Experimental Study

Effect of Thimerosal on Arrhythmia Induced by Coronary Ligation

The Involvement of ATP-dependent Potassium Channels

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SUMMARY

Thiol-modifying agents induce the release of nitric oxide (NO) from endothelial epithelium and the release of reactive oxygen free radicals in the vascular system. Moreover, thiol groups are essential for the functioning of the ATP dependent potassium channel (K-ATP). The effects of thiol-modifying agents and their molecular mechanisms on arrhythmia have not been widely studied. In this study, we investigated the effect of the hydrophilic SH-group-oxidizing substance thimerosal on the arrhythmia induced by reperfusion/ischemia after coronary artery ligation in rats. We studied the possible involvement of the K-ATP and NOS on the effect of thimerosal. Thimerosal pretreatment (3, 30 mg/kg dose iv. 10 minutes before coronary occlusion) significantly decreased the length of total arrhythmia, ventricular tachycardia, and the arrhythmia score. This effect of thimerosal was reversed by the K-ATP opener pinacidil but not by the K-ATP blocker glibenclamide. The inhibition of iNOS by L-NAME did not alter the antiarrhythmic effect of thimerosal. These data clearly suggest that the antiarrhythmic effect of thimerosal is dependent upon the blockage of K-ATP. (Int Heart J 2005; 46: 711-721)

Key words: Ischemia and reperfusion, Arrhythmia, ATP-dependent potassium channel blocker, Thimerosal

K^+ channels set the membrane potential and the excitability of most living cells.[1)] The activity of some K^+ channels is drastically altered by the oxidation of critical SH-groups of the channel protein. An interesting example is the loss of rapid inactivation of the voltage-gated K^+ channel RCK4 (Kv1.4) produced by oxidation of a thiol group; this residue is located near the intracellular inactivation (ball) domain of the channel.[2)] ATP-sensitive K^+ channels (K-ATP channels) in skeletal muscle,[3,4)] heart[5)] and pancreatic β-cells or insulin-secreting cell

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lines[6-8] are closed by thiol-oxidizing reagents, an effect that is in general reversed by the disulphide bond reducing agent 1,4-dithiothreitol (DTT). This modulation of K-ATP channel activity by thiol oxidation is of particular interest in heart and skeletal muscle. To the best of our knowledge, the effect of thiol-modifying agents on the coronary vascular K-ATP channel has not yet been studied.

Thimerosal is an agent that affects the SH group of molecules.[5,9] It affects the activity of various membrane channel proteins and intracellular enzymes.[9,10] In several studies, it was shown that it increases intracellular calcium and leads to calcium loading, like ATP dependent channel blockers.[11-13] Thimerosal causes long-lasting relaxation in smooth muscle[14] and has been suggested to produce endothelially-derived coronary dilatation.[15] It induces the release of NO from endothelial epithelium that stimulates the relaxation.[16,17] It was also reported that thimerosal leads to the release of reactive oxygen free radicals from HeLaS cells.[18] Thimerosal decreases the sensitivity of the ATP dependent potassium channel to ATP[5] and leads to the blockage of the channel.[6] The opening of the channel by the ATP dependent potassium channel opener analog P1075 was blocked by thimerosal.[19]

The involvement of opening or closing of the ATP dependent potassium channel and the mechanism to decrease myocardial ischemic or reperfusion induced damage or development of arrhythmias have been studied.[20-23] Although thimerosal was effective at blocking the ATP dependent potassium channel, only one study found that it is protective against reperfusion-induced arrhythmia.[24] No study was found indicating that the antiarrhythmia produced by thimerosal depends on blockage of the ATP dependent channel or that another factor is responsible for this protection.

The objectives of the present study were to investigate the effect of thimerosal on reperfusion-induced arrhythmia and the roles of the ATP dependent potassium channel and iNOS inhibition in this effect.

