A novel glutamate transporter blocker, LL-TBOA, attenuates ischaemic injury in the isolated, perfused rat heart despite low transporter levels

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Abstract

OBJECTIVES: Loss of glutamate from cardiomyocytes during ischaemia may aggravate ischaemia-reperfusion injury in open heart surgery. This may be due to reversal of excitatory amino acid transporters (EAATs). However, the expression of such transporters in cardiomyocytes is ambiguous and quantitative data are lacking. Our objective was to study whether EAATs were expressed in the rat heart and to study whether blocking of transporter operation during cardiac ischaemia could be beneficial.

METHODS: We used TaqMan real-time PCR and immunosolubilization followed by western blotting to unequivocally identify EAAT subtypes in rat hearts. We used a novel high-affinity non-transportable competitive inhibitor, named LL-TBOA [(2S,3S)-3-(3-(6-(6-(2-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)acetamido)hexanamido)-hexanamido)5-(4-(trifluoromethyl)benzamido)benzyloxy)aspartic acid], to block EAAT-mediated transport during global ischaemia and reperfusion of isolated rat hearts.

RESULTS: Rat hearts expressed EAAT subtypes 1 and 3, while subtypes 2 and 4 were not detected. Hearts were isolated and perfused with 1.6 µM LL-TBOA for 5 min before 30 min of induced global ischaemia and 60 min of reperfusion (n=8). Control hearts were perfused either with the solvent dimethylsulfoxide 3.5 mM (n=7) or with no pretreatment (n=8). Infarct size was evaluated by triphenyl tetrazolium chloride (TTC) staining. LL-TBOA reduced infarct size from 33 ± 14 to 20 ± 5% (mean ± SD) (P = 0.015). Dimethylsulfoxide alone had no effect (35 ± 2%). Reperfusion arrhythmias were reduced by LL-TBOA (P = 0.009), but not by dimethylsulfoxide alone.

CONCLUSION: Rat hearts express EAAT1 and EAAT3, but the mRNA levels are, respectively, ~25 and 200 times lower than in the brain. Addition of LL-TBOA has a beneficial effect against ischaemia-reperfusion injury.

Keywords: Myocardial protection • Ischaemia-reperfusion • Reperfusion arrhythmias

INTRODUCTION

Glutamate has been used as a supplement in cardioplegic solutions and organ preservation solutions to improve cardiac function in cardiac surgery and transplantation [1–5]. Although glutamate contributes marginally as an energy source in itself, it appears to be important for heart function as it takes part in metabolic processes including the maleate-aspartate shuttle, ammonia detoxification and activation of succinate oxidation [3,6,7]. In acute ischaemia, glutamate leaks from the myocardium [8] and this leakage is believed to aggravate the ischaemic damage [3,9]. Several mechanisms may contribute to the leak [8]: one possibility is diffusion through anion channels, another is leakage through damaged myocyte plasma membranes and a third is reversal of glutamate transporters [7–10] similar to that observed in cerebral ischaemia [11].

The identities of the proteins involved in cardiac glutamate transport are still being discussed. Low levels of mRNA encoding glutamate transporter (excitatory amino acid transporter (EAAT)) subtypes EAAT1 [10,12], EAAT3 [10,12,13] and EAAT5 [14] have been reported in normal hearts. EAAT1 and EAAT3 were found in both normal and hypertrophic hearts [10]. mRNA encoding for EAAT4 has not been detected in normal hearts [15]. EAAT2 is more controversial—its mRNA was not detected in normal hearts in one study [10], while another study found EAAT2 mRNA in both normal and hypertrophic hearts [16]. The detection of the corresponding proteins through immunocytochemistry and western blotting has been attempted [10,17,18], but reliable detection of glutamate transporters is difficult, particularly in the heart [19]. However, assessments of glutamate transport activity in sarcoplasmal vesicles and isolated myocytes suggests that the transporter proteins are expressed [6,20].

Here, we show that a novel blocker of the EAAT-type of transporters, LL-TBOA [(2S,3S)-3-(3-(6-(6-(2-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethoxy)ethoxy)acetamido)hexanamido)-hexanamido)5-(4-(trifluoromethyl)benzamido)benzyloxy)aspartic acid], protects the heart when given prior to global ischaemia. Further, by
immunoisolation and TaqMan real-time polymerase chain reaction (PCR), we firmly confirm the presence of EAAT1 and EAAT3 in normal rat hearts, although at a level much lower than in the brain. The cardiac presence of EAAT5 was confirmed by TaqMan real-time PCR.

