

CDP-choline prevents cardiac arrhythmias and lethality induced by short-term myocardial ischemia–reperfusion injury in the rat: involvement of central muscarinic cholinergic mechanisms

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Received: 10 December 2007 / Accepted: 10 April 2008 / Published online: 27 May 2008
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Abstract In the present study, we aimed to determine whether cytidine-5'-diphosphatecholine (CDP-choline or citicoline) can improve the outcome of short-term myocardial ischemia–reperfusion injury in rats. Ischemia was produced in anesthetized rats by ligation of the left anterior descending coronary artery for 7 min followed by a reperfusion period of 7 min. Reperfusion-induced ventricular tachycardia (VT), ventricular fibrillation (VF), survival rate, and changes in arterial pressure were evaluated. Saline (1 ml/kg), CDP-choline (100, 250, and 500 mg/kg), or lidocaine (5 mg/kg) was intravenously injected in the middle of the ischemic period. Intracerebroventricular (i.c.v.) mecamylamine (50 µg) or atropine sulfate (10 µg) pretreatments were made 10 min before the coronary occlusion period. Pretreatment with intravenous (i.v.) atropine methylnitrate (2 and 5 mg/kg; i.v.) or bilateral vagotomy was performed 5 min before the induction of ischemia. An *in vivo* microdialysis study was performed in the nucleus ambiguus area (NA); choline and acetylcholine levels were measured in extracellular fluids. In control rats, VT, VF, and lethality were observed in 85%, 60% and 50% of the animals, respectively. Intravenous CDP-choline produced a short-term increase in blood pressure and reduced the incidence of VT, VF, and lethality dose-

dependently when injected in the middle of the ischemic period. CDP-choline at doses of 250 and 500 mg/kg completely prevented death. Intracerebroventricular atropine sulfate pretreatment completely abolished the protective effect of CDP-choline, while mecamylamine pretreatment had no effect on the drug. CDP-choline increased the levels of extracellular choline and acetylcholine in the NA area. Bilateral vagotomy completely abolished the protective effect of CDP-choline in the reperfusion period. Moreover, the intravenous pretreatment with atropine methylnitrate produced dose-dependent blockade in the reduction of VT, VF, and mortality rates induced by CDP-choline. Neither of these pretreatments except mecamylamine affected the pressor effect of CDP-choline. Intracerebroventricular mecamylamine attenuated the increase in blood pressure induced by CDP-choline. In conclusion, intravenously injected CDP-choline prevents cardiac arrhythmias and death induced by short-term myocardial ischemia–reperfusion injury. Activation of central muscarinic receptors and vagal pathways mediates the protective effect of CDP-choline. The protective effect of CDP-choline is not related to its pressor effect.

Keywords CDP-choline · Myocardial ischemia-reperfusion · Cholinergic · Muscarinic · Central · Vagal

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Introduction

Cytidine-5'-diphosphatecholine (CDP-choline or citicoline) is an endogenously synthesized mononucleotide and intermediate product of membrane phosphatidylcholine (Weiss 1995). It is also used as a drug for the treatment of brain trauma and stroke in several European countries. After oral

or intravenous (i.v.) administration, it is rapidly hydrolyzed to cytidine and choline by membrane phosphodiesterases, resulting in increased plasma levels of these metabolites (Lopez G-Coviella et al. 1987, 1995; Savci et al. 2002, 2003; Wurtman et al. 2000). Both choline and cytidine are taken up by cells, mediate several physiological and pharmacological effects, and contribute to the intracellular resynthesis of CDP-choline (Weiss 1995; Cansev 2006). The effect of CDP-choline in ischemia and hypoxia is well documented (Adibhatla and Hatcher 2005). The proposed general mechanisms of CDP-choline's protective effects in ischemia are a lessening of membrane-phospholipid breakdown and stabilization of membrane integrity (Adibhatla and Hatcher 2005; Adibhatla et al. 2002; Adibhatla et al. 2006). We previously reported that CDP-choline exerts several cardiovascular and endocrine effects by activating central cholinergic transmission (Savci et al. 2002, 2003; Cavun et al. 2004; Cavun and Savci 2004). It increases blood pressure in normotensive animals and reverses hypotension in hemorrhagic shock (Savci et al. 2002, 2003; Yilmaz et al. 2006). It also affects the plasma levels of several pituitary hormones (Cavun et al. 2004; Cavun and Savci 2004). Those effects of CDP-choline are mainly mediated by activation of central nicotinic and/or muscarinic mechanisms. Recently, it was reported that intraperitoneal (i.p.) administration of choline protects ischemic myocardium by activating peripheral M₃-subtype muscarinic cholinergic receptors (Yang et al. 2005). On the other hand, activation of the central vagal pathway plays a very important role in protecting against cardiac arrhythmias or tissue necrosis induced during myocardial ischemia–reperfusion studies (Mioni et al. 2005; Kakinuma et al. 2005; Bernik et al. 2002; Ando et al. 2005). Moreover, the cytoprotective effect of cholinergic nicotinic receptor activation has already been demonstrated (Newman et al. 2002). Therefore, in view of these observations and the fact that CDP-choline exerts some of its effects by activating the central cholinergic system, we hypothesized that CDP-choline may have a protective effect on cardiac arrhythmias induced by short-term myocardial ischemia–reperfusion injury. The present study was designed to test this hypothesis. We also examined the involvement of the central and/or peripheral cholinergic mechanisms in these effects.