METHODS

Animals: Male Sprague-Dawley rats weighing 270-285 g were fed a standard laboratory rat food pellet and allowed to drink tap water *ad libitum*. All animals were treated in adherence to the guiding principles in the care and use of animals together with the protocol stipulated by the ethics committee of Hacettepe University, Turkey.

Coronary artery ligation and reperfusion: Rats were anesthetized with thiopental sodium (60 mg/kg) and then underwent a tracheotomy for artificial respiration. The left carotid artery was cannulated to measure blood pressure with a blood pressure transducer (Power Lab, AD Instruments, United Kingdom). After the

chest was opened in the fourth intercostal space, the heart was exposed and a loose loop of atraumatic silk was placed around the left main coronary artery, approximately 2 mm from its origin. The heart was returned to its origin and artificial respiration performed using a ventilator (0.9 mL/100 g body weight at a rate of 60 strokes/min). The animals were allowed to stabilize for 5 minutes before coronary ligation. The 6 minutes of ischemia was followed by 6 minutes of reperfusion. Reperfusion was produced by loosening the clamp.

At the end of the experiment the live animals were heparinized (500 IU/kg) and the heart was excised. The left coronary artery was retightened and the heart was first exposed in retrograde to 10 mL of isotonic NaCl solution and then 2 mL of ethanol for demarcation of the occluded and nonoccluded myocardium. The percentage of occluded ventricular myocardium under risk of infarction with respect to the whole ventricular myocardium was calculated.

Drug application: Thimerosal and L-NAME (Sigma, USA) were prepared in physiological saline and pinacidil in dimethyle sulfoxide/saline 1:1. Atropine (Biofarma TR) was given at a dose of 1 mg/mL/kg intravenously 10 minutes before ligation. Glibenclamide (Sigma, USA) was prepared in dimethyl sulfoxide and ethanol (1:1), and given intraperitoneally 25 minutes before ligation. Thimerosal was administered at doses of 3 and 30 mg/kg/mL, pinacidil 1 mg/kg/mL, L-NAME 20 mg/kg/mL, and glibenclamide 5 mg/kg/100 μL. The 30 mg/kg dose of thimerosal was combined with pinacidil, glibenclamide, and L-NAME individually. Atropine was used to decrease the hypotensive effect of pinacidil.

Recording: Bipolar ECGs were recorded using needle electrodes, and blood pressure was measured from the carotid artery using a blood pressure transducer (ADInstruments) and stored in a computer using the Power Lab System (ADInstruments). Arrhythmia duration and heart rate were determined from the ECGs. The incidence of arrhythmias was analyzed in accordance with the Lambeth Conventions,[25] as ventricular fibrillation (VF), ventricular tachycardia (VT), and other types of arrhythmias including extrasystoles, bigeminy, and salvos. An arrhythmia score was used to indicate the incidence and duration of arrhythmias, where 0 indicates no arrhythmia; 1 an arrhythmia duration of less than 10 seconds; 2 an arrhythmia duration of 11 to 30 seconds; 3 an arrhythmia duration of 91 to 180 seconds or reversible VF for less than 10 seconds; 5 an arrhythmia duration longer than 180 seconds or reversible VF for more than 10 seconds; and 6 an irreversible VF or death of the animal.

Statistical analyses: All data are expressed as the mean ± SE. Differences between groups were calculated using analyses of variance. After analyses of variances (LSD post hoc test), the treatment and control group were compared by Student's *t*-test (unpaired). The survival rate and incidence of arrhythmias were compared by the χ^2 method (Fisher exact test).

