Mildronate, an Inhibitor of Carnitine Biosynthesis, Induces an Increase in Gamma-Butyrobetaine Contents and Cardioprotection in Isolated Rat Heart Infarction

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Abstract: The inhibition of gamma-butyrobetaine (GBB) hydroxylase, a key enzyme in the biosynthesis of carnitine, contributes to lay ground for the cardioprotective mechanism of action of mildronate. By inhibiting the biosynthesis of carnitine, mildronate is supposed to induce the accumulation of GBB, a substrate of GBB hydroxylase. This study describes the changes in content of carnitine and GBB in rat plasma and heart tissues during long-term (28 days) treatment of mildronate [i.p. (intraperitoneal) 100 mg/kg/daily]. Obtained data show that in concert with a decrease in carnitine concentration, the administration of mildronate caused a significant increase in GBB concentration. We detected about a 5-fold increase in GBB contents in the plasma and brain and a 7-fold increase in the heart. In addition, we tested the cardioprotective effect of mildronate in isolated rat heart infarction model after 3, 7, and 14 days of administration. We found a statistically significant decrease in necrotic area of infarcted rat hearts after 14 days of treatment with mildronate. The cardioprotective effect of mildronate correlated with an increase in GBB contents. In conclusion, our study, for the first time, provides experimental evidence that the long-term administration of mildronate not only decreases free carnitine concentration, but also causes a significant increase in GBB concentration, which correlates with the cardioprotection of mildronate.

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INTRODUCTION

Mildronate (3-(2,2,2-trimethylhydrazinium)propionate dihydrate) was identified 20 years ago as an inhibitor of gamma-butyrobetaine hydroxylase,[1] an enzyme that catalyzes the synthesis of carnitine from gamma-butyrobetaine (GBB) in the liver.[2] Because carnitine is known to be an essential regulatory molecule during mitochondrial metabolism of long-chain fatty acids in myocardial energy metabolism,[3,4] the first pharmacologic application of mildronate was sought in cardiovascular pharmacology. It was found that mildronate ameliorates cardiac function during ischemia by modulating myocardial energy metabolism.[5,6] In a recent study it has been shown that a 10 day course of mildronate reduces myocardial infarct size without affecting hemodynamics,[5,7] but no additional analysis was performed to study the underlying biochemical mechanisms of the cardioprotective effect of mildronate.

By inhibiting the biosynthesis of carnitine, mildronate shifts the equilibrium of GBB hydroxylase and, thus, increases the concentration of its substrate, GBB, which is mostly known as a bioprecursor of carnitine.[4,8,9] The importance of GBB in mediating the cardioprotective effect of mildronate remains obscure. After 10 days of pretreatment with mildronate, elevated GBB content in heart tissues and protection against hydrogen peroxide–induced damage have been found in an isolated perfused rat heart model.[10] Although in some other studies it has been hypothesized that the beneficial pharmacologic effect of mildronate might be, at least partially, mediated by GBB accumulation in tissues as a result of the inhibition of GBB hydroxylase by mildronate,[10] the influence of mildronate administration on GBB content in comparison to the decreased carnitine concentration and its cardioprotective effect has not been evaluated. Therefore, we undertook this study to investigate the effect of long-term (28 days) mildronate administration on carnitine and GBB contents in rat plasma and heart tissues. The present study provides the first evidence of the time-dependent regulation of the GBB and mildronate contents in rat tissues, which correlated with the cardioprotection of mildronate.