Materials and methods

Adult male Wistar Albino rats (250–300 g; Experimental Animals Breeding and Research Center, Uludag University Medical Faculty, Bursa, Turkey) were used in the present study. They were housed under a 12-h light/dark cycle with food pellets and tap water available ad libitum. The surgical and experimental protocols were approved by the Animal

Care and Use Committee of Uludag University and were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Surgical and preparative procedures

After anesthesia with urethane (1.25 g/kg intraperitoneally; Fluka; Buchs, Switzerland), rats were placed in the supine position on a heated operating platform to maintain a rectal temperature of 37.5°C. Temperatures were monitored using a rectal probe throughout the study. The left common carotid artery (for monitoring blood pressure) and the left jugular vein (for drug administration) were cannulated with PE 50 tubing filled with heparinized saline (250 U/ml). During the arterial cannulation procedure, the vagus nerve and the cervical sympathetic trunk were separated very carefully. For blood pressure and heart rate monitoring, the arterial cannula was connected to a volumetric pressure transducer (BPT 300), which was attached to a DA100B general purpose transducer amplifier (Commat, Ankara, Turkey). Blood pressures of rats were recorded and analyzed using the MP100 system and AcqKnowledge software (BIOPAC Systems, CA, USA). Blood pressure is reported as mean arterial pressure (MAP; mmHg), and heart rate is expressed as beats per minute (BPM). For intracerebroventricular injection of drugs, a burr hole was drilled through the skull 1.5 mm lateral to the midline, 1.0 mm posterior to the bregma. A 22-gauge stainless steel hypodermic tubing was directed through the hole toward the lateral ventricle. The cannula was lowered 4.5 mm below the surface of the skull and was fixed to the skull with acrylic cement. After intubation of the trachea, the animals were ventilated with room air via a respirator for small rodents (CWE model SAR-830/AP, PA, USA) with a stroke volume of approximately 20 ml/kg and a rate of 70 strokes per minute. Needle electrodes were placed subcutaneously on the limbs, and an electrocardiogram (ECG) was continuously recorded. The chest was then opened by left thoracotomy, the pericardium was incised, the heart was exteriorized, and a loose loop (6/0 braided silk suture attached to a 10-mm micropoint reverse cutting needle) was placed around the left main coronary artery. The heart was replaced in the chest cavity with the ligature ends exteriorized, and any animal in which this procedure produced dysrhythmias or a sustained fall in mean arterial pressure to <60 mmHg was withdrawn from the study at this point.

Ischemia/reperfusion procedure

After an equilibration period of 20 min, the ligature was tied to start the ischemia; after a 7-min period of coronary occlusion, reperfusion was obtained by loosening the suture, and the animals were then monitored for a further 7 min.

The blood pressure, heart rate, occurrence of dysrhythmias, and lethality were recorded. Each rat underwent a single coronary occlusion. Rats subjected to sham ischemia underwent all surgical procedures experienced by the ischemic rats, except coronary ligation.

Evaluation of arrhythmias

The ECG was continuously recorded up to the seventh minute after reperfusion and was retrospectively analyzed for the incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF). All analyses were carried out in accordance with the Lambeth Conventions (Walker et al. 1988). Ventricular tachycardia was defined as four or more consecutive premature beats of ventricular origin, and ventricular fibrillation was defined as a signal in which individual QRS deflections could no longer be distinguished from one another and for which the rate could not be determined.

Bilateral cervical vagotomy

Five minutes before coronary occlusion, rats were subjected to bilateral cervical vagotomy or sham surgical procedures. For the cervical vagotomy, a cervical midline incision was performed, the vagus nerve was dissected carefully to avoid tissue damage, and cut bilaterally with microsurgery scissors. For the sham surgery, animals underwent the same procedure, except no nerves were severed.

Microdialysis study

Microdialysis probes were made by Murat Yalcin. The molecular weight cutoff of the dialysis membrane was 18,000 Da and the length was 1 mm. Rats were anesthetized with urethane (1.25 g/kg; i.p.) and placed in a stereotaxic frame. The skull was exposed and drilled over the nucleus ambiguus (coordinates: 12.8 mm posterior to the bregma, 2.0 mm lateral to the midline, and 9.8 mm vertical to the skull) according to Rat Brain Atlas (Paxinos and Watson 2005). Handmade probes were implanted and fixed with acrylic cement to the skull. The dialysis probe was perfused with artificial cerebrospinal fluid (pH 7.4) of the following composition: 148 mM NaCl, 3.0 mM KCl, 1.4 mM CaCl₂, 0.8 mM MgCl₂, 1.3 mM NaH₂PO₄, 0.2 mM Na₂HPO₄. The perfusion rate was adjusted to 2 µl/min. Dialysate samples were collected at 10-min intervals. Dialysis probe was perfused for the first 90-min period for stabilization. After this period, three consecutive samples were collected, and the mean value of these samples was used as the basal choline and acetylcholine levels of the rats. CDP-choline (250 mg/kg) or saline (1 ml/kg) was injected intravenously, and collection of dialysate samples was continued for the next 60 min.

