and weight-matched (777 ± 185) animals in oestrus compared to

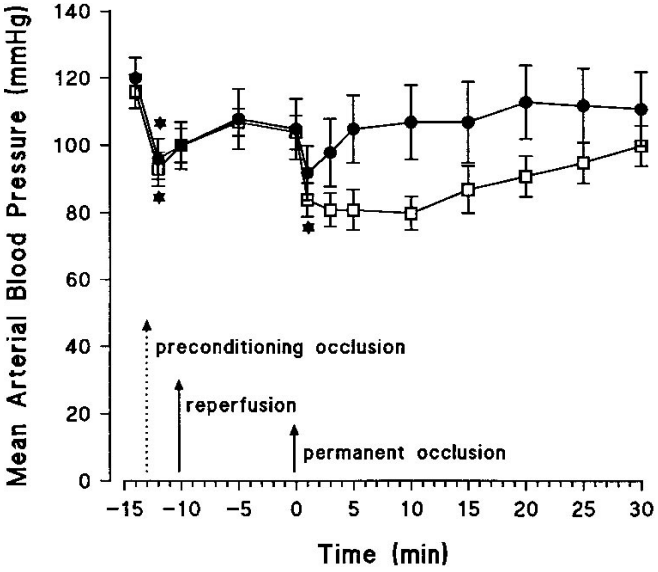

Fig. 4 Mean arterial blood pressure (MABP, mmHg) before and during coronary artery occlusion in preconditioned male (open squares) and female (closed circles) rats. * $P < 0.05$ significantly different from appropriate pre-occlusion value. In females, pressure is significantly higher than males at 3, 5, 10, 15 and 20 min post-occlusion.

males (1447 ± 231). There was no irreversible VF or mortality in females in oestrus compared with a 46 % incidence ($P < 0.05$) in male animals. These differences were not accompanied by any significant differences in either mean arterial blood pressure or heart rate in females in oestrus or non-oestrus.

Ischaemia-induced arrhythmias in hearts isolated from weight-matched male and female rats

In hearts isolated from female rats both the total number of VEBs (Fig. 5) and the percentage incidence of VT (Fig. 5) were significantly lower than that of hearts from male rats. The percentage incidence of VF was low, occurring in 19 and 0 % of hearts from male and female animals, respectively. Heart rate was not significantly different between male (225 ± 7 beats min^{-1}) and female (222 ± 6 beats min^{-1}) rats, and did not alter significantly from preligation values throughout the experimental protocol. Perfusion pressure was also not significantly different between male (38 ± 3 mmHg) and female (40 ± 4 mmHg) groups before the onset of the experimental protocols. Perfusion pressure increased significantly following coronary artery occlusion in both male and female groups by approximately 20 mmHg.

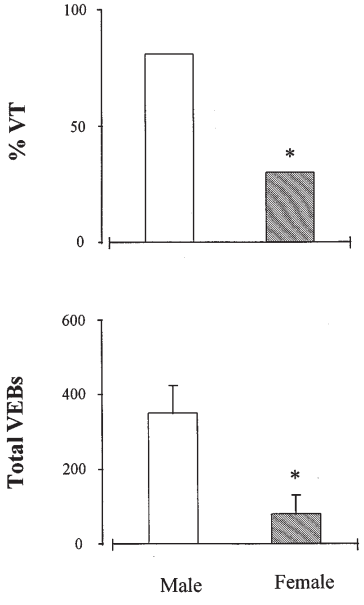

Fig. 5 The total number of ventricular ectopic beats (VEBs) and percentage incidence of VT during a 30 min period of coronary artery occlusion in hearts isolated from male (n = 16) and female (n = 10) rats. * $P < 0.05$ indicates significantly different from male control.

Evaluation of area at risk

In the age-matched studies, the body weight of females (230 ± 15 g) was significantly less than that of males (310 ± 22 g). The area of the myocardium at risk following myocardial ischaemia, expressed as a percentage of the whole heart weight, was, however, similar, i.e., 27 ± 1 % and 30 ± 3 % for male and female animals, respectively. In the weight-matched study, heart sizes were similar in male (1.15 ± 0.05 g) and female (1.13 ± 0.08 g) rats. There was no significant difference in the area of the myocardium at risk expressed as a percentage of whole heart weight in male (27 ± 4 %) and female (29 ± 5 %) weight matched hearts.