MATERIALS AND METHODS

Animal care

The protocols were approved by Norwegian Animal Health Authority and the animals received humane care in compliance with the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe no 123, Strasbourg 1985). Male Wistar rats (Scanbur AS, Nittedal, Norway) with a body weight of 250–350 g were used. All animals had conventional microbiological status. Environmental conditions regarding food (RM3 from Scanbur AS, Nittedal, Norway), water (ad libitum), humidity (55–60%) and light (12 h light and 12 h darkness) were the same for all animals. Animals were acclimatized for at least 4 days before the experiments.

Antibodies

Antibodies to EAAT1 (Anti-AS22, Ab#314), to EAAT2 (Anti-B12, Ab#150), to EAAT3 (Anti-C491, Ab#371) and to EAAT4 (Anti-D537, Ab#207) are the same as those described previously [18, 19, 21, 22] and have been raised against synthetic peptides corresponding to residues 522–541, 563–580 in EAAT1, 22–49, 52–54 in EAAT2, 57–76 in EAAT3 and 54–71 in EAAT4, respectively.

Glutamate uptake blocker

LL-TBOA (Fig. 1) was synthesized in a manner similar to that of TFB-TBOA [(2S, 3S)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate] [20]. The structure and purity (>95%) of the compound was confirmed by 400 MHz nuclear magnetic resonance and high pressure (or high performance) liquid chromatography. LL-TBOA inhibited glutamate uptake in the cell line-1 cells transiently expressing EAATs with IC50 values of 2.13 ± 0.31 µM (EAAT1), 0.50 ± 0.04 µM (EAAT2) and 7.37 ± 0.91 µM (EAAT3), respectively. The TBOA-family of compounds represents competitive inhibitors that block transporter operation. Thus, they do not mediate release of internal substrates due to heteroexchange [11].

Immunoisolation of EAAT-proteins

EAAT-antibodies were covalently immobilized on protein A-Sepharose Fast Flow as described previously [21]. Hearts from 10 adult rats were homogenized in 90 ml water with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM ethylenediaminetetraacetic acid. The homogenate was centrifuged (250,000 × g, 30 min, 4°C). The pellet was solubilized in 90 ml solubilization buffer (5.7 mg/ml sodium dodecyl sulphate (SDS); 50 mM Li-HEPES pH 7.5; 0.1 M LiCl) and centrifuged (Beckman JA20 rotor, 18,000 rpm, 20 min, 20°C). The supernatant was divided into four aliquots and made ‘antibody friendly’ by adding Triton X-100 to a final concentration of 3% (v/v) before incubation (60 min, room temperature) with the immobilized antibodies. Bound proteins were eluted with low pH-buffer as described [21] in a total volume of 0.5 ml from each of the four aliquots.

Electrophoresis and blotting

The purified proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting was performed as described previously [21]. Briefly, crude SDS-extracts of rat hippocampus and cerebellum were run as positive controls. After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes. The blots were blocked with 4% (w/v) powdered skimmed milk in Tris buffered saline with Tween 20 (TBST), incubated (overnight) with primary antibodies (0.3–1 µg/ml) in bovine serum albumin (10 mg/ml) in TBST, washed (3 × 10 min) with TBST and incubated (1 h) with secondary antibody (Sigma A-1949, 1: 30,000–50,000 in 4% (w/v) powdered skimmed milk in TBST (without azide) with secondary antibody. After washing (1 × 1 and 3 × 10 min in TBST without azide), they were incubated (5 min) with Pierce West DuraTM.

RNA extraction

Rat left ventricles were collected either after a brief wash-out period with Krebs–Henseleit buffer (n = 4) or after 20 min stabilization in the Langendorff apparatus, 30 min of global ischaemia and 60 min of reperfusion (n = 4). Rat brains (hippocampus region) and left ventricles were collected from another four rats for comparison of EAAT expression between tissues. Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Inc., Hilden, Germany) with an additional phenol-chloroform (Sigma-Aldrich Norway AS, Oslo, Norway) extraction step and in-column DNase treatment (QIAGEN). The quantity and quality of RNA was evaluated in Agilent 2100 bioanalyzer with adsorption at 260 nm. A 260/280 ratio between 1.7 and 2.1 was accepted. The extracts were stored at −80°C until usage.

cDNA synthesis

One microgram of RNA was reverse transcribed using random hexamers for priming (3 min at 70°C) followed by a modified First Strand cDNA Synthesis Protocol with Superscript III (Life Technologies, Oslo, Norway) and RNasin (Promega, Madison, USA) enzymes (10 min at 25°C, 50 min at 42°C and 4 min at 94°C).

