The effect of prolonged administration of propranolol and timolol on the growth and the heart of growing rabbits

K. L. EVEMY, P. CUMMINS AND W. A. LITTLER

British Heart Foundation Department of Cardiovascular Medicine, Clinical Research Block, Queen Elizabeth Hospital, University of Birmingham, Birmingham, U.K.

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Summary

1. The effect of prolonged administration of β-adrenoreceptor-blocking agents was studied in growing rabbits. Equivalent doses of propranolol and timolol were given parenterally for 8 weeks.

2. Animals given propranolol had a significantly lower heart rate than that in the controls. After subcutaneous injection, plasma propranolol levels were twice those obtained after intraperitoneal injection.

3. Growth rate of animals given propranolol intraperitoneally was significantly less than controls, whereas animals given propranolol subcutaneously or timolol intraperitoneally grew normally. Reduced growth rate was associated with reduced food intake and was not related to the β-adrenoreceptor-blocking activity of the drugs.

4. At the end of the experiment the heart and other organs were examined. β-Adrenoreceptor blockade produced no significant changes in ventricular size or water content, and no significant changes in ultrastructure were found.

Key words: β-adrenoreceptor, heart, propranolol, timolol.

Introduction

β-Adrenoreceptor-blocking agents now have an established place in the long-term management of a variety of cardiovascular disorders. Despite widespread use of these agents for well over a decade, the effects of their prolonged administration in therapeutic doses have only recently been studied in normal animals. Administration of propranolol and practolol to young rabbits for 6 weeks has been shown to cause changes in myocardial structure and growth rate (Vaughan Williams, Raine, Cabrera & Whyte, 1975). A reduction in ventricular weight was accompanied by an increase in ventricular water content. These changes in gross structure were subsequently associated with changes at the ultrastructural level (Vaughan Williams, Tasgal & Raine, 1977). The relative volume of mitochondria was reduced and was replaced by an equivalent increase in sarcoplasm, while relative volume of vascular elements was increased and interfibrillar volume decreased. These changes in the heart were not dependent on detectable levels of drug in the circulation, since administration was discontinued 24 h before examination. In addition, a significant reduction in growth rate occurred after 4 weeks of administration.

The antihypertensive effect of β-adrenoreceptor antagonists has been shown to persist long after administration of the drug has ceased (Zacharias & Cowen, 1970; Wilson, Morgan & Morgan, 1976). Propranolol has also been shown to have a protective effect on hypoxic myocardium up to 72 h after administration (Nayler, Yepez, Fassold & Ferrari, 1978). Such effects, which are not directly dependent on detectable serum drug levels, may be related to the changes in myocardial structure observed in rabbits. It has been suggested that these effects of β-adrenoreceptor antagonists on the myocardium may be of relevance in considering the efficacy of such agents in angina pectoris. Changes in relationship between capillary and muscle cell wall offering a shorter pathway for the diffusion of oxygen, and changes in mitochondrial volume leading to
reduced oxygen consumption, could explain the beneficial effect of prolonged β-adrenoreceptor blockade in myocardial ischaemia (Vaughan Williams et al., 1977). A reduced rate of growth of the heart in relation to body weight during long-term β-adrenoreceptor blockade may also be of clinical relevance in the treatment of hypertrophic obstructive cardiomyopathy in infants (Vaughan Williams et al., 1977).

These effects on the heart and on growth were seen in rabbits weighing less than 1 kg (about 3–4 weeks old) when the experiment commenced. The sympathetic nervous system of the rabbit is not fully developed until at least the age of 4 weeks (Friedman, Poole, Jacobowitz, Seagren & Braunwald, 1968). Changes in the heart have therefore been seen at an age when the heart has been under sympathetic control for only a very short time.

The present work reports the effect of prolonged administration of propranolol and timolol on growth rate and myocardial structure in rabbits aged 12–15 weeks at the start of the experiment when the sympathetic nervous system is fully developed. Neither of these non-cardioselective β-adrenoreceptor-blocking drugs possesses intrinsic sympathomimetic activity and, in addition, timolol has no membrane-stabilizing activity (Hall, Robson & Share, 1975).

**Methods**

**Relative potency of timolol and propranolol**

The potency of propranolol relative to timolol was measured. Log dose–response curves were obtained for inhibition of the chronotropic response to isoprenaline before and after administration of antagonist. Bolus injections of isoprenaline were given via a flexible plastic cannula in an ear vein. Heart rate was recorded after injection of antagonist. Further experiments were performed 3 and 6 h after administration of antagonist in order to obtain an estimate of the duration of functional β-adrenoreceptor blockade. The relative potency of the two antagonists was measured by comparing the pA2 in vivo (Bowman, Rand & West, 1968) of antagonists by the method of Vaughan Williams, Bagwell & Singh (1973).