Discussion

In this study, preconditioning in anaesthetised 3 month old male rats had a pronounced antiarrhythmic effect (reduced number of ectopic beats, VT number and incidence of VF). This is in accord with previous studies in this species (12, 27). In 16 month old male animals the antiarrhythmic effect of preconditioning activity was similar to that in young (3 month old) rats. A recent study using rat isolated hearts reported that the ability of preconditioning to attenuate ischaemia-reperfusion induced arrhythmias is lost by the age of 24 month (1). In this study preconditioning failed both to suppress early ischaemia-reperfusion induced arrhythmias or improve the recovery of contractile function form a period of ischaemia and reperfusion. If the present results and those of Abete et al. (1) are considered together, it could be suggested that the antiarrhythmic action of preconditioning in the rat is lost between 16 and 24 months of age. In our study 16 month old control animals did not exhibit enhanced arrhythmic activity during sustained coronary artery occlusion compared to 3 month old control animals. It has been shown previously that at the age of 24 months there is an *increased* incidence of reperfusion and reoxygenation-induced arrhythmias (16). This suggests that at 16 month the rats were insufficiently aged to exhibit enhanced arrhythmic activity in response to coronary artery occlusion. This is in agreement with a previous study which also failed to show increased arrhythmia severity before the age of 24 months (9). It has been suggested that many of the changes observed within the cardiovascular system with ageing are due to the concomitant development of myocardial hypertrophy (29). The absence of hypertrophy in the 16 month old animals used in the present study may explain the failure to observe an increased susceptibility to ischaemia-induced arrhythmias. The results of the present study demonstrate that the ability of ischaemic preconditioning to protect the heart from ischaemia-induced arrhythmias is still evident in middle-aged rats.

Gender did not influence the anti-arrhythmic effect of a 3 min temporary coronary artery occlusion on ectopic activity. In contrast to their male counterparts, female rats did not exhibit irreversible VF; thus, it was not possible to elucidate whether or not preconditioning had any effect on this serious arrhythmia or on mortality in female animals. However, these results do show for the first time that female rats can be protected from VT and less severe forms of ischaemia-induced arrhythmias by preconditioning.

The significantly lower arrhythmic activity observed in female control animals is in agreement with previous studies which have also shown a reduction in ischaemia-induced arrhythmic activity in female compared to male animals (21, 14). In contrast to these findings, there is a report in dogs that the incidence of lethal ventricular fibrillation is not influenced by gender (18). The dog study, however, was a retrospective analysis of control male and female dogs in which infarct size determination was the main end-point and in which animals had been administered lidocaine prophylactically just prior to coronary artery occlusion. Our study was a prospective one which focussed specifically upon arrhythmogenesis and in which experiments in male and female animals were both randomised and time-matched. In our study, since the rats were initially age-matched it could have been argued that the smaller body weight, and hence heart size, of the female animals could have been responsible for the reduced ischaemia-induced arrhythmic activity, especially as the arrhythmic activity is directly proportional to body weight, with larger animals exhibiting more pronounced arrhythmic activity (25). We addressed this possibility by weight matching male and female animals and also by carrying out experiments in isolated hearts. In both rat hearts *in vivo* and isolated perfused hearts, when matched by weight, the severity of ischaemia-induced arrhythmias was still significantly lower in female than in male animals, suggesting that the lower arrhythmic activity in age-matched female animals was not simply a result of the smaller heart size.

In anaesthetised female rats the attenuation in arrhythmic activity observed in both weight and age-matched animals was most pronounced during the oestrus state which, in the rat, is preceded by peak plasma oestrogen levels (26) suggesting that hormonal factors could be responsible. Certainly, oestrogen has been shown to influence the cardiovascular system in many ways and a cardioprotective role has been suggested (20). Oestrogen has been shown to alter vascular tone and produce vasodilation in coronary arteries (20), to induce negative cardiac inotropic effects (19, 22) and to reduce ST-segment depression in postmenopausal women suffering from exercise induced angina (20). There is evidence, recently reviewed (7), to suggest that these actions of oestrogen may be attributed to a calcium channel blocking effect. Interestingly, a recent study in rat isolated hearts has shown that 17 beta-oestradiol administration improves contractile function following global ischaemia and reperfusion (11). Since this cardioiprotective effect of 17 beta-oestradiol was independent of a significant improvement in coronary flow a direct effect of the hormone on the cardiac myocyte was postulated. In anaesthetised rats 17 beta-oestradiol administration was recently found to reduce myocardial necrosis following regional ischaemia and reperfusion of the heart (23).

Of interest in the present study was the observation that in rat isolated hearts, where the influence of circulating hormones is removed, attenuated arrhythmic activity was still present in female compared to male hearts. This suggests either that circulating oestrogen is not responsible for the lower arrhythmic activity in female hearts or that the anti-arrhythmic action of this hormone is maintained for some time after its removal. Further study to investigate the possible mechanisms involved in the protective action of oestrogen upon the ischaemic heart is necessary.

In conclusion, we show that maturation and gender do not influence the antiarrhythmic effect of myocardial ischaemic preconditioning in anaesthetised rats. However, female animals do exhibit a lower level of arrhythmic activity during sustained coronary artery occlusion compared to males. This attenuation in ischaemia-induced arrhythmias is most pronounced during the oestrus state indicating the possible importance of hormonal influences.

▶

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