HPLC measurement of choline and acetylcholine levels

Dialysate samples were injected into a high-performance liquid chromatography (HPLC) system with an immobilized enzyme reactor and an electrochemical detector (Jasco 840 EC). Acetylcholine and choline were separated on a cation exchange column (from B.A.S.). An enzyme reactor containing acetylcholinesterase and choline oxidase converted choline to hydrogen peroxide. Hydrogen peroxide was then electrochemically detected with a platinum electrode at +0.500 V. The mobile phase consisting of 0.05 M Na₂HPO₄ (pH 8.5) and antibacterial Kathon (0.5%) was delivered by an HPLC pump (Jasco PU 980). The flow rate was 1.0 ml/min. Chromatograms were completed within 6 min.

Drugs and treatments

All drugs used throughout the study were purchased from Sigma-Aldrich Chemie GmbH (Germany). CDP-choline was partly purchased from Sigma-Aldrich Chemie GmbH and also was a gift from Grupo Ferrer Internacional S.A., Spain. The chemicals were dissolved in saline (0.9% NaCl). Intravenous injection volumes were 0.1 ml/kg, and intracerebroventricular injection volumes were 10 µl. Saline, CDP-choline (100, 250, and 500 mg/kg) and lidocaine (5 mg/kg) were intravenously injected in the middle of the ischemia period. Intracerebroventricular mecamylamine (50 µg) or atropine sulfate (10 µg) pretreatments were made 10 min before the coronary occlusion period. Atropine methylnitrate (2 and 5 mg/kg; i.v.) and methyllycaconitine (1 mg/kg; i.v) pretreatments were administered 5 min before induction of the ischemia.

Statistics

The incidences of VT, VF, and lethality were compared by using Fisher's exact probability test. Mean arterial pressure values and extracellular choline and acetylcholine changes were analyzed by means of repeated measures of analysis of variance (two-way analysis of variance) with a post hoc Tukey's test. A *P* value <0.05 was considered significant.

Results

Effect of CDP-choline treatment on short-term myocardial ischemia/reperfusion-induced cardiac arrhythmias, arterial pressure, and survival in rats

Coronary reperfusion following the 7-min ischemic period produced the immediate occurrence of severe ventricular

arrhythmias (VT in 11 of 13 rats; VF in 8 of 13 rats) and caused the death of 7 of 13 rats within the 7-min reperfusion period in the saline control group (Table 1). Intravenous injection of CDP-choline (100, 250, and 500 mg/kg) 3.5 min before reperfusion (i.e., 3.5 min after coronary occlusion) reduced the incidence of arrhythmias and lethality dose-dependently (Table 1). No episode of VF occurred with a dose of 250 mg/kg, and complete survival was obtained with doses of 250 and 500 mg/kg. A dose of 250 mg/kg of CDP-choline had antiarrhythmic and survival benefits comparable to those of lidocaine (5 mg/kg; i.v.), which was used as a reference antiarrhythmic compound (Table 1).

In the control group, the arterial pressure of the living rats tended to decrease during the reperfusion period following the occlusion; however, it did not reach to statistically significant levels (Fig. 1A). Intravenous injection of CDP-choline in the middle of the ischemic period caused a prompt increase in arterial pressure in a dose-dependent manner (Fig. 1A). The pressor effect was temporary. The increase in arterial pressure after treatment with CDP-choline reached its maximum within 1 min and returned to control values at the beginning of the reperfusion period (Fig. 1A). CDP-choline did not change the heart rate under these conditions (Fig. 1B).

Effect of intracerebroventricular atropine sulfate or mecamylamine pretreatments on the protective effect of CDP-choline in myocardial ischemia–reperfusion injury

As our previous results showed that CDP-choline enhances central cholinergic transmission and exerts its cardiovascular and endocrine effects through the activation of central nicotinic or muscarinic receptors (Savci et al. 2002, 2003;

Table 1 Effect of CDP-choline or lidocaine treatments on the incidence of cardiac arrhythmias and mortality induced by myocardial ischemia–reperfusion injury in anesthetized rats

Treatment (i.v.)	Incidence of		
	VT	VF	Mortality
Sham	0/8	0/8	0/8
Saline (1 ml/kg)	11/13*	8/13*	7/13*
CDP-choline (100 mg/kg)	4/12**	5/12	5/12
CDP-choline (250 mg/kg)	1/12**	0/12**	0/12**
CDP-choline (500 mg/kg)	3/12**	1/12**	0/12**
Lidocaine (5 mg/kg)	1/8**	0/8**	0/8**

Treatments were performed in the middle of the ischemic period, that is, 3.5 min before the reperfusion. The sham treatment group underwent all surgical procedures except ligation of the coronary artery.

