A prospective study of the effects of prolonged timolol therapy on α - and β -adrenoceptor and angiotensin II receptor mediated responses in normal subjects

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Aims Long-term treatment with β_1 -selective adrenergic antagonists gives rise to cross-sensitisation of cardiac β_2 -adrenoceptor responses, with no corresponding alteration in β_1 -adrenoceptor responses. We performed a prospective randomised double-blind placebo-controlled cross-over study of the effects of nonselective β -blockade with timolol on α -adrenergic and angiotensin II receptor mediated responses in normal subjects. We also wished to study the time course of β_1 - and β_2 -adrenergic responses after withdrawal of timolol.

Methods Six healthy males received timolol 10 mg twice daily or placebo for 14 days. On day 11 of treatment, vascular α_1 -, α_2 - and angiotensin II receptor responses were assessed by measuring the blood pressure increases in response to intravenous phenylephrine, α -methylnoradrenaline and angiotensin amide respectively, following one dose of timolol 10 mg (to block the β -adrenergic effects of phenylephrine and α -methylnoradrenaline). Both systolic and diastolic blood pressure increased in response to each of these drugs, but these increases were not different on timolol treatment or placebo. Following cessation of treatment with timolol or placebo, β_1 - and β_2 -adrenoceptor mediated responses were assessed by measuring the heart rate responses to treadmill exercise and intravenous salbutamol infusion respectively. Half each of the subjects underwent this 2 days and 3 days respectively, after the end of treatment.

Results Both exercise-induced and salbutamol-induced tachycardia were not different following placebo or 3 days following the end of timolol treatment. However, 2 days following timolol treatment, both were attenuated; the reduction in salbutamol-induced tachycardia was significant, whilst the reduction in exercise tachycardia did not reach statistical significance. We also measured metabolic responses to exercise and to salbutamol infusion. Exercise induced a rise in plasma potassium and noradrenaline. Salbutamol produced a fall in plasma potassium, a rise in plasma glucose and insulin and also a rise in plasma noradrenaline. All of these changes were not different following placebo or 3 days after the end of timolol treatment; by contrast, 2 days following timolol treatment, all were significantly attenuated, with the exception of the rise in plasma glucose. In addition, the rise in both plasma glucose and insulin in response to an oral load of 75 g glucose were not different post-placebo, 2 or 3 days post-timolol.

Conclusions These results show that, following 14 days of nonselective β -adrenoceptor blockade with timolol, there is evidence of residual β -adrenoceptor blockade 2 days after drug withdrawal; this finding is in contrast with the known plasma profile of timolol (half-life 3–6 hours), but is consistent with our previous observations of the slow speeds of association and dissociation of timolol with β -adrenoceptors *in vitro*. There is no evidence, in this study, of β -adrenergic sensitisation following timolol withdrawal, nor of cross-regulation of vascular α_1 -, α_2 - or angiotensin II receptors in response to nonselective β -adrenoceptor blockade.

Keywords: nonselective β -adrenoceptor blockade, α -adrenoceptors, β -adrenoceptors, angiotensin II receptors

Introduction

β -Adrenoceptor antagonists are widely used in the treatment of patients with ischaemic heart disease and hypertension.

The drugs most frequently employed are the β_1 -selective antagonists, e.g. atenolol, metoprolol and bisoprolol. We and others have shown, both *in vitro* and *in vivo*, that both patients and normal healthy subjects on treatment with β_1 -selective blockers exhibit cross-sensitisation of β_2 -adrenergic responses, with no change in β_1 -adrenergic responses even after washout of the drug [1–4]. This cross-talk between

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β_1 - and β_2 -adrenoceptors appears to be tissue-specific. It occurs in cardiac tissue, but not in internal mammary artery or saphenous vein [5]. It has also been shown that chronic β_1 -adrenoceptor blockade induces cross-desensitisation of cardiac muscarinic M_2 receptor responses [4].