MATERIALS AND METHODS

Chemicals

1-Aminoanthracene (1-AA) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) were purchased from Aldrich (USA). Methanol was purchased from Merck (Germany). Acetone, acetic acid, chloroform, diethyl ether, potassium dihydrogen phosphate, potassium hydrogen orthophosphate, and ammonium acetate were purchased from Acros Organics (Belgium) and used without further purification. Mildronate dihydrate was obtained from JSC Grindeks (Latvia). Sodium pentobarbital solution (Narkodorm-n) was purchased from Alvetra (Germany). Carnitine, GBB, NaCl, $CaCl_2$, $MgCl_2 \times 6H_2O$, $NaHCO_3$, KH_2PO_4, glucose, ethylenediamine tetraacetic acid (EDTA), methylene blue, and triphenyltetrazolium chloride were purchased from Sigma (USA). The solid-phase extraction was performed on LC-SAX cartridges (3 mL) supplied by Supelco (USA).

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Animals

Male Wistar rats weighing 200–250 g were housed under standard conditions (21°–23°C, 12 hours light–dark cycle) with unlimited access to food (R3 diet, Lactamin AB, Sweden) and water. The experimental procedures were carried out in accordance with the guidelines of the European Community and local laws and policies and were approved by Latvian Animal Protection Ethical Committee, the Food and Veterinary Service, Riga, Latvia.

Tissue Extract Preparation

Mildronate (100 mg/kg) was administered i.p. (intraperitoneal) daily for up to 28 days. Control rats received saline. Rats were decapitated 24 hours after the last administration. Blood was collected into citrate-containing vacutainers and centrifuged; then the obtained plasma was stored frozen (−80°C) until analyzed. Hearts were excised and also stored frozen (−80°C). Rat hearts were homogenized at 1500 rpm for 1 min with a glass Teflon homogenizer in ice-cold distilled water at wt/vol ratio 1:5. The homogenate was spun at 14,000*g* for 10 min at 4°C. The supernatant was then decanted, but the pellet was homogenized in the same volume of distilled water as before. The obtained homogenate was spun at 14,000*g* for 10 min at 4°C. The supernatants were combined and stored frozen (−80°C) until analyzed.

Solid Phase Extraction and Derivatization of Samples

An aliquot (100 μL) of plasma or tissue homogenate sample was loaded on preconditioned Supelco SAX cartridge (conditioned with 2 mL of methanol and 2 mL of water). Elution was performed with 3 mL of 0.01 mol/L KH_2PO_4 buffer (pH = 3.5). Eluate was collected into a 5 mL volumetric flask, and 0.01 mol/L KH_2PO_4 (pH = 3.5) was added up to the mark. An aliquot (1 mL) of this solution was used in the derivatization reaction.

The derivatization of samples was performed using a modified method described by Longo et al.[11] In brief, 1-AA was dissolved in acetone (16 mg/mL) and EDC was dissolved in 0.01 mol/L KH_2PO_4 buffer (pH = 3.5) (160 mg/mL). Using vortex mixing after the addition of each component, 20 μL of 1 mol/L HCl, 100 μL of 1-AA solution, and 100 μL of EDC solution were added to 1 mL of sample. The mixture was incubated at room temperature for 20 minutes, and the excess reagent was removed by washing the sample with 5 mL of diethylether. A 300 μL aliquot of the aqueous phase was then transferred into a test tube and 700 μL of 0.01 mol/L K_2HPO_4 buffer (pH = 9.1) were added to adjust the pH of the samples to 7. After mixing, the mixture was washed with 5 mL of chloroform. A 500 μL aliquot of the final aqueous phase was transferred into a chromatographic vial and diluted with 500 μL of 0.01 mol/L KH_2PO_4 buffer (pH = 3.5); then 30 μL of this solution were injected into the high-performance liquid chromatography (HPLC) system.

HPLC Analysis of Samples

The HPLC system consisted of a Waters 2695 Separations Module, Waters 474 Scanning Fluorescence Detector, and JETSTREAM 2 Column-Thermostat. Millennium 4.0 software was used for data acquisition and processing. The mobile phase was 80% 0.1 mol/L ammonium acetate buffer (pH 3.5) and 20% acetonitrile. Chromatographic separation was performed at a flow rate of 0.8 mL/min, using ZORBAX Bonus-RP, 5 μm, 4.6 × 250 mm column. The excitation and emission wavelengths of the spectrofluorimeter were 248 nm and 418 nm, respectively.