VT ventricular tachycardia, VF ventricular fibrillation

* $P < 0.05$, vs. sham group; ** $P < 0.05$, vs. saline group

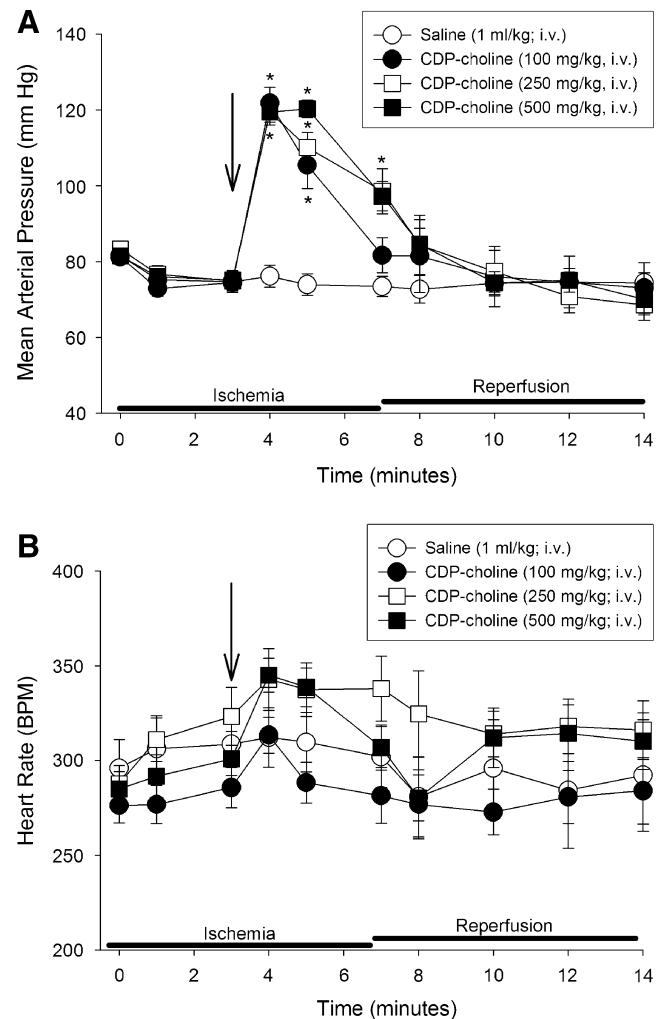


Fig. 1 Cardiovascular effects of CDP-choline under short-term myocardial ischemia–reperfusion conditions. Rats were subjected to ischemia for 7 min and reperfusion for 7 min. Mean arterial pressure (A) and heart rate (B) of the animals were monitored during both periods. Arrows in the figures represent the treatment time point. Data are presented as the mean \pm SEM in mean arterial pressure and beats per minute in heart rate. * $P < 0.05$, significantly different from the saline group

Cavun et al. 2004; Cavun and Savci 2004), we aimed to investigate whether activation of either of those receptors might be involved in the protective effect of CDP-choline during the reperfusion period. Therefore, we pretreated rats with atropine sulfate (10 μ g; i.c.v.), a nonselective muscarinic receptor antagonist or mecamylamine (50 μ g; i.c.v.), a nonselective nicotinic receptor antagonist, 13.5 min (in the middle of the stabilization period) before CDP-choline injection. These pretreatments themselves did not alter the incidence of VT, VF, or mortality rates of animals (Table 2) observed during reperfusion period. Atropine sulfate pretreatment greatly abolished the protective effect of CDP-choline (250 mg/kg; i.v.) on the occurrence of both cardiac arrhythmias (VT and VF) and lethality induced by short-term myocardial ischemia–

Table 2 Influence of pretreatment with atropine sulfate or mecamlamine on the incidence of VT, VF and mortality following treatment with CDP-choline

Pretreatment (i.c.v.)	Treatment (i.v.)	Incidence of		
		VT	VF	Mortality
Saline	Saline	7/7	6/7	6/7
Saline	CDP-choline	1/7*	0/7*	0/7*
Atropine sulfate	Saline	5/8	6/8	6/8
Atropine sulfate	CDP-choline	8/8	7/8	7/8
Mecamlamine	Saline	7/12	9/12	8/12
Mecamlamine	CDP-choline	1/7*	0/7*	0/7*

Intracerebroventricular administration of saline (10 μ l), atropine sulfate (10 μ g), or mecamlamine (50 μ g) was performed 10 min before the coronary occlusion period. Saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) was injected 3.5 min before the reperfusion period.

VT ventricular tachycardia, VF ventricular fibrillation

* $P < 0.05$ vs "saline + saline" and "mecamlamine + saline" groups

reperfusion injury (Table 2). However, mecamlamine pretreatment did not influence the protective effect of CDP-choline under these conditions (Table 2).