Whether similar cross-regulatory effects occur following nonselective β -adrenoceptor blockade is not known, but this question is of considerable clinical importance. It has not been possible to study patients on nonselective β -adrenoceptor blockers up till now because such drugs are prescribed relatively infrequently. We have recently demonstrated *in vitro* that timolol blocks both β_1 - and β_2 -adrenoceptors, with a higher affinity for the β_2 - than the β_1 -adrenoceptor in human atrium, and that the times both of onset and of offset of blockade with this drug are slow [6]. We therefore conducted a prospective randomised placebo-controlled cross-over study in healthy subjects, to examine the effects of nonselective β -adrenoceptor blockade with timolol on cardiovascular and metabolic β -adrenergic subtype responses following withdrawal. We wished also to take this opportunity to look for effects of β -adrenoceptor blockade on receptors coupled to different G-proteins from the β -adrenoceptors, and we therefore examined vascular α_1 - and α_2 -adrenoceptor and angiotensin II receptor responses during treatment with timolol and placebo.

Methods

Subjects

Six non-smoking healthy men aged 22–41 years, without contraindications to β -adrenoceptor blockade, took part in the study. The study was approved by the district medical ethics committee and was in accord with the Helsinki Convention (Tokyo and Venice amendments). Subjects gave written informed consent.

Procedure

Before commencing drug treatment, the subjects exercised on a treadmill, and a level of exercise was found for each subject that produced a stable heart rate of about 150 beats min^{-1} . We have found this heart rate response to be highly reproducible within each subject. Subjects then commenced a 14 day course of treatment with either timolol (10 mg twice daily) or matched placebo. The treatment order was double blinded and randomized according to a Latin square design. A washout period of at least 1 month was given between each treatment.

Responses to phenylephrine, α -methylnoradrenaline and angiotensin amide

On day 11 of treatment, subjects attended having had a light breakfast excluding all caffeine-containing drinks. They were given 10 mg timolol 90 min prior to testing (to block the β -adrenergic effects of phenylephrine and α -methylnoradrenaline.) An intravenous cannula was inserted into a forearm vein; they were then rested for 30 min, and the resting heart rate and blood pressure were noted. An infusion of phenylephrine was commenced at $0.5 \mu\text{g kg}^{-1}$

min^{-1} , and the infusion rate increased by $0.25 \mu\text{g kg}^{-1} \text{min}^{-1}$ every 10 min. Heart rate and blood pressure were recorded every 2 min, and the infusion was stopped once an increase in systolic blood pressure of 20 mmHg had been achieved.

Following a recovery period of at least 30 min, once basal heart rate and blood pressure had been restored, an infusion of α -methylnoradrenaline was commenced at $0.125 \mu\text{g kg}^{-1} \text{min}^{-1}$, and the infusion rate doubled every 10 min. Again, heart rate and blood pressure were recorded every 2 min, and the infusion was stopped once an increase in systolic blood pressure of 20 mmHg was achieved.

After another recovery period of at least 30 min, once basal heart rate and blood pressure had been restored, an infusion of angiotensin amide was commenced at $5 \text{ ng kg}^{-1} \text{min}^{-1}$, and the infusion rate increased by $5 \text{ ng kg}^{-1} \text{min}^{-1}$ every 10 min. Once again, heart rate and blood pressure were recorded at 2 min intervals, and the infusion was stopped once an increase in systolic blood pressure of 20 mmHg was achieved.

Glucose tolerance tests

Subjects underwent standard glucose tolerance testing following withdrawal of timolol or placebo. Three subjects underwent this 2 days post-drug withdrawal and four underwent this 3 days post-withdrawal (one subject underwent testing in both ways). A 75 g glucose load was given orally, following an overnight fast; blood samples were taken before this, and at 30 min intervals for 2 h following administration of glucose, for measurement of plasma glucose and insulin levels.

Responses to exercise and to salbutamol

Three subjects underwent this 2 days post-drug withdrawal and four underwent this 3 days post-withdrawal (one subject underwent testing in both ways). Subjects attended having had a light breakfast but excluding all caffeine-containing drinks. Two intravenous cannulae were inserted into forearm veins, and subjects were then rested for 30 min and resting heart rate was noted. They then walked on the treadmill at the previously determined level of exercise for 2 min, and the heart rate was measured at the end of this time. Blood samples were taken for measurement of plasma potassium and noradrenaline before exercise, at peak exercise, and at 5 min after exercise.