Isolated Rat Heart Infarction Model

Rats weighing approximately 300 g were randomly divided into 4 groups. The first group received water by mouth (control group), and the second, third, and fourth groups received 100 mg/kg mildronate by mouth for 3, 7, and 14 days, respectively. The last administration was performed 24 hours before the experiment.

Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and heparin (1 IU/g) was administered concomitantly. Hearts were excised and retrogradely perfused via the aorta at a constant pressure of 50 mm Hg, with oxygenated Krebs-Henseleit buffer (content in mmol/L: NaCl 118, $CaCl_2$ 2.52, $MgCl_2$ 1.64, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 10.0, EDTA 0.05) pH 7.3–7.5 at 37°C.

A saline-filled latex balloon connected to a pressure transducer was inserted into the left ventricle, and baseline end-diastolic pressure was set at 5–10 mm Hg. Heart rate, left-ventricle end-diastolic pressure, and left-ventricle developed pressure were recorded continuously. Coronary flow was measured using an ultrasound flow detector (HSE) and PowerLab8/30 system from ADInstruments.

A 5-0 polypropylene suture (Surgipro II, Syneture) was passed under the left anterior descending coronary artery and threaded through a small plastic tube to permit reversible occlusion of the coronary artery. Hearts were adapted for 20 minutes and occlusion was performed for 60 minutes by constricting threads through a plastic tube. Successful occlusion was confirmed by coronary flow decrease of about 40 percent. Reperfusion was achieved by releasing the threads.

At the end of the 150 minute reperfusion, the left anterior descending coronary artery was religated and the risk zone was delineated with 0.1% methylene blue solution in Krebs-Henseleit buffer infused via the aortic root. Hearts were sectioned transversely from the apex to the base in 5 slices of 2 mm thickness and incubated in 1% triphenyl-tetrazolium chloride in phosphate buffer (pH 7.4, 37°C) for 10 minutes to stain viable tissue red and necrotic tissue white. Afterward, the right ventricle was cut off and photos of the left-ventricle slices were made with a Minolta 7D photo camera. Computerized planemetric analysis of photographs was performed using Image-Pro Plus 4.5.1 software to determine the area at risk (AR) and area of necrosis (AN) expressed as percentage of the left ventricle (LV). Obtained values were then used to calculate the infarct size (IS) as percentage of risk area according to the formula IS = AN/AR × 100%.

Statistical Methods

Concentrations of carnitine, GBB, and mildronate are expressed as means ± SEM (standard error of the mean). Experimental groups were compared by one-way ANOVA (analysis of variance), followed by post hoc Dunnett's test,

using myocardial infarction control as a reference group. *P* values less than 0.05 were considered to be significant. Correlation analysis was performed by the Pearson's correlation test.

RESULTS

Influence of Mildronate Administration on Carnitine and GBB Contents in Rat Plasma and Heart Tissues

Mildronate was administered (i.p., 100 mg/kg/daily) to rats for 4 weeks; hearts and blood samples were collected at different time points, and plasma and heart tissue homogenate samples were prepared for analysis by solid-phase extraction. The extracts were then analyzed for carnitine, GBB, and mildronate content. The HPLC setup with precolumn derivatization demonstrated a valley-to-valley separation of both GBB and mildronate and a baseline separation of carnitine in a single run. The assignment of peaks was performed by analysis of reference standards and both spiked and blank plasma samples during the method validation step.