Intracerebroventricular atropine sulfate pretreatment did not affect the increase in arterial pressure induced by i.v. injection of CDP-choline (250 mg/kg), while meca-

mylamine attenuated the pressor effect of the drug (Fig. 2A).

Effect of CDP-choline administration on the extracellular choline and acetylcholine levels at the nucleus ambiguus

We next sought to determine if i.v. administration of CDP-choline can increase the choline and/or acetylcholine levels in the nucleus ambiguus (NA), which is the main pool of vagal motor neurons in the brainstem area controlling heart function. CDP-choline (250 mg/kg; i.v.) increased both the choline [treatment-time, $F(6,48)=17.80$, $P < 0.001$] and acetylcholine [treatment-time, $F(6,48)=7.98$, $P < 0.001$] levels at the NA. Maximum increases were 47% and 67% for the choline and acetylcholine levels, respectively (Fig. 3A,B).

Effect of bilateral cervical vagotomy on the preventive effect of CDP-choline on cardiac arrhythmias and death induced by myocardial ischemia/reperfusion injury

We also investigated whether the vagal cholinergic pathway is involved in the cardioprotective effect of CDP-choline by performing a bilateral cervical vagotomy before the treatment with CDP-choline (250 mg/kg). The protective effect of CDP-choline was completely prevented by the

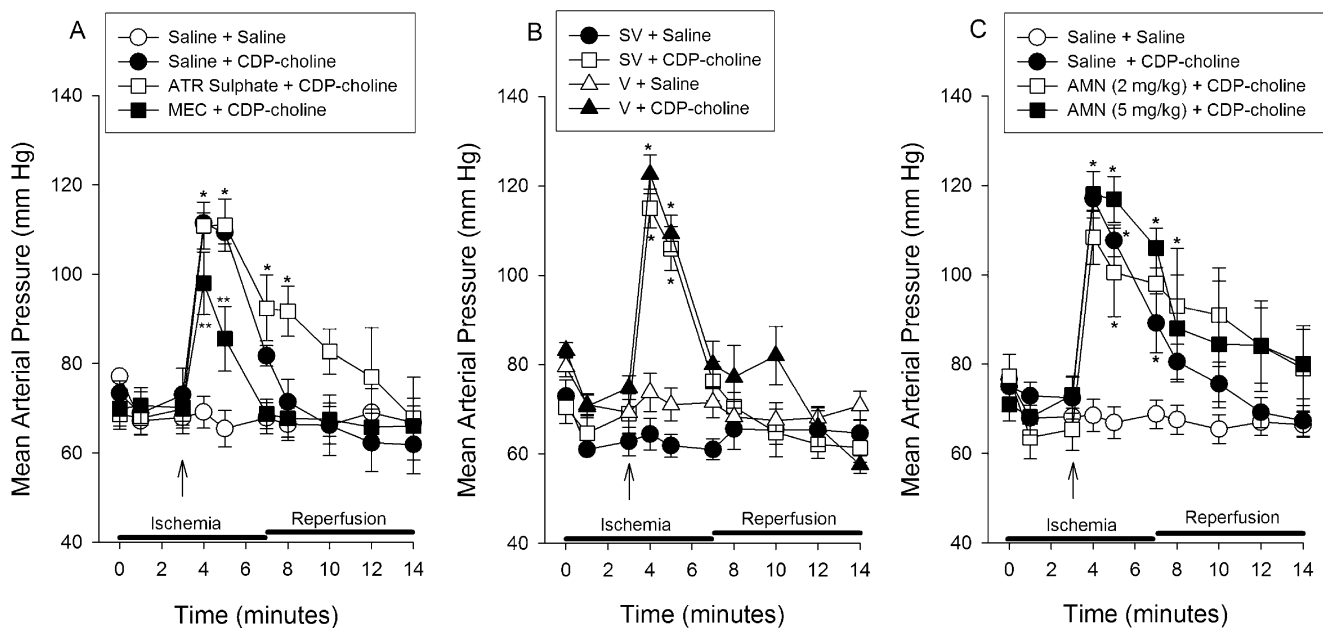


Fig. 2 Effect of CDP-choline on blood pressure in rats: **A** pretreated with atropine sulfate or mecamlamine, **B** underwent bilateral vagotomy, **C** pretreated with atropine methylnitrate under myocardial ischemia-reperfusion conditions. Rats were subjected to ischemia for 7 min and reperfusion for 7 min. Mecamlamine (50 μ g; i.c.v.) or atropine sulfate (10 μ g; i.c.v.) pretreatments were made 10 min before the coronary occlusion period. Bilateral cervical vagotomy or atropine methylnitrate (2 and 5 mg/kg; i.v.) pretreatments was performed 5 min

before the coronary occlusion period. Arrows in the figures represent the second injection (saline or CDP-choline) time point. Data are presented as the mean \pm SEM in mean arterial pressure. * $P < 0.05$, significantly different from their control values; ** $P < 0.05$, significantly different from "saline + CDP-choline" group. ATR atropine, AMN atropine methylnitrate, MEC mecamlamine, SV sham vagotomy, V vagotomy