Figure 1: Chemical structure of LL-TBOA.
Real-time PCR

Real-time PCR was carried out using the 5-3 nucleic acid chain termination primer method, which involves the release and subsequent direct detection of a fluorescent reporter dye of probes. Primers and probes were designed using the Primer Express software and custom made (Life Technologies, Oslo, Norway) on the basis of published rat cDNA sequences. All primers and probes were designed to span exon–exon junctions, where appropriate (Table 1). 18S rRNA was purchased from Applied Biosystems and used as an endogenous control gene.

Reactions took place in MicroAmp Optical 96-well plates (Life Technologies, Oslo, Norway) using 2 µl of cDNA, 12.5 µl of Universal Master Mix (Life Technologies, Oslo, Norway), at a final concentration of 900 nM and probes at a final concentration of 200 nM. The total reaction volume was 25 µl, and the samples were run in duplicates. The PCR reaction had a standard amplification scheme: one cycle of 2 min at 50°C (AmpErase UNG activation) and one cycle of 10 min at 95°C (Gold AmpliTaq activation, AmpErase UNG inactivation), followed by 45 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. ABI 7900 Sequence Detection System (Life Technologies, Oslo, Norway) was used to run PCR reactions. Relative quantification of gene expression was carried out following the comparative Ct method (User Bulletin #2; Stockholm, Sweden). Relative gene expression in the heart was calculated in percentage of the expression in the brain (Table 1). 18S rRNA was purchased from Applied Biosystems and used as an endogenous control gene.

Isolated heart perfusion

Rats were anesthetized with 5% sodium pentobarbital 60 mg/kg intraperitoneally, and 200 IU Heparin (Leo, Pharma AS/2750 Ballerup, Denmark) was injected into the femoral vein. After anaesthesia, hearts were rapidly excised through a median sternotomy, and placed in ice-cold, modified Krebs–Henseleit Buffer containing (mmol/l): NaHCO3 25, KCl 4.7, KH2PO4 1.2, MgSO4/7H2O 1.2, glucose/1H2O 11.1, CaCl2 1.8 (Merck KGaA, 64 271 Darmstadt, Germany), NaCl 118.5. The aorta was cannulated on a Langendorff system and perfused with warm (37°C), oxygenated (95% O2, 5% CO2) Krebs–Henseleit buffer at constant pressure (70 mmHg). A fluid-filled latex balloon was inserted into the left ventricle for measurement of pressures. Left ventricular end diastolic pressure (LVEDP) was set at 5 mmHg during stabilization by filling fluid in the balloon, and was not changed afterwards. Left ventricular developed pressure (LVDP) was calculated as left ventricular systolic pressure (LVSP)—LVEDP. Coronary flow (CF) was measured by timed collections of the coronary effluent. Heart rate (HR) and arrhythmias (ventricular fibrillation or asystolia) were registered. The absence of pressure-generating beating due to arrhythmias was defined as LVDP <4 mmHg. Hearts with LVSP ≤60 mmHg, CF ≤6 or ≥15 ml/min or HR ≤220 beats/min after 20 min stabilization were excluded from the study.

All hearts were subjected to 20 min of stabilization. Thereafter, the hearts were either perfused with 1.6 µM of LL-TBOA into a sidearm of the aortic cannula for 5 min (n = 8), the solvent of LL-TBOA dimethylsulphoxide (DMSO) 3.5 mM (n = 7) or time-matched perfusion with buffer only (n = 8). The concentration of LL-TBOA used was high to ensure complete saturation of all glutamate-binding sites on the EAATs in spite of competition from endogenous amino acids (glutamate and aspartate). After that, all hearts were subjected to 30 min of global ischaemia by clamping the inflow tubing (thus, LL-TBOA was present during ischaemia), and 60 min of reperfusion with buffer only (no LL-TBOA). During reperfusion, several hearts had reperfusion arrhythmias in the form of asystolia or ventricular fibrillation. Data on cardiac performance during reperfusion were excluded from hearts without any electrical activity, hearts with HR > 500 beats/min or hearts with LVDP ≤4 mmHg.

Infarct size

After 60 min of reperfusion, infarct size was measured by sectioning the heart in 1 mm sections, which were incubated for 20 min at 37°C in 1% TTC solution. After fixing in 4% formaldehyde for 60 min, the sections were scanned on both sides and imported into Adobe Photoshop, where calculation of infarct area was performed by a person blinded to the protocol.