**Experimental methods**

Male, littermate, New Zealand white rabbits weighing 2–3 kg (12–15 weeks old) received either timolol maleate (0·2 mg/kg) or propranolol hydrochloride (2 mg/kg), dissolved in water, twice daily intraperitoneally. A further group received the same dose of propranolol subcutaneously. Controls received an equivalent volume of water either subcutaneously or intraperitoneally. Administration was continued for 8 weeks. Animal weight and food intake were measured twice weekly.

The electrocardiogram was recorded on unsedated, loosely restrained animals before and during administration.

Arterial pressure, recorded on a Devices Polygraph M19, was measured directly from an ear artery in the unsedated animal. A subsequent pressure measurement by this method could not be performed in two animals, pressure being measured via femoral arteriotomy performed under light halothane anaesthesia. Animals were allowed to recover from this anaesthetic before recordings were made.

At various times during the experiment venous blood was withdrawn, the plasma or serum separated and stored at −20°C for subsequent analysis of serum alkaline phosphatase, aspartate transaminase and albumin levels. Plasma propranolol was assayed by gas–liquid chromatography with the modified method of Jack & Reiss (1974).

Administration was discontinued 24 h before animals were killed by a sharp blow to the back of the neck. Hearts were immediately excised, atria dissected free and the ventricles blotted dry and weighed. Approximately 100 mg of ventricle was accurately weighed and then dried at 60–70°C to constant weight. Samples were taken from the apex of the left ventricle and fixed for electron microscopy. The lungs, liver and right kidney were removed, blotted dry and weighed. Samples of liver were fixed in 10% formalin in sodium chloride solution (pH 7·0) and stained either with haematoxylin and eosin or with periodic acid–Schiff for light microscopy.

Specimens for electron microscopy were prefixed for 5 min at 4°C in 1% paraformaldehyde, sucrose (0·2 mol/l), sodium phosphate buffer (0·2 mol/l, pH 7·4), fixed in 4% glutaraldehyde for 2 h and post-fixed in 1% osmium tetroxide for 1 h (Mackay, Littler & Sleight, 1978). After dehydration specimens were embedded in Epon resin. Ultra-thin sections (60–150 nm) were cut, mounted on copper grids and stained with uranyl acetate in 50% ethanol and Reynolds lead citrate.
Sections were examined on a Siemens Emiskop 102 or a Zeiss EM9 electron microscope. Volumetric analyses were performed by using the method of Weibel (1963). Nine blocks were prepared from the left ventricular apex of each heart, and sections taken from randomly selected blocks from each heart. Between 10 and 15 electron micrographs were taken from randomly selected sites on each section. Each electron micrograph was then placed beneath a transparent grid and the number of points overlying each structure were counted. The structure beneath each point was assigned to one of the seven categories being considered. When a total of eight micrographs had been counted from each block in this way, the examination of further micrographs produced no significant change in the mean count of each structure. Sixteen pictures at a final magnification of $\times 12,000$ were examined from each heart. Approximately 10,000 points were counted for each group studied.

Results

Experimental groups

The animals were studied in two groups. In the first group nine animals received propranolol intraperitoneally, nine received timolol intraperitoneally and there were nine control animals. In the second group eight received propranolol subcutaneously, five received propranolol intraperitoneally, five received timolol intraperitoneally and there were nine control animals. One animal given propranolol intraperitoneally died of respiratory infection after 1 week and one animal from the timolol group died at 7 weeks, after anaesthesia and femoral arteriotomy.

Relative potency of antagonists and duration of $\beta$-adrenoceptor blockade

After intraperitoneal administration timolol was found to have approximately 20 times the potency of propranolol. Because results from individual animals showed considerable variation a ratio of 10:1 was used when calculating equivalent doses. After subcutaneous administration propranolol possessed three times the potency as when given intraperitoneally. Three hours after intraperitoneal administration of antagonists, the inhibition of the chronotropic response to isoprenaline was approximately 60% of that obtained within 30–90 min of administration. At 6 h inhibition was less than 20% and in some animals $\beta$-adrenoceptor blockade could not be demonstrated. There was no difference between the duration of $\beta$-adrenoceptor blockade obtained with intraperitoneal timolol and propranolol.