Subjects then rested for 15 min, and heart rate and blood pressure were rechecked prior to commencement of intravenous salbutamol injection. Bolus injections of salbutamol were given over 2 min, and after a further three minutes the next dose was given. Injections started at $0.5 \mu\text{g kg}^{-1}$, and the dose was increased by doubling increments up to $4 \mu\text{g kg}^{-1}$. Heart rate and blood pressure were recorded at 1 min and 3 min after the end of each injection. Peak responses to each injection were seen at the 1 min recording and these readings were used for further analysis. Blood samples were taken for measurement of plasma potassium, glucose, insulin and noradrenaline before the start of the salbutamol injections and at 5 min intervals for 40 min.

Methods of assessment

Heart rate was calculated from the R–R intervals averaged over 15 s from a continuously monitored electrocardiogram. Blood pressure was measured with an automated sphygmomanometer (Datascope, USA). Potassium was determined with an ion selective electrode, glucose by the hexokinase method and insulin by an immunoenzymetric assay (Medgemix, Belgium). Noradrenaline was measured by high performance liquid chromatography with electrochemical detection (method modified from Moyer & Jiang [7]).

Statistics

Systolic and diastolic blood pressure responses to phenylephrine, α -methylnoradrenaline and angiotensin amide in each subject during timolol treatment or placebo were fitted to a quadratic equation, and the dose of each drug required to increase systolic blood pressure by 20 mmHg (SBP₂₀) and diastolic blood pressure by 10 mmHg (DBP₁₀) was determined from this; SBP₂₀s and DBP₁₀s were compared during timolol therapy and placebo by paired Student's *t*-test. Changes in heart rate and in plasma potassium and noradrenaline concentrations induced by exercise were compared (2 and 3 days after timolol treatment and after placebo) by Kruskal–Wallis one-way analysis by ranks. Heart rate, systolic and diastolic blood pressure were determined for each subject after each course of treatment, before and after the maximal dose of salbutamol; these were compared between the different treatments by Kruskal–Wallis one-way analysis by ranks. For changes in the metabolic variables during salbutamol infusion (plasma potassium, glucose, insulin and noradrenaline), and also for plasma glucose and insulin responses during the glucose tolerance tests, areas under the concentration–time curve were determined for each subject after each course of treatment; these were compared between the different treatments by Kruskal–Wallis one-way analysis by ranks. A *P* value <0.05 was taken as significant in all tests. All results are expressed as mean $\pm$ s.e. mean.

Results

Responses to phenylephrine, α -methylnoradrenaline and angiotensin amide

Basal values of systolic and diastolic blood pressure were not significantly different on timolol or placebo. Both systolic and diastolic blood pressure increased in response to phenylephrine, α -methylnoradrenaline and angiotensin amide. These increases were not significantly different during timolol therapy or placebo (Figure 1, Table 1). Heart rate fell in response to each of these drugs, but again not differently during timolol treatment or placebo (data not shown).

Glucose tolerance tests

Both plasma glucose and insulin rose in response to an oral 75 g glucose load (Figure 2), but these responses were not

significantly different post-placebo, 2 or 3 days post-timolol treatment.

Responses to exercise

The heart rate increase in response to exercise was not significantly different post-placebo or 3 days post-timolol therapy; there was a reduction in exercise tachycardia 2 days post-timolol therapy which did not reach statistical significance (Table 2). Pre-exercise heart rate was not different following each course of treatment.

Plasma potassium and noradrenaline both rose following exercise (Table 2). Values at baseline, at peak exercise and 5 min post-exercise were not significantly different after placebo, 2 or 3 days post-timolol.