Analysis of control and mildronate-treated rat plasma samples is shown in Figure 1 and Table 1. The average concentrations of free carnitine and GBB in saline-treated animal plasma were 72.6 ± 4.7 and 2.3 ± 0.1 μmol/L, respectively (Table 1). As can be seen in Figure 1C, mildronate concentration in rat plasma increased gradually, reaching a plateau (more than 30 μmol/L) after 14 days of treatment. In turn, carnitine concentration in rat plasma decreased during the first week of treatment with mildronate (Figure 1A). After that it stabilized at about 25 μmol/L concentration (Table 1). In contrast, the GBB concentration increased considerably and also reached a plateau at about 12 μmol/L after 10 days of treatment with mildronate (Figure 1B and Table 1). As it can be seen in Figure 2A, during the 14 day treatment with mildronate, the concentration of free carnitine in plasma decreased about 3-fold, but GBB concentration increased more than 5-fold.

The analysis of heart tissue extracts revealed that, consistent with the concentration changes in plasma samples, the free carnitine and GBB concentrations in heart tissues also changed with mildronate treatment (Table 1). As can be seen in Figure 3B, the concentration of free carnitine was reduced 2-fold (from 1894 ± 40 up to 995 ±195 nmol/g) after only 3 days of treatment and reached a 2.5-fold decrease (to 745 ± 60 nmol/g) after 10 days of treatment with mildronate. At the same time, the GBB concentration increased about 7-fold (ie, from 32.8 ± 4.9 nmol/g up to 226 ± 26; Fig. 3B and Table 1). Also, in heart tissue extracts we observed the gradual

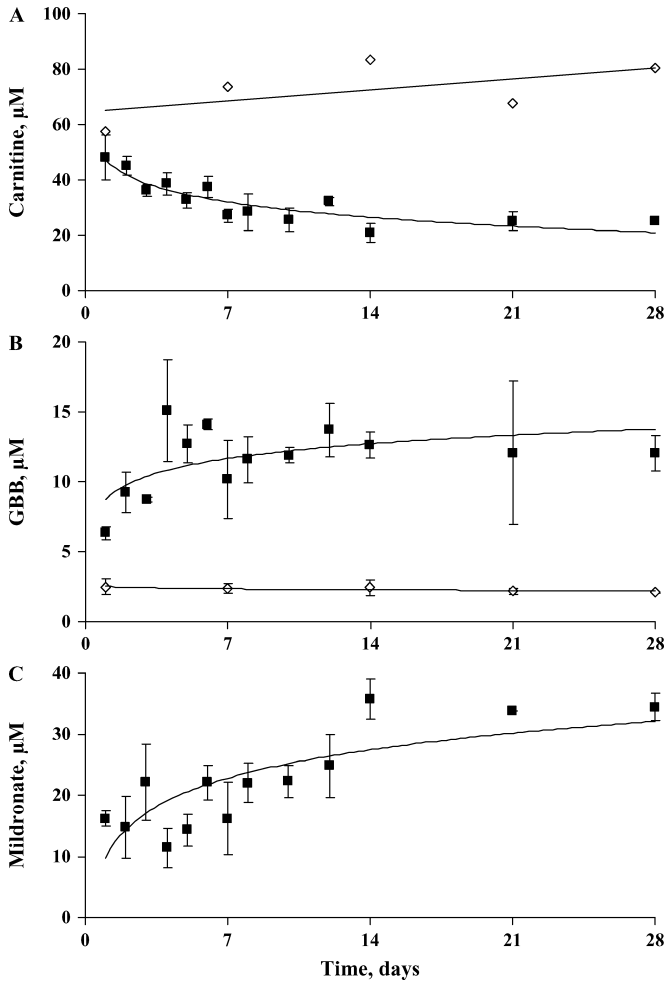

FIGURE 1. Mildronate administration (100 mg/kg i.p. daily) induced changes in rat plasma. A, carnitine; B, GBB; and C, mildronate concentration. Each value represents the mean ± SEM of 3 animals, control (open symbol) and mildronate treated (filled symbol). Logarithmic trendline is calculated.

increase in mildronate concentration during the first 14 days of treatment (ie, we found 674 ± 106 nmol/g and 866 ± 82 nmol/g of mildronate in samples obtained after 1 week and 2 weeks of treatment, respectively).