Heart rate and arterial pressure

Mean heart rate of both groups given propranolol was significantly less than that of the controls at 4, 6 and 8 weeks (Fig. 1). The difference was also significant when recordings were made 5–7 h after injection of propranolol. There was no significant difference between the control animals and test animals during the experiment in the timolol group, although the initial heart rate of this group was higher than that of the controls. No significant difference was seen in mean arterial pressure between control and test animals either before or after administration.

Effects on the heart

There was no significant difference in wet ventricular weight or in water content of hearts from control and test groups of animals. The water content of skeletal muscle was similarly unaltered.

Cardiac ultrastructure was examined in five controls, in seven animals given propranolol intraperitoneally and in four given propranolol subcutaneously. Four intracellular structures (mitochondria, myofibrils, nuclei and sarcoplasm) and three extracellular structures (collagen, vasculature and extracellular space) were examined. Results are shown in Table 1. There was no significant difference, in either the relative volume of individual organelles or total intracellular and extracellular volume, between controls and test animals.

Plasma propranolol levels

A wide range of propranolol levels was observed in plasma taken from animals throughout the experimental period. Peak values obtained 30–90 min after injection ranged from 20 to 120 ng/ml after intraperitoneal injection and 60 to 180 ng/ml after subcutaneous injection. Four hours after injection the mean values were 20 and 30 ng/ml respectively.

Organ weights

No difference was found between wet weight corrected for body weight of liver, kidney or lung of control and test animals. No histological differences were observed when liver from the
controls and from animals given propranolol intraperitoneally was examined or when glycogen content was assessed histologically. Liver function studies on plasma from all test groups showed no difference from the controls.

Growth and food intake

Fig. 2 shows the growth rates of each group. Animals in the first trial grew at the same rate as animals in the second trial given the same drug:

### TABLE 1. Effect of prolonged administration of propranolol on cardiac ultrastructure

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 5)</th>
<th>Intraperitoneal propranolol (2 mg/kg twice daily) (n = 7)</th>
<th>Subcutaneous propranolol (2 mg/kg twice daily) (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myofibrils</td>
<td>34·9 ± 1·9</td>
<td>32·8 ± 2·0</td>
<td>30·7 ± 0·8</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>19·6 ± 0·5</td>
<td>18·9 ± 1·0</td>
<td>18·6 ± 1·5</td>
</tr>
<tr>
<td>Nuclei</td>
<td>1·6 ± 0·4</td>
<td>1·2 ± 0·3</td>
<td>2·6 ± 0·9</td>
</tr>
<tr>
<td>Saroplasm</td>
<td>16·9 ± 0·4</td>
<td>15·7 ± 0·7</td>
<td>14·7 ± 0·9</td>
</tr>
<tr>
<td>Total intracellular</td>
<td>73·0 ± 2·4</td>
<td>67·9 ± 3·7</td>
<td>66·6 ± 2·4</td>
</tr>
<tr>
<td>Intravascular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>6·8 ± 1·8</td>
<td>8·3 ± 1·5</td>
<td>9·6 ± 1·4</td>
</tr>
<tr>
<td>Extravascular</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>space</td>
<td>2·0 ± 0·2</td>
<td>3·3 ± 0·7</td>
<td>3·5 ± 0·7</td>
</tr>
<tr>
<td>Total extracellular</td>
<td>27·0 ± 2·4</td>
<td>32·1 ± 3·7</td>
<td>33·4 ± 2·4</td>
</tr>
</tbody>
</table>
Prolonged β-adrenoreceptor blockade on the heart

**Fig. 2.** Effect of prolonged administration of propranolol and timolol on rabbit body weight. ■, Control; ●, intraperitoneal propranolol (2 mg/kg twice daily); ○, subcutaneous propranolol (2 mg/kg twice daily); ▲, intraperitoneal timolol (0.2 mg/kg twice daily). Results are means ± SEM. Statistical comparisons were by Student’s *t*-test for unpaired data: *P < 0.02; **P < 0.01: test vs control animals.

the results of both trials have therefore been combined. There was no significant difference after 8 weeks in either mean weight or weight increase, between control animals given timolol or propranolol subcutaneously. Weight increase in animals given propranolol intraperitoneally was significantly less than controls from week 1, and by the end of week 8 a 23% reduction in weight was observed compared with that in the controls.

Food intake of groups given timolol intraperitoneally and propranolol subcutaneously was not significantly different from that of the controls. However, animals given propranolol...
intraperitoneally began to eat less after week 2 and continued to eat significantly less throughout the remaining period.