RESULTS

ST segment elevation and QRS changes were seen following coronary ligation in all treated animals. Following ligation, frequent arrhythmias usually appeared in the fourth minute of ligation and gradually increased toward the fifth

Table I. Heart Rate and (HR) and Mean Arterial Blood Pressure (BP) in Anesthetised Rats

			Basal		Occlusion			Reperfusion	
	Dose mg/kg	n1	HR	BP	HR	BP	n2	HR	BP
Control		10	415 ± 14	185 ± 12	367 ± 34	115 ± 10	8	347 ± 31	124 ± 21
Thimerosal	3	9	375 ± 11	198 ± 8	342 ± 31	144 ± 17	9	377 ± 9	182 ± 18*
Thimerosal	30	16	416 ± 10	190 ± 10	373 ± 25	135 ± 14	15	363 ± 30	162 ± 15
Pinacidil	1	8	409 ± 15	113 ± 9***	366 ± 44	97 ± 12	7	351 ± 65	98 ± 16
Thimerosal +	30	8	406 ± 21	138 ± 19*	316 ± 46	86 ± 12	6	257 ± 34	68 ± 16*
Pinacidil	1								
Thimerosal +	30	10	393 ± 11	152 ± 9	368 ± 32	112 ± 14	10	379 ± 23	150 ± 11
Glibenclamide	5								
Thimerosal +	30	7	400 ± 14	169 ± 11	423 ± 25	117 ± 17	7	405 ± 18	168 ± 19
L-NAME	20								
Thimerosal mg +	30	5	370 ± 31	131 ± 20**	371 ± 33	84 ± 8	2	435 ± 25	141 ± 35
Pinacidil+	1								
Atropine	1								

Results are mean ± S.E. of the animals surviving the given period (n1, and n2 means the number of these animals, respectively).
Heart rate (HR) and mean arterial blood pressure were measured before coronary artery ligation (Basal), 5 minutes after coronary ligation (Occlusion), and 5 minutes after the release of occlusion (Reperfusion).
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ Compared to the corresponding control value.

Table II. The Incidence of Arrhythmias During Reperfusion After 6 Minute Coronary Artery Ligation in Anesthetized Rats

	Dose mg/kg	N	Survived n/%	Incidence of arrhythmia				
				None	VF	VT	Other	Brady
Control		10	9/90	0/0	1/10	8/80	9/90	1/10
Thimerasol	3	9	9/100	0/0	0/0	7/78	9/100	0/0
Thimerosal	30	16	10/100	2/12	1/6	12/75	13/81	3/18
Pinacidil	1	8	8/100	1/12	1/12	6/75	7/87	3/37
Thimerasol +	30	8	6/75	1/12	3/37	6 /75	7/87	5/62
Pinacidil	1							
Thimerosal +	30	10	10/100	1/10	0/0	8/80	9/90	0/0
Glibenclamide	5							
Thimerosal +	30	7	7/ 100	0/0	0/0	7/100	7/100	
L-NAME	20							0/0
Thimeraso+ +	30	5	3/60	0/0	5/100	5/100	5/100	3/60
Pinacidil +	1							
Atropine	1							

N = Total number of animals at the beginning of reperfusion; None = no arrhythmia developed; VF = ventricular fibrillation; VT = ventricular tachycardia; Other = ventricular extrasystole, bigeminy, and salvo.

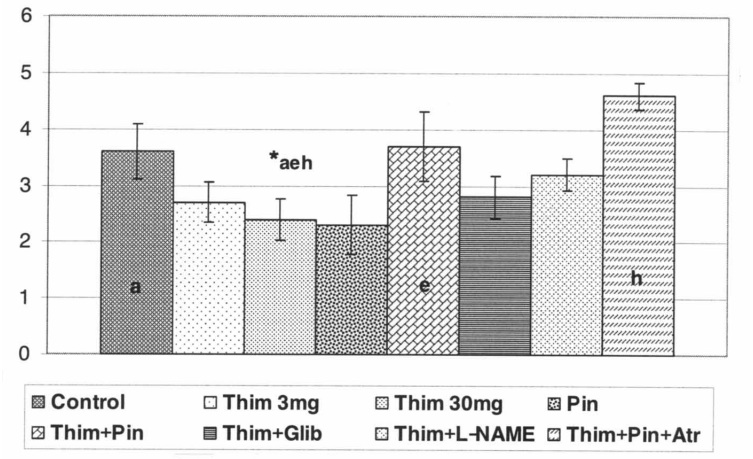

Figure 1. The score of arrhythmia according to Lambeth Convention.
*$P < 0.05$; Different from (a) control, (e) thim + pin, (h) thim + pin + atr.