There were no significant differences in rat body and heart weights among saline and mildronate treatment groups. The statistical analysis of all data sets confirmed that the treatment with mildronate resulted in highly significant changes of carnitine and GBB content in blood plasma and heart tissues.

TABLE 1. Effect of Mildronate Treatment

Time, Days	Plasma (μmol/L)			Heart (nmol/g)			Area of Necrosis, % of Area at Risk
	Carnitine	GBB	Mildronate	Carnitine	GBB	Mildronate	
0	72.6 ± 4.7	2.3 ± 0.1	—	1894 ± 39	32.8 ± 4.9	—	33.6 ± 2.3
3	36.2 ± 2.2	8.7 ± 0.16	22.2 ± 6.2	995 ± 195	127 ± 22.6	598 ± 135.8	32.3 ± 2.5
7	27.0 ± 2.2	10.2 ± 2.8	16.2 ± 6.0	998 ± 111	215 ± 34.8	674 ± 106	27.7 ± 2.0
14	20.7 ± 3.3	12.6 ± 1.0	35.7 ± 3.3	745 ± 59.5	224 ± 16.1	866 ± 82.4	22.4 ± 1.7

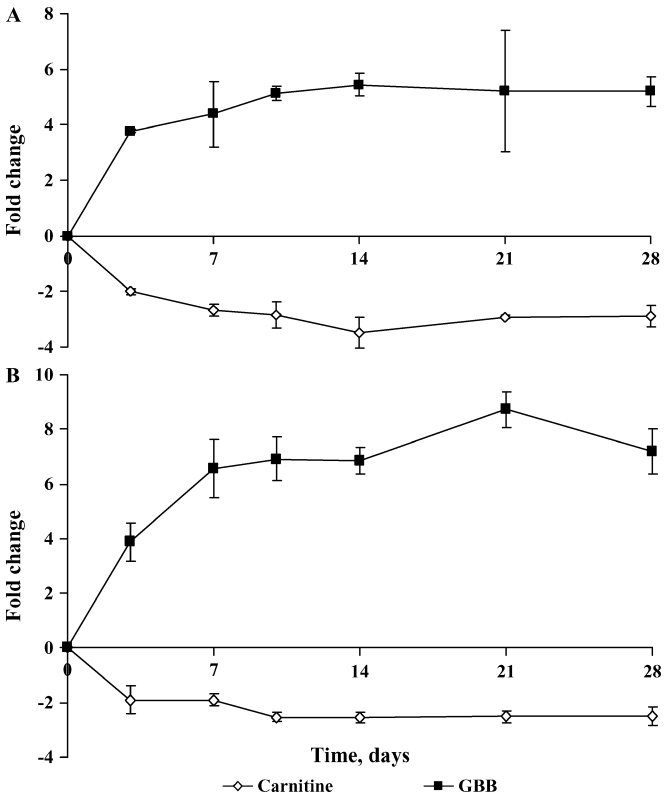

FIGURE 2. The mildronate administration (100 mg/kg i.p. daily) induced changes in carnitine (filled symbol) and GBB (open symbol) concentrations in rat plasma (A) and heart (B). Each value represents the mean ± SEM of 3 animals.