In order to determine whether this effect of prolonged administration of propranolol intraperitoneally was due to the β-adrenoreceptor-blocking properties of the drug, or to some other property, a further trial was conducted. Rabbits were given the (+)- or (−)-isomers of propranolol. Two groups of 10 littermates (weight 2–3 kg) were given either (+)- or (−)-propranolol (1 mg/kg) twice daily intraperitoneally. Four littermates served as controls. A dose of the (+)-isomer (1 mg/kg) was shown to have no β-adrenoreceptor-blocking activity as judged by inhibition of the chronotropic response to isoprenaline. A similar dose of the (−)-isomer showed a highly significant inhibition of this response.

Growth of animals given the (−)-isomer was not different from those given the (+)-isomer and from the controls. All three groups grew at the same overall rate as did control animals in the previous groups.

Discussion

The effects of prolonged administration of propranolol and timolol to growing rabbits was studied by using the intraperitoneal route of administration. Twice-daily intraperitoneal administration was chosen as being more reliable in terms of dosage and time of administration than the oral route. The dose of drugs used compares with a standard therapeutic dose in man and with that used in a previous study with propranolol in rabbits (Vaughan Williams et al., 1975). In this latter study, the regimen was shown to produce intermittent β-adrenoreceptor blockade throughout the day with daily variations in intensity and duration. Alterations in growth and in ventricular weight and water content were seen after 6 weeks administration even though effective β-adrenoreceptor blockade was not achieved for a part of each 24 h period. Although the magnitude of such changes appeared to be related to duration of blockade, it is relevant that changes occurred despite intermittent blockade.

In the present study propranolol (2 mg/kg) and timolol (0.2 mg/kg) given intraperitoneally achieved significant inhibition of isoprenaline tachycardia within 30 min of administration and antagonism was detectable 6 h later.

After administration of propranolol by either route, a significant reduction in heart rate was seen compared with that in the controls. This difference between control and test animals was not seen after administration of timolol. It is possible this was due to an inherently faster rate in this group. Rabbits aged 7–8 weeks given propranolol (1 mg/kg) twice daily for 5–7 weeks underwent no change in heart/weight ratio (Nayler, Yepez, Slade & Fassold, 1977). Younger rabbits (600–900 g) given twice this dose of propranolol subcutaneously for a similar period showed significant changes in ventricular weight and water content (Vaughan Williams et al., 1975).

Effects on the heart and other organs

No significant difference was found between the ventricular weights of control and test hearts, and the water content of skeletal and cardiac muscle was similarly unaltered. No significant differences in wet weights of lung, liver and kidney were noted. Animals in this study were between 12 and 15 weeks old at the start of the experiment. When these experiments were repeated in older, nearly adult rabbits, no changes were seen in heart weight (Vaughan Williams, 1977). Thus in rabbits both age and dose of antagonist are important factors in determining the effects of long-term β-adrenoreceptor blockade. Changes in the heart with long-term β-adrenoreceptor blockade have only been shown to occur in very young rabbits given propranolol (2 mg/kg) twice daily or an equivalent dose of practolol. It remains to be shown whether the changes occur in older animals given larger doses of β-adrenoreceptor-blocking agents that are sufficient to produce effective β-adrenoreceptor blockade throughout the 24 h period.

Effects on cardiac ultrastructure

Although no changes in gross cardiac structure were seen after prolonged β-adrenoreceptor blockade, this did not exclude changes which may have occurred at the ultrastructural level. Highly significant changes in cardiac morphometry have previously been shown in young rabbits after prolonged β-adrenoreceptor blockade. In the present study, no significant difference was found between the volume composition of test and control hearts. Normal cardiac ultrastructure has also been seen in young male rabbits given propranolol (1 mg/kg) twice daily subcutaneously for 5–7 weeks (Nayler et al., 1977). However, the statistical assessment of results used in the present study differs from that used previously. The volume composition of each heart examined was estimated by the point-counting technique of
Prolonged \( \beta \)-adrenoreceptor blockade on the heart

Weibel (1963). When a number of hearts in each group had been examined, it was then possible to compare the mean of each result of one group with that in another by Student's \( t \)-test for unpaired samples. Previous studies (Vaughan Williams et al., 1977) were based on experiments with only three animals in each group. Electron micrographs from different hearts in the same group were then pooled, and point counting was performed on electron micrographs chosen at random from each group. The total number of points counted on a particular organelle in a test group was then compared with the control group by a 2 \( \times \) 2 Chi-squared test. Although this test assumes that each individual point counted is statistically independent of the others, this is not the case in points taken from the same micrograph or from the same animal. This difference in statistical analysis might explain why highly significant results were obtained previously with only small numbers of animals in each group.