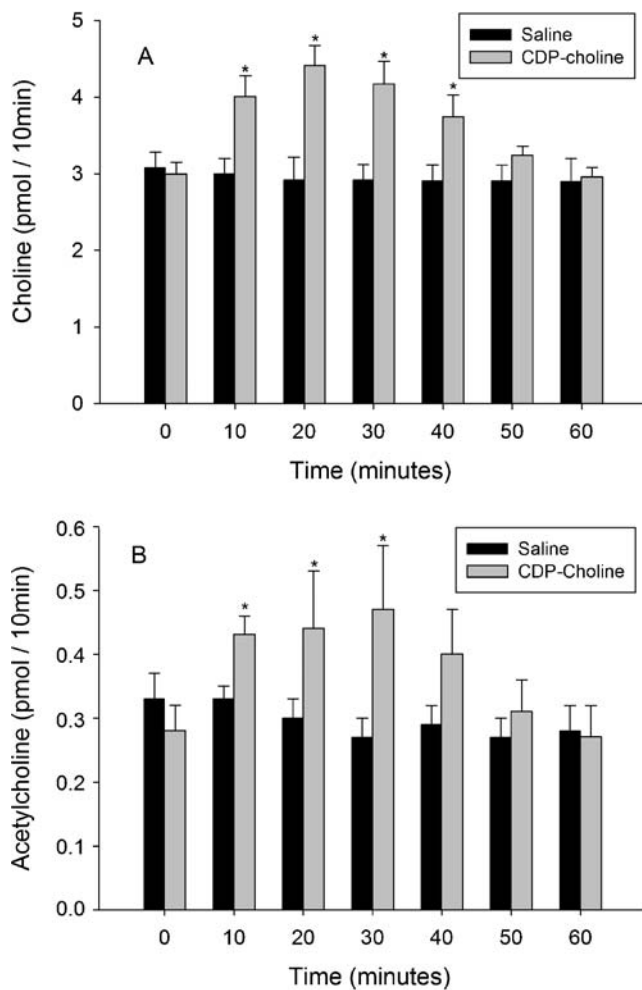


Fig. 3 Effect of CDP-choline treatment on the extracellular choline (A) and acetylcholine (B) levels at the nucleus ambiguus. CDP-choline (250 mg/kg) or saline (1 ml/kg) was injected intravenously and collection of dialysate samples was continued for the next 60 min. * $P < 0.05$, significantly different from saline group

bilateral cervical vagotomy (Table 3). Bilateral cervical vagotomy did not influence the pressor effect of CDP-choline under those conditions (Fig. 2B).

Effect of i.v. injected atropine methylnitrate treatment on the preventive effect of CDP-choline on cardiac arrhythmias and death induced by myocardial ischemia/reperfusion injury

To determine whether activation of peripheral muscarinic receptors through activation of the efferent vagal pathway is involved in the protective effect of CDP-choline, rats were pretreated with atropine methylnitrate (2 and 5 mg/kg; i.v.), which can not cross the blood brain barrier, before CDP-choline (250 mg/kg; i.v.) administration. Atropine methylnitrate at the dose of 2 mg/kg did not significantly alter the protection induced by CDP-choline on the occurrence of VT, VF, and lethality (Table 4) during the reperfusion

period; however, when the dose increased to 5 mg/kg, atropine methylnitrate pretreatment inhibited the protective effect of CDP-choline (Table 4). Although the mortality rates of rats pretreated with the higher dose of atropine methylnitrate seemed to be lower than those observed in saline or 2 mg/kg atropine methylnitrate-pretreated rats, they were not statistically significant. This pretreatment, at either dose, did not block the increase in blood pressure in response to CDP-choline (250 mg/kg; i.v.) injection (Fig. 2C).

We, additionally, wanted to examine if the activation of alpha7-nicotinic receptors located on the membranes of inflammatory cells can be involved in the cardiovascular effects of CDP-choline observed during reperfusion period. To clarify this point, rats were pretreated with methyllycaconitine (MLA; 1 mg/kg; i.v.) 5 min before CDP-choline (250 mg/kg; i.v.) administration. This pretreatment did not affect the reduced incidence of VT (1/8 vs 1/6; saline + CDP-choline vs MLA + CDP-choline), VF (0/8 vs 0/6; saline + CDP-choline vs MLA + CDP-choline), and lethality (0/8 vs 0/6; saline + CDP-choline vs MLA + CDP-choline) by CDP-choline pretreatment.