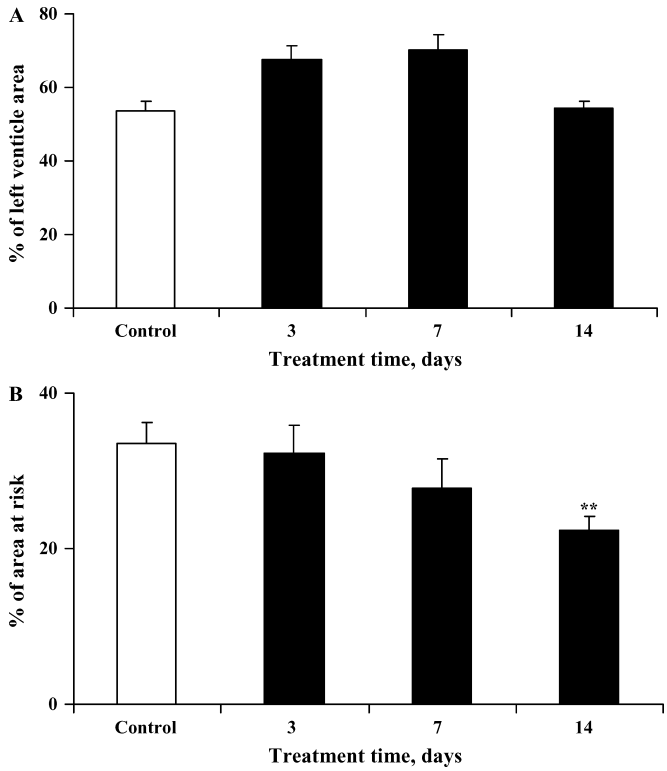

FIGURE 3. Values of the area at risk as percentage of the left-ventricle area (A) and necrotic area as percentage of the area at risk (B) in the control (open bar) and mildronate-treated groups (black bar). Mildronate 14 day treatment group vs control group by Dunnett's test; $P = 0.003$ (**). Represented values ± SEM are average of at least 6 animals.

The possible correlation of carnitine and GBB concentration and respective mildronate concentration in plasma and heart tissues was evaluated by the use of Pearson's correlation analysis test. Highly significant correlation was found between the mildronate and free carnitine concentration in plasma ($r = -0.544$, $P < 0.0001$) and heart ($r = -0.682$, $P < 0.0001$). We also calculated the correlation between concentrations of all tested compounds in plasma samples and tissue extract samples. Mildronate, GBB, and free carnitine plasma concentrations were highly correlated with those in heart tissue, with correlation values of 0.71 and 0.73, for carnitine and GBB, respectively ($P < 0.05$).

Hemodynamic Effects

There were no significant differences in the basal heart rate, developed pressure, or coronary flow among any of the experimental groups. Averaged group data in an isolated rat heart experimental model under normoxic conditions show that mildronate treatment (3–14 days) did not induce any significant changes in hemodynamic parameters [eg, developed left-ventricular pressure, heart rate, blood flow, and left ventricular contractility (±dp/dt) values].

During occlusion of left coronary artery, the coronary flow in all experimental groups was decreased from 10 mL/min to 6 mL/min. Moreover, the decrease in developed left-ventricular pressure of 53% (from 93 mm Hg to 44 mm Hg) was observed. The heart rate during the occlusion period did not change significantly. In reperfusion stage, coronary flow, developed left-ventricular pressure, and ±dP/dt values were recovered to about 80% of control level. There were no significant differences between control and mildronate treated groups.

Cardioprotective Effect of Mildronate in Isolated Rat Heart Infarction Model

We evaluated the cardioprotective effect of mildronate administration (3-, 7- and 14-day treatment) in an isolated rat heart experimental model. As it can be seen in Figure 3A, the values for the area at risk were similar in hearts from all experimental groups. After a 3 day treatment with mildronate the necrotic area of the infracted rat hearts did not differ from that found in control hearts (Fig. 3B). After 14 days of treatment the cardioprotective effect of mildronate was statistically significant (ie, the necrotic zone in mildronate-treated hearts was 22 ± 2% as compared with 34 ± 2% in control rat hearts; Fig. 3B).