Fixation artifact could have obscured any small difference that existed between test and control animals. Sections showing recognizable artifact were discarded but tissue shrinkage, due to the relatively high osmolality of fixative, is a possibility. This artifact has, however, been demonstrated by using fixatives with osmolality close to that of tissue (Eisenberg & Mobley, 1975), and is therefore dependent on factors other than carrier osmolality. We used an established technique (Mackay et al., 1978) which did not appear to produce significant shrinkage in previous work where changes in ultrastructure were demonstrated (Vaughan Williams et al., 1977). The technique is also suitable for use in examining specimens obtained from cardiac biopsy and the potential clinical application of the method was considered when the technique was chosen. It is possible that shrinkage eliminated differences in the volumes of areas of high water content, although the lack of any difference in water content of control and test hearts makes this unlikely. Electron-micrographs did not show evidence of shrinkage of vascular and perivascular compartments.

Effects on growth

Only animals given propranolol (2 mg/kg) twice daily intraperitoneally showed a significant reduction in weight increase during prolonged administration. This was apparent in both trials after week 1 and by week 8 animals were on average 380 g lighter than the control animals. Intraperitoneal injections \textit{per se} did not cause the reduction as control animals injected intra-peritoneally were unaffected. At \textit{post mortem} the peritoneal cavity of some animals showed a mild fibrinous peritonitis related to injection sites, but these animals did not show reduced growth or food intake when compared with those which had normal peritoneal cavities. No animals developed diarrhoea or other evidence of gastrointestinal upset. Although there was some variation in food intake between members of the same group, animals growing slowly ate correspondingly less food. Such a simple relationship might be explained by a reduction of appetite in the slow growing animals.

The effect on growth was unrelated to the \( \beta \)-adrenoreceptor-blocking properties of propranolol because the growth of animals given propranolol subcutaneously was not significantly less than that of the controls. Moreover, these animals had higher plasma drug levels and a slower resting heart rate than those given propranolol intraperitoneally. Propranolol given subcutaneously was shown in separate experiments to have three times the potency of the drug given intraperitoneally. It was possible that the effects related to a local action of propranolol on the gastrointestinal tract. However, when the \((-)\)-isomer was given intraperitoneally (1 mg/kg), growth was no different from that in the controls. This dose of isomer (equivalent to 2 mg/kg of the racemate) has \( \beta \)-adrenoreceptor-blocking properties but less membrane-stabilizing activity (Barrett & Cullum, 1968). The effect on growth is therefore unlikely to be due to a local \( \beta \)-adrenoreceptor-blocking effect on the gastrointestinal tract. A dose of the \((+)-\)-isomer, shown to have no \( \beta \)-adrenoreceptor-blocking activity in rabbits, failed to produce any reduction of growth. Although this suggests that membrane-stabilizing activity was not responsible for the reduced growth rate seen with the racemate, in order to prove this point it would have been necessary to dose the animals with 2 mg/kg daily.

It is possible that the effect is related to levels of a specific metabolite of propranolol present only when the drug is given intraperitoneally at the higher dose of 2 mg/kg. After intraperitoneal administration, the drug is absorbed through the peritoneum into the hepatic portal vein and the liver is presented with a sudden surge of the drug (Fitzgerald & O'Donnell, 1971). One metabolite of propranolol, 4-hydroxypropranolol, has been found free in plasma of animals and man after oral but not after intravenous administration. The size of such hepatic surges would depend on the dose injected, the higher the surge the more likely it is that the main metabolic pathway would be saturated, allowing hydroxylation to produce
sufficient 4-hydroxypropranolol and possibly other metabolic products to appear free in the plasma.

In man gastrointestinal symptoms have been reported with propranolol. Anorexia, abdominal cramps and diarrhoea were reported in 22 out of 121 patients taking a median dose of 160–200 mg/day for angina pectoris (Amsterdam, Gorlin & Wolfson, 1969). It is possible that propranolol or one of its metabolites has an effect on appetite. Although the adrenergic nervous system may have a role in the hypothalamic control of appetite (Leibowitz, 1970), neither propranolol nor its metabolites have been shown to suppress appetite.

The results presented here serve to emphasize that changes in cardiac structure after long-term $\beta$-adrenoreceptor blockade are seen only in very young animals, at a time when sympathetic control of the heart is not fully developed. Once this control has developed, such regimens do not appear to affect either ventricular mass, water content or ultrastructure. It remains to be seen whether the changes previously observed in very young rabbits are seen in older animals with higher doses of antagonist sufficient to produce not just intermittent, but significant levels of $\beta$-adrenoreceptor blockade throughout each 24 h period.

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