Statistics

Infarct size was evaluated by the Mann–Whitney U-test. Cardiac function (left ventricular pressures, HR and CF) was evaluated by two-way analysis of variance. Occurrence of arrhythmias was calculated with a χ2 test, by scoring pressure-generation as 1 and no pressure generation as 0, adding all registration time points of each heart in each group throughout reperfusion for one test. P < 0.05 was considered significant. Data are shown as individual data or mean ± standard deviation (SD).

RESULTS

Immunoblotting

Brain tissue contains very high concentrations of EAAT1s [11]. EAATs are readily detected with anti-EAAT antibodies on
immunoblots even when intact tissue has been homogenized directly in SDS-sample buffer and subjected to electrophoresis without prior purification (Fig. 2, lane 4). In contrast, attempts to detect EAATs directly in crude heart SDS extracts failed (data not shown). This suggests that the EAAT levels in the heart are considerably lower than those in the brain. To circumvent the detection problem, EAAT-proteins were first isolated from solubilized heart tissue using immobilized antibodies. The extracts obtained in this way contained EAAT1 and EAAT3 proteins at concentrations that were readily detectable (Fig. 2) while EAAT2 and EAAT4 remained below detection limit even in these extracts (Fig. 2). We did not attempt to identify EAAT5 in heart extracts due to lack of a functional antibody.

**Real-time PCR**

RNA was extracted from brains and hearts in the same animals, and amplified with real-time PCR using species-specific primers and probes against EAAT1–4 to evaluate relative organ expression. EAAT5 was not included as it is not expressed in rat hippocampus. As seen on immunoblots, the brain expression of EAAT1–4 were much higher than that in the heart. EAAT4 was not detectable in the heart, while only trace amounts of EAAT2 were found (Fig. 3A). The cardiac expression of EAAT1 was <1% of that in the brain, while EAAT3 was above 3% of brain levels (Fig. 3A). In other experiments, RNA was extracted from rat left ventricle before and after Langendorff-perfusion with induced global ischaemia and reperfusion. Relative gene expression of EAAT 1, 3 and 5 were found, but only trace amounts of EAAT2 and no EAAT4 (Fig. 3B), confirming the experiments shown in Fig. 3A. Ischaemia and reperfusion did not significantly influence gene expression of any EAAT (EAAT1–4: Fig. 3B; EAAT5: not shown).

**Left ventricular performance**

Left ventricular end-diastolic pressure was set to 5 mmHg at the end of stabilization, and was not influenced by perfusion with DMSO or LL-TBOA. LVEDP increased at the start of postischaemic reperfusion in ischaemic controls, and thereafter gradually decreased without reaching the starting level. LL-TBOA did not significantly attenuate this increase (Table 2). Perfusion with DMSO did not significantly influence LVEDP compared with ischaemic controls. LVSP was not different between groups (results not shown). There were no significant differences observed in LVDP, HR or CF between groups (Table 2).

**Reperfusion arrhythmias**

During reperfusion, arrhythmias in the form of asystolia or ventricular fibrillation were observed. In ischaemic controls, this was seen in 5/8, 5/8, 4/8, 4/8, 3/8, 2/8, 2/8 and 2/8 after 5, 10, 15, 20, 30, 40, 50 and 60 min reperfusion, respectively. In hearts perfused with LL-TBOA, the occurrence of arrhythmias was reduced to 3/8, 3/8, 2/8, 2/8, 1/8 and 0, 0 and 0 at the same time points. For DMSO-perfused hearts, the corresponding values were 4/7, 4/7, 3/7, 3/7 and 2/7 for the remaining time points. When this was calculated and evaluated throughout the whole reperfusion period, the reduction of reperfusion arrhythmias evoked by LL-TBOA was...
**DISCUSSION**

Glutamate has been used successfully as a supplement to cardio-pug solutions and organ preservation solutions to improve cardiac function in open heart surgery and transplantation [1–5].

Glutamate supplementation may lead to neurotoxicity. A beneficial effect similar to that of adding exogenous glutamate may be achieved through blocking glutamate transport by using pharmacological agents such as LL-TBOA. The only known biological effect of LL-TBOA is to bind to EAATs with much higher affinity than glutamate in such a way that both forward and reversed transport are blocked [20, 23]. The TBOA-type of compounds do not interact with the neutral amino acid transporter ASCT2 [24]. This means that LL-TBOA should prevent glutamate and aspartate from crossing the plasma membranes (in any direction) through the EAATs. Thus, unless LL-TBOA has unknown actions in addition to blocking EAATs, the cardioprotective effects are due to the prevention of amino acid leak from the cardiomyocytes to the amino acid-free perfusion solution. The beneficial effects of preventing leak from the cardiomyocytes are in good agreement with studies showing beneficial effects of adding glutamate in clinical or experimental studies [1–5].