Discussion

These data show that intravenously injected CDP-choline reduces the incidence of VT and VF and the mortality rate of rats in a short-term myocardial ischemia-reperfusion study. Intracerebroventricular atropine sulfate pretreatment and bilateral cervical vagotomy completely blocked the protective effect of CDP-choline, while intracerebroventricularly administered mecamylamine had no effect on the protection afforded by CDP-choline. On the other hand, intravenously given atropine methylnitrate dose-dependently inhibited CDP-choline's protective effects under these conditions. CDP-choline increased the levels

Table 3 Influence of bilateral vagotomy on the ability of CDP-choline to reduce cardiac arrhythmias and mortality in the reperfusion period

Surgical preparation	Treatment (i.v.)	Incidence of		
		VT	VF	Mortality
Sham vagotomy	Saline	6/8	5/8	6/8
Sham vagotomy	CDP-choline	1/8*	1/8*	0/8*
Bilateral vagotomy	Saline	8/8	6/8	6/8
Bilateral vagotomy	CDP-choline	7/8	7/8	5/8

Bilateral vagotomy or a sham operation was performed 5 min before the coronary occlusion period. Saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) was injected 3.5 min before the reperfusion period. VT ventricular tachycardia, VF ventricular fibrillation

* $P < 0.05$ vs. "sham vagotomy + saline" group

Table 4 Influence of pretreatment with atropine methylnitrate on the effects of CDP-choline on VT, VF, and mortality during reperfusion period

Pretreatment (i.v.)	Treatment (i.v.)	Incidence of		
		VT	VF	Mortality
Saline	Saline	8/8	7/8	6/8
Saline	CDP-choline	1/8*	0/8*	0/8*
AMN (2 mg/kg)	Saline	4/5	4/5	4/5
AMN (2 mg/kg)	CDP-choline	2/8*	2/8*	0/8*
AMN (5 mg/kg)	Saline	6/8	7/8	3/8
AMN (5 mg/kg)	CDP-choline	3/9	5/9	3/9

Saline (1 ml/kg; i.v.) or atropine methylnitrate (2 and 5 mg/kg; i.v.) pretreatments were performed 5 min before the coronary occlusion period. Saline (1 ml/kg; i.v.) or CDP-choline (250 mg/kg; i.v.) was injected 3.5 min before the reperfusion period.

VT ventricular tachycardia, VF ventricular fibrillation, AMN atropine methylnitrate

* $P < 0.05$, significantly different from corresponding control groups

of extracellular choline and acetylcholine in the NA area. The pressor effect of CDP-choline was partially blocked by pretreatment with mecamylamine, but neither vagotomy nor atropine pretreatments changed the cardiovascular effect of the drug.

In rats that were applied myocardial ischemia–reperfusion injury, a very high incidence of severe ventricular arrhythmias and lethality occurred during reperfusion period. Intravenous administration of CDP-choline in the middle of the ischemic period was able to abrogate the development of serious arrhythmias, resulting in a high survival rate. The effect was dose-dependent. The occurrence of VT was significantly reduced starting at a dose of 100 mg/kg, and no episode of VF occurred with a dose of 250 mg/kg. Virtually complete survival was obtained with i.v. doses of 250 and 500 mg/kg CDP-choline. Indeed, 250 mg/kg CDP-choline was as effective as lidocaine, which was used as a positive reference for an antiarrhythmic effect during ischemia-reperfusion.

Our findings demonstrate that the protective effect of CDP-choline was mediated by activation of the brain cholinergic system through stimulation of the vagal pathway as well as activation of central muscarinic cholinergic receptors because: (1) i.c.v. pretreatment with atropine sulfate completely blocked the prevention of VT, VF, and lethality induced by CDP-choline, while i.c.v. mecamylamine did not alter these responses to CDP-choline; (2) the extracellular choline and acetylcholine levels in the NA area increased after i.v. CDP-choline administration; (3) bilateral cervical vagotomy greatly abolished the protective effect of the drug.

We previously reported that intravenously injected CDP-choline is able to increase choline levels in both plasma and

brain, i.e., in the lateral cerebral ventricle and hypothalamus (Savci et al. 2003). The increase in brain choline levels enhances the synthesis and release of acetylcholine and stimulates central cholinergic transmission (Blusztajn and Wurtman 1983). In agreement with this knowledge, our previous reports showed that the cardiovascular and endocrine effects of CDP-choline injected either centrally or peripherally are mediated by activation of central cholinergic receptors (Cavun et al. 2004; Cavun and Savci 2004; Savci et al. 2002, 2003). Our data from the present study are consistent with those previous findings and reinforce the idea that CDP-choline works as a cholinergic agent. Moreover, the present results are also in good agreement with previous reports demonstrating that the activation of central vagal pathway abolishes ventricular arrhythmias in cats during the reperfusion period after 20 min of coronary occlusion (Zuanetti et al. 1987), prevents ventricular fibrillation and sudden death in dogs with a healed myocardial infarction (Vanoli et al. 1991), and protects the myocardium from reperfusion injury (Mioni et al. 2005; Kakinuma et al. 2005; Bernik et al. 2002; Ando et al. 2005), as i.c.v. pretreatment with the nonselective muscarinic cholinergic antagonist, atropine sulfate, or bilateral cervical vagotomy before the CDP-choline injection completely blocked the protective effect of CDP-choline under reperfusion conditions. However, i.c.v. pretreatment with mecamylamine, a nonselective nicotinic cholinergic antagonist, did not affect CDP-choline's effects on the incidence of VT, VF, and mortality during the reperfusion period.