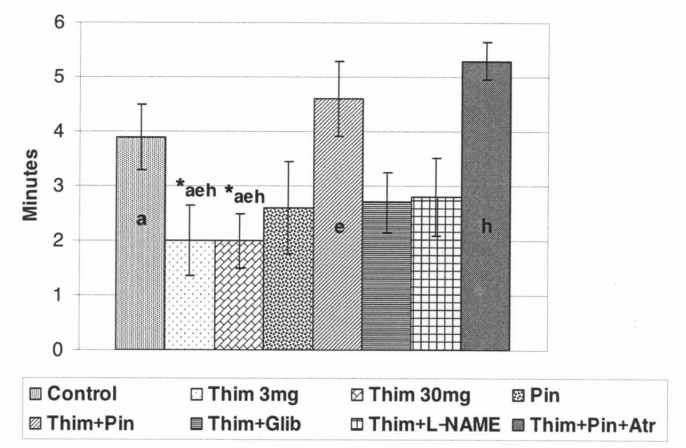

Figure 2. Arrhythmic period during reperfusion.
*$P < 0.05$; different from (a) control, (e) thim + pin, (h) thim + pin + atr

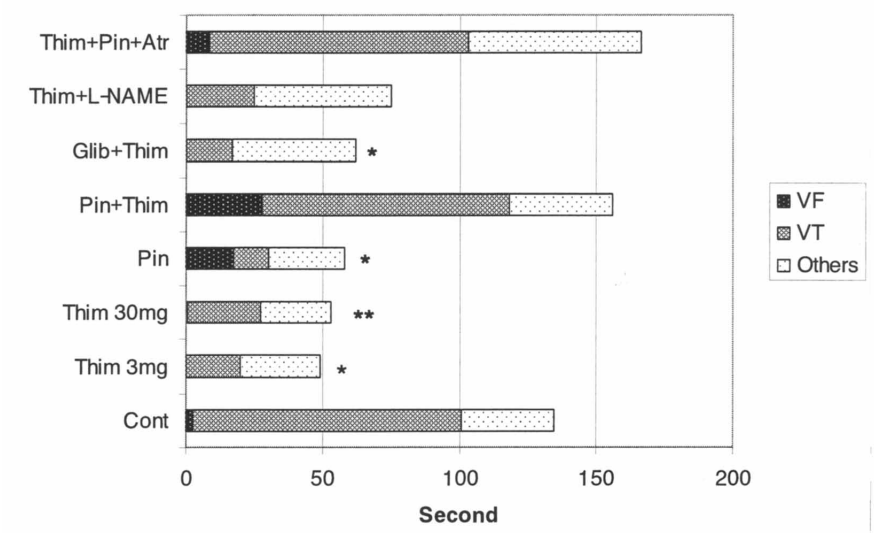

Figure 3. The type and duration of arrhythmias during 6 minutes of reperfusion.
*$P < 0.05$ **$P < 0.01$: The difference of total arrhythmia from control.