Responses to salbutamol

Heart rate increased in response to intravenous salbutamol. Salbutamol-induced tachycardia was not different after placebo and 3 days post-timolol, but was significantly reduced 2 days post-timolol (Figure 3, Table 3). Pre-infusion heart rate was not significantly different after each course of treatment. Systolic blood pressure did not change during salbutamol infusion after either treatment. Diastolic blood pressure fell in response to salbutamol; the fall was not significantly different post-placebo and 2 or 3 days post-timolol, although there appeared to be a trend towards a lesser fall 2 days post-timolol (Figure 3, Table 3).

Basal plasma potassium, glucose, insulin and noradrenaline were not different after timolol and placebo. In response to salbutamol, potassium fell whilst glucose, insulin and noradrenaline rose (Figure 4). None of these responses was significantly different post-placebo and 3 days post-timolol; 2 days post-timolol, the potassium, insulin and noradrenaline responses were significantly attenuated, whilst the increase in plasma glucose was not significantly different.

Discussion

We have shown that, in normal subjects, 14 days treatment with the nonselective β -adrenoceptor blocker timolol gave rise to persistence of β -adrenoceptor blockade 2 days after withdrawal of the drug; this persistent β -adrenoceptor blockade disappeared by 3 days post-timolol withdrawal. Measured 3 days post-withdrawal, neither the haemodynamic nor the metabolic responses to β -adrenergic stimulation were different after timolol. However, at 2 days post-withdrawal, cardiac β -adrenergic responses appeared to be diminished. There may also have been a reduced vasodilator response to salbutamol, as manifested by a smaller fall in diastolic blood pressure. The hypokalaemia, hyperinsulinaemia and increase in plasma noradrenaline in response to β_2 -adrenergic stimulation were also attenuated at 2 days, with no change in glucose tolerance or in plasma glucose response to β_2 -adrenoceptor stimulation. Finally, we have shown that vascular α_1 - and α_2 -adrenoceptor and angiotensin II receptor mediated responses were unaltered by timolol therapy.

There was no evidence of β -adrenergic subtype sensitisation following nonselective β -blocker withdrawal, in this study. This is in marked contrast with our previous results

Figure 1 Following pre-treatment with 10 mg timolol, subjects on either timolol therapy or placebo received increasing doses of phenylephrine, α -methylnoradrenaline and angiotensin amide intravenously, and blood pressure responses were measured. Full details are as in Methods. Blood pressure responses are shown to (a) phenylephrine, (b) α -methylnoradrenaline and (c) angiotensin amide. Values plotted are mean $\pm$ s.e. mean. Systolic ($\square$) and diastolic ($\blacksquare$) blood pressure responses on timolol therapy, systolic ($\diamond$) and diastolic ($\blacklozenge$) blood pressure responses on placebo.

Table 1 Total doses of phenylephrine, α -methylnoradrenaline and angiotensin amide required to increase systolic blood pressure by 20 mmHg (SBP₂₀) and diastolic blood pressure by 10 mmHg (DBP₁₀).

	Phenylephrine ($\mu\text{g kg}^{-1}$)	α -methylnoradrenaline ($\mu\text{g kg}^{-1}$)	Angiotensin II (ng kg^{-1})
SBP ₂₀ ^s			
Timolol	16.8 $\pm$ 2.1	4.9 $\pm$ 1.0	187.5 $\pm$ 48.0
Placebo	13.7 $\pm$ 1.1	5.7 $\pm$ 0.7	160.5 $\pm$ 50.0
DBP ₁₀ ^s			
Timolol	9.5 $\pm$ 1.6	4.2 $\pm$ 0.4	108.0 $\pm$ 37.0
Placebo	8.8 $\pm$ 1.1	2.8 $\pm$ 0.5	86.0 $\pm$ 20.0

Following pre-treatment with 10 mg timolol, subjects on either timolol therapy or placebo received increasing doses of phenylephrine, α -methylnoradrenaline and angiotensin amide intravenously, and blood pressure responses were measured. Full details are as in Methods. SBP₂₀, DBP₁₀: dose of drug required to increase systolic blood pressure by 20 mmHg and diastolic blood pressure by 10 mmHg respectively. Values were not significantly different between timolol therapy and placebo, for each drug tested.