In the present study, we have repeated our previous observations on CDP-choline-induced extracellular choline and acetylcholine increases in the brain by demonstrating nearly 47% and 67% increases in choline and acetylcholine levels, respectively, in the extracellular area of the NA. It is very well known that both NA and the dorsal motor nucleus of the vagus (DmnX) are very important brainstem nuclei which control the heart (Cheng et al. 2004). Although recent anatomical evidence may imply that in rats, these nuclei and dual vagal cardiac pathways could play different roles in controlling cardiac function (Cheng et al. 2004), the NA is generally considered as an important vagal motor neuron in cardiac control (Jones 2001). In light of these reports and taking into account of our data showing that central muscarinic activation is mainly responsible for the CDP-choline-induced protection and increase in choline and acetylcholine levels in NA after CDP-choline, we suggest that CDP-choline may enhance the activity of cardiac neurons that have already been activated by ischemic injury and activated efferent vagal pathway to protect the myocardium against reperfusion injury. Indeed, our data demonstrating the dose-dependent blockade of the CDP-choline-induced responses by peripherally injected

atropine methylnitrate pretreatment supported this hypothesis and implicated that efferent vagal pathway is involved in the protective effect of CDP-choline.

Reperfusion injury after myocardial ischemia is considered an inflammatory reaction because toxic substances, including proinflammatory cytokines [e.g., tumor necrosis factor (TNF), interleukin-1, interleukin-6, interleukin-8] are released systemically after the blood flow is restored. Recently, it was demonstrated that activation of the cholinergic anti-inflammatory pathway either chemically or electrically inhibits cytokine synthesis, suppresses TNF- α release, and attenuates organ damage (Bernik et al. 2002; Kawashima and Fuji 2003). These effects are mediated by activation of the α 7-subtype of nicotinic receptors located on the membranes of macrophages (Kawashima and Fuji 2003). Therefore, the inhibition of TNF- α release, through activation of the central vagal pathway and/or ganglionic transmission after CDP-choline administration, could still be the one of the mechanisms which mediate CDP-choline's protective effect. However, our results showing that pretreatment of rats with the α -7 nicotinic receptor antagonist methyllycaconitine did not block the protective effect of CDP-choline ruled out this possibility. We believe that the dose of methyllycaconitine (1 mg/kg; i.v.) was enough to block the α -7 nicotinic receptors on macrophages, as the reported (Stegelmeyer et al. 2003) maximal serum concentrations (\sim 2 μ M) of i.v. injected twice dose of methyllycaconitine (2 mg/kg) was almost 90-fold higher than its recently reported IC₅₀ values (24.0 \pm 3.4 nM) to block the nicotine mediated attenuation of proinflammatory mediator release in activated macrophages (De Jonge et al. 2005). It was also reported that the i.v. pretreatment of methyllycaconitine with the dose of 0.174 mg/kg blocked the antinociceptive effect of choline (32 mg/kg; i.v.) in the late phase of formalin test in mice (Wang et al. 2005). Finally, as methyllycaconitine is a very potent toxin and antagonist at peripheral, not only α -7, but also α -1 nicotinic receptors whose inhibition may cause in neuromuscular blockade and death (Dobelis et al. 1999), we did not consider to use higher doses of the drug for this study.

In the present study, intravenously injected CDP-choline increased arterial pressure dose-dependently. However, the results suggested that the protective effects of CDP-choline in the reperfusion period were not mediated by the drug's pressor effect because: (1) the increases in blood pressure were very short-lived at all doses injected so that the blood pressure of rats returned to control levels by the beginning of the reperfusion period; (2) the atropine sulfate and atropine methylnitrate pretreatments and bilateral cervical vagotomy did not change the blood pressure response to CDP-choline, while they blocked the protective effect of the drug; and (3) mecamylamine partially blocked the

pressor response; however, it did not affect the protective effect of CDP-choline on the myocardium. On the other hand, as far as the pressor response of CDP-choline is concerned, the results are in very good agreement with our previous cardiovascular data in which we showed that activation of the central nicotinic receptor is involved in the pressor effect of CDP-choline (Savcı et al. 2003).

Collectively, our data demonstrate that CDP-choline exerts strong protection against cardiac arrhythmias and lowers mortality rates induced by myocardial ischemia–reperfusion by activating central muscarinic receptors through increased brainstem cholinergic transmission and by activating efferent vagal pathways. The pressor effect of the drug is not involved in its protective effect during reperfusion period. CDP-choline has been used in the treatment of several ischemic brain diseases in several countries. It is very well tolerated and safe drug that no serious side effects have been reported in any series of patients treated with citicoline (Secades and Lorenzo 2006). The present results further support the potential usefulness of CDP-choline in ischemic myocardial diseases.

Acknowledgment This study was supported by the grant from Scientific Research Foundation of Uludag University (2003/31). We are grateful to Cagatay Buyukuysal (Uludag University Faculty of Medicine, Department of Bioistatistic) for his valuable help with the statistical analysis. We would like to thank Dr. Daryl Henderson for his kind help in editing our manuscript.

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