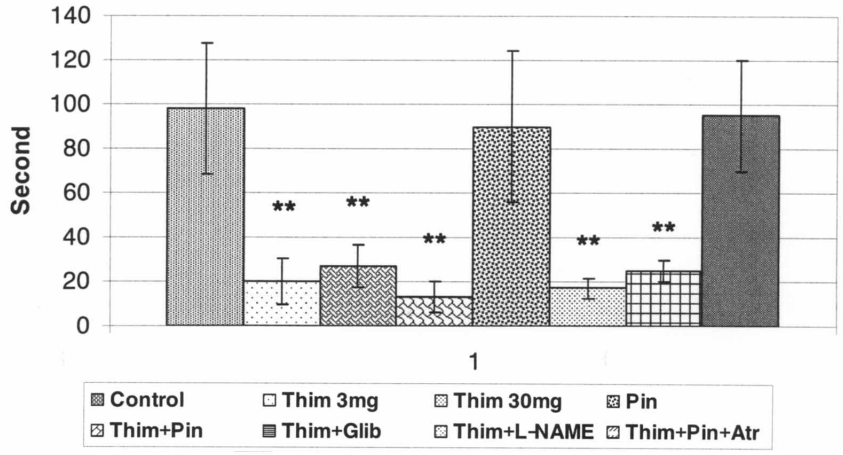

Figure 4. The length of ventricular tachycardia observed during 6 minutes of reperfusion.
**$P < 0.01$; different from control.

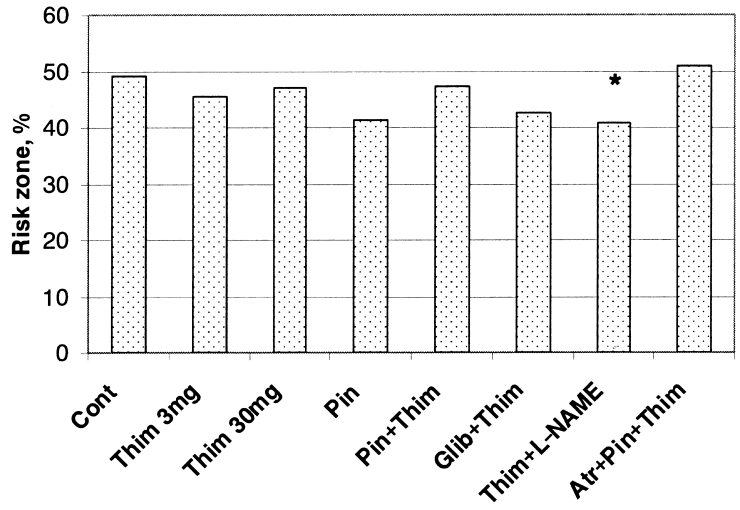

Figure 5. Risk of infarct area. *$P < 0.05$; compared with control.

minute of ischemia. Severe arrhythmias usually started between 10-30 seconds (mean = 21.7 seconds, $n = 68$) following reperfusion. There were no significant differences in the start of arrhythmias following reperfusion; but the arrhythmia ended earlier in the thimerosal pretreated animals in comparison with the control and thimerosal + pinacidil combined groups, (268 ± 29, 296 ± 43 in the control and combination group; 130 ± 40, 136 ± 30 in the 3 and 30 mg/kg thimerosal groups, respectively). There were no significant differences in the incidence or total length of bradycardia among the groups.

Blood pressure decreased significantly following coronary ligation, recovered gradually in the late phase of ischemia, and reached the control value at the end of reperfusion (Table I). During basal conditions, that is, before coronary ligation, the blood pressure was lower in the pinacidil and pinacidil + thimerosal pretreated animals. The pressure also continued to remain at a low level in the following minutes of ischemia and during reperfusion in the animals pretreated with pinacidil alone and with thimerosal. Atropine was not effective at decreasing the hypotensive effect of pinacidil. There were no significant differences in heart rate between the groups before and after coronary ligation and reperfusion (Table I).

Although thimerosal at either dose and pinacidil pretreatment alone did not affect the incidence of arrhythmias or survival rate during 6 minutes of reperfusion, the total length of arrhythmia and the length of ventricular tachycardia dur-

ing reperfusion were significantly decreased in these groups (Table II). Thimerosal at a dose of 30 mg/kg was more effective than the lower dose on the arrhythmia score when compared with the control (Figure 1). Both doses of thimerosal decreased the arrhythmic period during reperfusion (Figure 2). The combination of thimerosal with glibenclamide or L-NAME did not influence its effect on the total length of arrhythmia (Figure 3) or ventricular tachycardia (Figure 4). Otherwise, the combination of thimerosal with pinacidil abolished its antiarrhythmic effect. The total length of arrhythmia, the length of ventricular tachycardia, the arrhythmia score, and the arrhythmic period were increased in the pretreatment of thimerosal with pinacidil. The combination of atropine with pinacidil + thimerosal was not effective at decreasing the severity of arrhythmia during reperfusion. The total length of arrhythmia and the arrhythmia score were similar to those in the control and thimerosal + pinacidil combined groups.