**Infarct size**

After 60 min of reperfusion, hearts of ischaemic control animals had an infarct size of $33 \pm 14\%$ of the left ventricle (mean ± SD).

This was reduced to $20 \pm 5\%$ by LL-TBOA. DMSO in itself did not influence infarct size compared with ischaemic controls ($35 \pm 2\%$) (Fig. 5).

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**Table 2:** Rat hearts were isolated and subjected to perfusion with 30 min of global ischaemia and 60 min reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>Preischaemic</th>
<th>5-min reperfusion</th>
<th>30-min reperfusion</th>
<th>60-min reperfusion</th>
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<tr>
<td><strong>LVEDP (mmHg)</strong></td>
<td></td>
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<tr>
<td>ISCH</td>
<td>5 ± 2</td>
<td>62 ± 15 (3/5)</td>
<td>27 ± 16 (5/3)</td>
<td>24 ± 11 (4/2)</td>
</tr>
<tr>
<td>LL-TBOA</td>
<td>5 ± 1</td>
<td>35 ± 11 (5/3)</td>
<td>14 ± 6 (7/1)</td>
<td>14 ± 6 (8/0)</td>
</tr>
<tr>
<td>DMSO</td>
<td>5 ± 1</td>
<td>61 ± 17 (3/4)</td>
<td>34 ± 11 (5/2)</td>
<td>28 ± 7 (5/2)</td>
</tr>
<tr>
<td><strong>LVDP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISCH</td>
<td>132 ± 15</td>
<td>30 ± 15</td>
<td>90 ± 15</td>
<td>85 ± 13</td>
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<tr>
<td>LL-TBOA</td>
<td>131 ± 13</td>
<td>75 ± 17</td>
<td>100 ± 13</td>
<td>93 ± 13</td>
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<tr>
<td>DMSO</td>
<td>132 ± 7</td>
<td>79 ± 8</td>
<td>91 ± 7</td>
<td>86 ± 7</td>
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<tr>
<td><strong>CF (ml/min)</strong></td>
<td></td>
<td></td>
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<tr>
<td>ISCH</td>
<td>11 ± 2</td>
<td>8 ± 3</td>
<td>7 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>LL-TBOA</td>
<td>11 ± 1</td>
<td>9 ± 1</td>
<td>8 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>DMSO</td>
<td>12 ± 2</td>
<td>10 ± 2</td>
<td>8 ± 1</td>
<td>8 ± 1</td>
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<tr>
<td><strong>HR (beats/min)</strong></td>
<td></td>
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<tr>
<td>ISCH</td>
<td>285 ± 20</td>
<td>172 ± 48</td>
<td>215 ± 33</td>
<td>211 ± 21</td>
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<tr>
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<tr>
<td>DMSO</td>
<td>269 ± 17</td>
<td>273 ± 35</td>
<td>257 ± 32</td>
<td>241 ± 31</td>
</tr>
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</table>

The hearts were either control perfused (ISCH, n = 8), perfused with the glutamate transporter blocker LL-TBOA (1.6 µM) 5 min prior to global ischaemia (LL-TBOA, n = 8) or its solvent DMSO 3.5 mM 5 min prior to global ischaemia (DMSO, n = 7). The effects on LVEDP, LVDP, HR and CF are shown in mean ± SD. Values from the hearts without pressure generating beating during reperfusion were excluded. Included/excluded hearts are denoted in brackets at each time point in the LVEDP column. There were no significant differences between groups.

LVEDP: left ventricular end-diastolic pressure; LVDP: left ventricular developed pressure; HR: heart rate; CF: coronary flow.

**Figure 4:** The rat hearts were isolated and subjected to perfusion with 30 min of global ischaemia and 60 min reperfusion. The hearts were either control perfused (ISCH, n = 8), perfused with the glutamate transporter blocker LL-TBOA (1.6 µM) 5 min prior to global ischaemia (LL-TBOA, n = 8) or its solvent DMSO 3.5 mM 5 min prior to global ischaemia (DMSO, n = 7). During reperfusion, arrhythmias in the form of asystolia or ventricular fibrillation were observed. LL-TBOA reduced the occurrence of reperfusion arrhythmias as averaged throughout the reperfusion period. DMSO per se did not influence this. Values are shown as absolute numbers of the hearts with reperfusion arrhythmias/total amount of observations during reperfusion.