As can be seen in Figure 3A the analysis of the contents of mildronate, GBB, and carnitine in rat blood plasma also revealed that in this experimental setup, the mildronate administration had induced a decrease in carnitine concentration and an increase in GBB concentration similar to that found in

28 day treatments (data not shown). The Pearson's correlation analysis showed that there is a statistically significant correlation between mildronate and GBB concentrations found in rat blood plasma and necrotic zone in infarcted hearts (Fig. 4). Thus, the *r* value for correlation of necrotic zone value and mildronate and GBB concentration found in corresponding rat plasma samples was -0.59 ($P < 0.001$) and -0.52 ($P < 0.05$), respectively (Fig. 4A and B). However, we did not find any correlation between carnitine concentrations in rat blood plasma and the necrotic zone value of the corresponding heart ($r = -0.093$, $P = 0.68$).

DISCUSSION

This is the first report of the time-dependent effects of mildronate on GBB and carnitine concentrations, and it demonstrates the time dependency of mildronate's cardioprotection during ischemia-reperfusion injury. After a 3 day treatment of mildronate the necrotic area of the infracted rat hearts did not differ from that found in control hearts, although we observed some reduction of carnitine concentration and an increase in GBB concentration. After 7 days of treatment we detected a slight, but not significant, infarct size reducing effect of mildronate. After 14 days of treatment the infarct size in mildronate-treated rat hearts was significantly decreased (Fig. 3B). The analysis of respective plasma and heart tissue samples revealed that changes in GBB, carnitine, and mildronate concentrations started to level off after 7 days of treatment and reached a plateau around day 14 of mildronate administration. We did not find any significant effect of mildronate on functional parameters of heart (left-ventricular pressure and heart rate), which indicates that the reduction of the infarction zone in mildronate-treated hearts is not a result of the reduced work load.

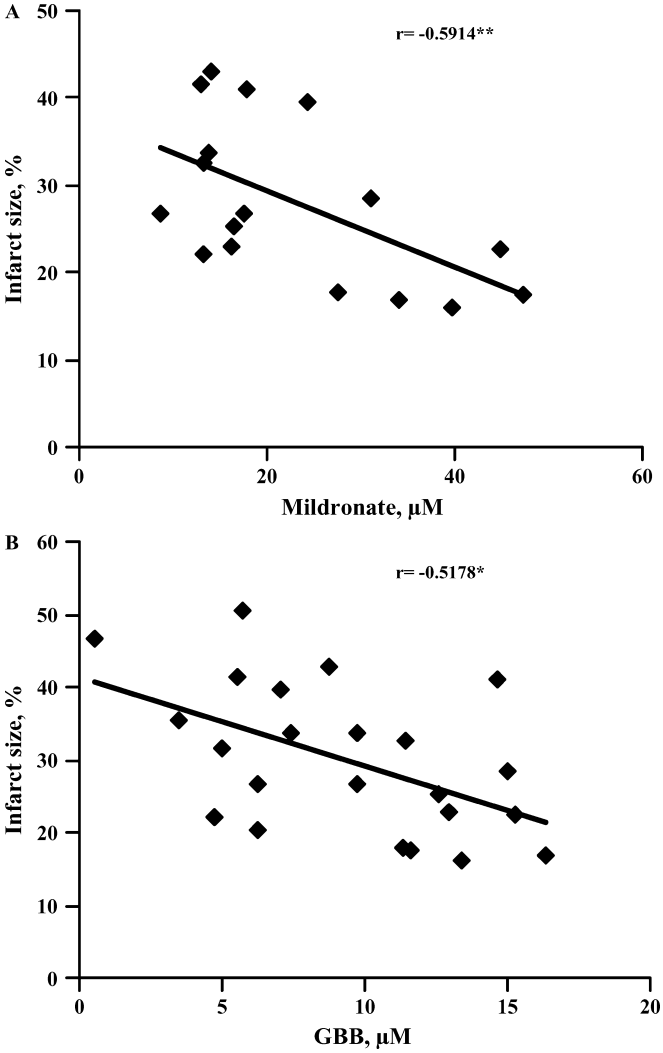

FIGURE 4. Pearson *r* value of infarct size/mildronate (A) and infarct size/GBB (B) concentration correlation in mildronate-treated rats. *$P < 0.05$, **$P < 0.001$.