using the β_1 -selective antagonist bisoprolol. In a similar prospective study in normal subjects, we found that 14 days treatment with bisoprolol sensitised cardiac β_2 -adrenergic responses, with no change in cardiac β_1 -adrenergic responses, when measured 3 days after withdrawal; vascular β_2 -adrenergic vasodilatation, as manifested by salbutamol-induced fall in diastolic blood pressure, was unchanged, but there was an augmented hypokalaemic response to salbutamol and impairment of glucose tolerance [3]. The plasma half-life of timolol is considerably shorter (3–6 h) than that of bisoprolol (12 h), and we therefore expected any

β -adrenoceptor sensitisation which might occur to be seen earlier and possibly disappear more quickly. Surprisingly, β -adrenergic responses were diminished at 2 days, at a time when plasma timolol levels should have declined to negligible levels [8–10]; these responses had returned to normal by 3 days. It is possible that it takes longer than 3 days to develop β -adrenergic hypersensitivity following withdrawal of timolol, and further studies with longer time points would be needed to confirm or refute this. It could also be argued that our study did not have the power to detect a small degree of β -adrenergic hyperresponsiveness

Figure 2 Post-placebo and either 2 or 3 days following timolol therapy, subjects underwent a standard glucose tolerance test. (a) Plasma glucose and (b) plasma insulin responses are shown to 75 g glucose orally. Values plotted are mean $\pm$ s.e. mean. Responses post-placebo (■), 2 days (◆) and 3 days (◇) post-timolol therapy.

induced by timolol. However, in our previous study of similar design and using the same number of subjects, a marked sensitisation was observed, in all subjects tested, of cardiac β_2 -adrenergic responses to salbutamol three days following withdrawal of the β_1 -selective blocker bisoprolol

Figure 3 Post-placebo and either 2 or 3 days following cessation of timolol therapy, subjects received increasing doses of salbutamol intravenously, and heart rate and blood pressure responses were measured. Full details are as in Methods. (a) Heart rate and (b) blood pressure responses are shown to salbutamol. Values plotted are mean $\pm$ s.e. mean. Responses post-placebo (■), 2 days (◆) and 3 days (◇) post-timolol therapy. Salbutamol-induced tachycardia was significantly reduced 2 days post-timolol therapy (*).

[3]. We have calculated that, in order to detect the same degree of difference in the present study, only five subjects would be required. Therefore at the very least, our study demonstrates that if any β_1 - or β_2 -adrenergic hypersensitivity occurs, it is much more difficult to demonstrate *in vivo* with

Table 2 Heart rate, plasma potassium and noradrenaline responses to treadmill exercise.

<i>Heart rate (beats min⁻¹)</i>			
	<i>Post-placebo</i>	<i>2 days post-timolol</i>	<i>3 days post-timolol</i>
Before exercise	61.5 ± 3.1	54.7 ± 4.7	60.8 ± 3.0
Peak exercise	147.7 ± 4.9	133.7 ± 7.5	154.5 ± 1.9
5 min post-exercise	67.7 ± 4.2	65.0 ± 9.7	74.8 ± 4.7
<i>Plasma potassium (mmol l⁻¹)</i>			
Before exercise	4.12 ± 0.09	4.37 ± 0.20	4.13 ± 0.06
Peak exercise	4.85 ± 0.10	5.10 ± 0.06	4.88 ± 0.22
5 min post-exercise	4.28 ± 0.10	4.63 ± 0.13	4.28 ± 0.09
<i>Plasma noradrenaline (nmol l⁻¹)</i>			
Before exercise	1.13 ± 0.06	0.84 ± 0.25	1.02 ± 0.14
Peak exercise	5.01 ± 0.68	4.31 ± 1.06	4.91 ± 0.55
5 min post-exercise	1.80 ± 0.23	1.23 ± 0.17	2.10 ± 0.29

Post-placebo and either 2 or 3 days after cessation of timolol therapy, subjects underwent treadmill exercise for 2 min, at a level of exercise previously determined to produce a heart rate of 150 beats min⁻¹, and heart rate, plasma potassium and noradrenaline responses were determined. Full details are as in Methods. Values were not significantly different post-placebo, 2 and 3 days post-timolol therapy.