DMSO per se did not alter arrhythmias compared with ischaemic controls, but hearts perfused with DMSO had a higher occurrence of arrhythmias than LL-TBOA hearts (Fig. 4).
interpretation is that LL-TBOA served to enhance stunning of viable cardiomyocytes, rendering the viable tissue less efficient. The antiarrhythmic effects are in accordance with reduced occurrence of arrhythmias in patients where glutamate was added to the cardioplegic solution [5]. In studies using other models, L-glutamate supplementation has improved left ventricular performance [1–3]. Cell death was not an end-point in other studies with L-glutamate [1–5]. The effects of LL-TBOA reported here were not due to the solvent, DMSO. The concentration of DMSO used influenced neither postischaemic function nor infarct size.

Our findings confirm the expression of EAAT1, EAAT3 and EAAT5 in normal rat hearts and are in accordance with previous studies [10, 12–17]. The expression of EAAT1 and 3 were much lower than that seen in the brain. However, the protective effect of LL-TBOA presented here suggests that the two transporters are not only present, but that they play important roles in cardiac pathophysiology. It is of course possible that LL-TBOA acts on other targets in addition to the EAATs. Although the present study does not rule out this possibility, the findings presented here makes previous reports on upregulation of glutamate uptake activity [7, 10], and of EAAT2 expression in hypertrophied hearts in particular [10], all the more interesting.

Although it is clear from this and the cited studies that EAAT1 and EAAT3 are expressed in the heart, their precise localizations have not been determined. EAAT3 has been detected in sarcosomal vesicle preparations by western blotting [10, 17]. This agrees well with the expectation that the EAAT3 transporter is localized on the plasma membrane. In contrast, EAAT1 has been reported to be localized on the inner mitochondrial membrane where it was suggested to transport glutamate into mitochondria [18]. This, however, is unexpected because EAAT1 is known as a plasma membrane protein [11]. To our knowledge, the possible

Figure 5: At the end of reperfusion of the hearts described in the legend to Fig. 4, the hearts were sampled, sectioned and stained with TTC. Images were imported into Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA.), and representative sections from one heart in each group are shown in the upper panel. Note that TTC stains viable tissue red, while necrotic tissue remains unstained. When the infarct area in all hearts in each group was calculated as percentage of the ventricles minus cavities, LL-TBOA reduced infarct size compared with ischaemic controls (lower panel), while DMSO did not. Individual infarct calculations plus mean value of each group are shown.
mitochondrial localization has not been confirmed by others or by use of true negative controls such as knockout animals. Mitochondria represent a common site for cross-reactivity in immunocytochemical procedures. On the other hand, if Ralphe et al. [18] are correct, the observed protection of LL-TBOA in the present study is likely to be due to EAAT3 and not to EAAT1. The fact that EAAT3 expression levels were four-fold higher than those of EAAT1 further strengthens this argument.

In the present study, we did not try to detect EAAT5 at protein level, as we do not have access to good antibodies to rat EAAT5. At gene expression level the presence of EAAT5 in rat hearts was confirmed. However, EAAT5 is unlikely to be relevant to the effects observed in the present study because the cardiac EAAT5 expression level was even lower than that of EAAT1 and because EAAT5 is reported to behave as a slow-gated anion channel with little glutamate transport activity [25].

In conclusion, the normal young adult Wistar rat hearts express EAAT3, EAAT1 and EAAT5. Addition of the glutamate transporter blocker LL-TBOA before ischaemia is cardioprotective. The natural conclusion based on available information is that LL-TBOA exerts its cardioprotective effect by acting on the EAATs (EAAT3 in particular), but the strong effect of LL-TBOA despite relatively low transporter expression levels raises the question whether there are additional unknown LL-TBOA targets after all. Regardless of target, LL-TBOA may represent a lead compound for development of new drugs to reduce ischaemia-reperfusion injury.

Limitations of the present study

In the study, we are assuming that LL-TBOA worked through blocking the reversal of glutamate transport in the cardiomyocytes. In order to confirm this assumption, measurements of glutamate and aspartate could have been performed in hearts at the beginning and the end of ischaemia in the presence and the absence of LL-TBOA.

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Conflict of interest: none declared.

REFERENCES