As expected, the present study demonstrated that mildronate treatment brought about a decrease in free carnitine contents in rat plasma and heart tissues (Table 1 and Fig. 2A and B). The thus far reported concentrations of carnitine found in plasma and other tissues after mildronate administration vary depending on dosage and treatment used.[5,12,13] Nevertheless, all studies have reported 2- to 3-fold decreases in carnitine concentration after a 10 day treatment with mildronate.[1,5,13] In agreement with these findings we found that the concentration of free carnitine in plasma and heart reached a plateau at 2.5- to 3-fold lower concentration than in control samples after a 7–10 day administration of mildronate (Fig. 2A and B).

We show here that the concentration of mildronate found in plasma reaches a plateau at more than 30-μmol/L concentration after 14 days of treatment (Fig. 1C). The K_i for GBB hydroxylase enzyme inhibition by mildronate is determined to be 16 μmol/L.[14] As it can be seen in Figure 1C, this concentration of mildronate in plasma is already reached after a single administration of mildronate. Thus, our data show that even after a single administration of a 100 mg/kg dose the plasma concentration of mildronate is efficient for inhibition of GBB hydroxylase. In addition, the estimated K_i for inhibition of reabsorption of free carnitine in kidneys and free carnitine transport into cardiac cells by mildronate is found to be 52.2 and 1340 μmol/L, respectively.[15] Therefore, we have obtained evidence that after the induction of changes in contents of carnitine and GBB afforded by a single administration of mildronate, additional treatment for up to 10 to 14 days is necessary for the manifestation of the cardioprotective effect of mildronate. This could bring about a new equilibrium of carnitine homeostasis–dependent intermediary metabolism and result in improved cardiac cell survival under ischemic conditions.

It is interesting that if we summed up the free carnitine, GBB, and mildronate concentration in samples after mildronate treatment, then the sum was close to that of free carnitine and GBB concentrations in control animals. Our data show that by decreasing the carnitine contents in tissues, mildronate does not change the relative proportion of carnitine-related trimethylammonium compounds in the tissue environment, which could have some consequences for buffering systems and intermediary metabolism.

In our experimental setup of the isolated rat heart, no mildronate, GBB, or carnitine were included in the perfusion

buffer, which supports the notion that long-term mildronate treatment and consequent changes in GBB and carnitine concentrations could induce preconditioning-like changes in cardiac cell functioning that improve their survival under ischemic conditions.[16] This could include the modulation of cardiac cell energy metabolism pathways by switching from the fatty acid oxidation to glucose metabolism, as suggested before.[5,6,16]

It has been hypothesized that the antiischemic mechanism of action of mildronate could result from the mildronate-induced accumulation of GBB and possible related molecules.[10] The possible acetylcholine-like activity of carnitine and GBB metabolites was investigated decades ago.[17–19] However, in a recent study we showed that only methyl-ester of GBB, but not the parent GBB, possesses acetylcholine-like activity in vivo and in vitro.[20] Also, in the present study we did not observe any changes in basal heart rate after the administration of mildronate and increased levels of GBB. Therefore, it seems unlikely that acetylcholine-like effects of GBB could contribute to the activity of mildronate. However, as continuation of the present study, we have started a series of experiments that aim to elucidate the role of GBB as a possible antiischemic agent in experimental heart infarction model.

CONCLUSION

Our study provides experimental evidence that, in concert with the well-known inhibitory effect on carnitine content, the administration of mildronate causes a significant increase in GBB concentration in rat plasma and heart tissues, which correlates with the cardioprotective effect afforded by mildronate. However, further studies are needed to confirm the potential activity of GBB in experimental heart infarction or as a biomarker of pharmacologic activity of mildronate.

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