Table 3 Heart rate and diastolic blood pressure responses to intravenous salbutamol.

<i>Heart rate (beats min⁻¹)</i>			
	<i>Post-placebo</i>	<i>2 days post-timolol</i>	<i>3 days post-timolol</i>
Basal	66.0 ± 3.4	61.0 ± 5.5	66.0 ± 2.4
After 4 µg kg ⁻¹ salbutamol	117.0 ± 7.2	75.3 ± 7.4*	114.8 ± 8.0
<i>Diastolic blood pressure (mmHg)</i>			
Basal	67.5 ± 1.8	71.0 ± 3.5	66.3 ± 2.5
After 4 µg kg ⁻¹ salbutamol	53.1 ± 3.7	62.0 ± 1.5	52.3 ± 6.0

Post-placebo and either 2 or 3 days following cessation of timolol therapy, subjects received increasing doses of salbutamol intravenously, and heart rate and blood pressure responses were measured. Full details are as in Methods. **P* < 0.05 as compared with post-placebo.

timolol than with atenolol or bisoprolol [2, 3]. By contrast, the β -adrenoceptor blockade seen 2 days post-timolol withdrawal is a large effect (Figures 3 and 4) which was observed in each of the three subjects tested in this way, and cannot be attributed to a simple type I statistical error.

This study demonstrates that β -adrenoceptor blocker effects may correlate poorly with predicted plasma levels of β -adrenoceptor blocking drugs. It is likely that, after disappearance from the circulation, timolol remained for longer within tissues; thus, the pharmacokinetic and pharmacodynamic profiles of timolol may differ, so that plasma timolol levels (which we could not measure in the present study) may not accurately reflect the degree of β -adrenoceptor blockade induced by this drug. Some β -adrenoceptor blockers (e.g. atenolol and propranolol) have been shown to be taken up and released by sympathetic nerve endings [11, 12], and this can produce persistent β -adrenoceptor blockade with very low plasma levels. On the other hand, we have recently demonstrated *in vitro* that the rates of

association and dissociation of blockade by timolol in human atrial appendage are slow, owing probably to its high lipophilicity as well as its β_2 -adrenoceptor affinity [6]. This phenomenon may well explain our present *in vivo* findings of persistent β -adrenoceptor blockade long after its predicted disappearance from the circulation.

In conclusion, nonselective β -adrenoceptor blockade with timolol for 14 days causes persistent β -adrenoceptor blockade for much longer than predicted from its kinetics in plasma. At 2 days post-withdrawal, β -adrenergic responses are reduced, particularly those mediated through cardiac β_2 -adrenoceptors, and these responses have normalised by 3 days post-withdrawal. Our study provides no evidence of β -adrenergic sensitisation following timolol withdrawal; at the very least, if it occurs, it is much more difficult to demonstrate than with the β_1 -selective antagonists atenolol and bisoprolol. These findings may have important therapeutic implications for the choice of β -adrenoceptor blocker in patients at risk of myocardial infarction, in whom the secretion of adrenaline will cause selective stimulation of cardiac β_2 -adrenoceptors.

Figure 4 Post-placebo and either 2 or 3 days following cessation of timolol therapy, subjects received increasing doses of salbutamol intravenously. Full details are as in Subjects and Methods. Plasma (a) potassium, (b) glucose, (c) insulin and (d) noradrenaline responses to intravenous salbutamol. Values plotted are mean $\pm$ s.e. mean. Responses post-placebo (■), 2 days (◆) and 3 days (◇) post-timolol therapy. All these metabolic responses, with the exception of the glucose response, were significantly attenuated 2 days post-timolol therapy (*).

Finally, our study provides no evidence to suggest that cross-regulation of other G protein-linked receptors in the cardiovascular system (α -adrenoceptors and angiotensin II receptors) occurs in response to nonselective β -adrenoceptor blockade.

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