The risk of infarct zone was not found to be different among the groups, except for the thimerosal + L-NAME group which was different compared to the control (Figure 5).

DISCUSSION

The present results demonstrate that thimerosal pretreatment decreased the length of ventricular tachycardia during ischemia and reperfusion, which agrees with the results of previous research.[24] This effect of thimerosal was independent of the dose, the first time such an effect was observed.

The K-ATP channel is closed under normal metabolic conditions; it opens in ischemia or hypoxia, when the intracellular level of ADP increases and that of ATP decreases. Channel opening renders the cell unexcitable and will salvage ATP to maintain structural integrity of the cell.[26-29] On the other hand, ischemia reduces the redox potential of the cell and decreases the level of reduced glutathione;[30] thereby thiol groups of K-ATP channels may be oxidized resulting in channel closure. Therefore, we hypothesized that thimerosal may increase NO release and/or block the KATP channels to inhibit arrhythmia induced by ischemia/reperfusion.

In this study, the antiarrhythmic effect of thimerosal on ischemia/reperfusion-induced arrhythmia was abolished when it was pretreated with pinacidil but not with glibenclamide. An *in vitro* study found that thimerosal blocked ATP dependent potassium channel opening by the opener P1075,[19] while other studies have reported an effect similar to ATP dependent potassium channel blockers, ie, that it increases calcium-loading to the cell.[12-14] Pinacidil acts as an opener and has the opposite effect of ATP dependent potassium channel blockers on the action potential duration.[20,31] Pinacidil alone decreased the severity of arrhyth-

mia, like thimerosal. This result highlights the controversy with respect to the underlying mechanism of antiarrhythmia induced by both openers or blockers, since there is no change in the antiarrhythmia induced by thimerosal when it is combined with glibenclamide. Lepran, *et al*[20] suggested that pretreatment with either pinacidil or glibenclamide may offer a significant protective effect during the acute phase of experimental myocardial infarction.

The finding that the antiarrhythmia induced by thimerosal is possibly related to the modulation of ATP-dependent potassium channels is supported by *in vitro* studies.[5,6,9] However, an additional factor may also be involved in this antiarrhythmia. Various studies have shown that both thimerosal[15-17] and pinacidil[32,33] induce coronary vasodilation. Both drugs can be expected to have a synergic effect on reperfusion-induced arrhythmia because of their vasodilatory effects. However, this was not supported by the present results.

The possibility of NO-dependent EDRF-released induction of vasodilation in the antiarrhythmia produced by thimerosal was also investigated in this study. No other studies have described the effect of thimerosal on NO release. Only one study reported that thimerosal activates endogenous NO formation.[33] The relation between NO and ATP-dependent potassium channel modulation was examined in various studies. An increase in nitric oxide activity may activate KATP, which plays a major role in the shortening of AP.[34] The effect of pinacidil on infarct size reduction is blocked by L-NAME, although L-NAME does not block the increase in coronary flow.[35] Ockalli, *et al*[36] suggested that the diazoxide-induced anti-ischemic effect via opening of mitoK(ATP) channels was NO-dependent. This suggestion supports our result showing that ATP-channel blockage is effective in antiarrhythmia induced by thimerosal. L-NAME does not alter the effect of thimerosal on reperfusion-induced arrhythmias. The iNOS inhibition by L-NAME may lead to the closing of the channels and may be synergistic to the action of thimerosal.[34,36]

Conclusion: Thimerosal decreased the arrhythmic period and lethal arrhythmia induced by reperfusion independently of the dose. This antiarrhythmic effect depends on blockage of the ATP dependent potassium channel. The inhibition of iNOS by L-NAME is not effective at reversing the influence of thimerosal.

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