Amino Acids (2015) 47:1559–1565
DOI 10.1007/s00726-015-1997-y

ORIGINAL ARTICLE

L-Glycyl-L-glutamine provides the isolated and perfused young and middle-aged rat heart protection against ischaemia–reperfusion injury

Amer Almashhadany[1] · Othman A. Alghamdi[1] · Thomas Van der Touw[1] · Graham L. Jones[1] · Nicola King[2]

Received: 19 December 2014 / Accepted: 23 April 2015 / Published online: 7 May 2015

Abstract The amino acids glycine and glutamine have been implicated in myocardial protection of the much studied young adult heart. This study aimed to determine whether such protection could be enhanced using the dipeptide, L-glycyl-L-glutamine (gly-gln) in both young hearts and in middle-aged hearts representative of a more clinically relevant age group. Hearts from 8-week-old and 36-week-old rats were perfused in the Langendorff mode for 20 min, before 40 min global normothermic ischaemia and 30 min reperfusion. Where present, 0.5, 2, or 5 mM gly-gln was added 10 min into baseline perfusion, was present throughout ischaemia and was washed out after 10 min reperfusion. Reperfusion damage was assessed from the release of lactate dehydrogenase. Metabolic fitness was assessed from the time to ischaemic contracture and the accumulation of lactate and thiobarbituric acid reactive substances during ischaemia. The presence of 5 mM gly-gln significantly improved the post-ischaemic rate pressure product (RPP) and decreased reperfusion damage in both the 8 (RPP in control on reperfusion 5527 ± 957 vs. 10,320 ± 795 mmHg beat min^{-1} in 5 mM gly-gln, $n = 6 \pm$ SE, $p < 0.05$) and 36-week-old (RPP in control on reperfusion 1964.33 ± 876.3 vs. 4008 ± 675 mmHg beat min^{-1}, $n = 6 \pm$ SE, $p < 0.01$) hearts. Five mM gly-gln also increased the time to ischaemic contracture and was able to protect against the rise in lactate that occurred in the controls during ischaemia. These results suggest that gly-gln has good potential as a combatant against ischaemia–reperfusion injury in both the young adult and middle-aged populations.

Keywords L-Glycyl-L-glutamine · Middle-aged · Ischaemia–reperfusion injury · Myocardial protection

Introduction

Reperfusion of the ischaemic myocardium is associated with the undesirable consequences of calcium overload and free radical generation (Andreadou et al. 2009; Halestrap and Pasdois 2009; Schaffer et al. 2014). The amino acids glutamine and glycine have shown promise as protective agents against ischaemia–reperfusion injury (Jiang et al. 2011; Khogali et al. 1998; Liu et al. 2007; Nadtochiy et al. 2009); however, their usefulness may be limited by poor solubility and low thermostability (Zhang et al. 2013). In contrast, the dipeptide L-glycyl-L-glutamine (gly-gln) is more stable (Zhang et al. 2013) and is already being used in the treatment of intestinal diseases including short small bowel and intestinal fistula (Song et al. 2004).

The expression and activity of amino acid transporters have been linked to the efficacy of amino acids as cardioprotective agents (King et al. 2004, 2006, 2011; Schaffer et al. 2014). Many amino acids share common transporters where adding individual amino acids simultaneously to a tissue increases competition rather than uptake (Broer 2014). This problem can be solved by exploiting the properties of a family of transporters, which, in addition to the uptake of di- and tri- peptides, are responsible for the transport of various important compounds such as ACE

Handling Editor: F. Galli.

✉ Nicola King
nicola.king@plymouth.ac.uk

[1] School of Science and Technology, University of New England, Armidale, NSW 2351, Australia

[2] School of Biomedical and Healthcare Sciences, Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth University, Plymouth PL4 8AA, UK

inhibitors and β-lactam antibiotics (Smith et al. 2013). One member of this family, PEPT2 (SLC15A2) which accepts gly-gln as a substrate, has been shown to be functionally expressed in the heart (Lin and King 2007). This raises the possibility of harbouring the activity of PEPT2 to facilitate the simultaneous uptake of glycine and glutamine in the form of gly-gln. This dipeptide readily undergoes intracellular hydrolysis to release the constituent amino acids (Zhang et al. 2013).

Many investigations into myocardial protection utilise young adult animals. Ageing is associated with deteriorating mitochondrial fitness, increasing possibility of comorbidities and a reduction in the efficacy of myocardial protection approaches (Jahangir et al. 2007; Pantos et al. 2007), all of which may increase vulnerability to ischaemia–reperfusion. Therefore, the aim of this study was to investigate the cardioprotective potential of three different concentrations of gly-gln in two age groups comprising young adult and middle-aged rats. This substantially extends the work published by Qi et al. (2009) who used young rats and only a single gly-gln concentration.

Materials and methods

Animals

Eight-week-old and 36-week-old male Wistar rats were used for these experiments (Central Animal House, University of New England, NSW, Australia 2351). All animals were sacrificed by stunning and cervical dislocation. The heart was then dissected and processed for aortic cannulation. These experiments were performed with the approval of the Animals Ethics Committee (AEC09/152 and EC10/036) of the University of New England. This investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Materials

All chemicals including amino acids were bought from Sigma (Australia 14 Anella Ave, Castle Hill NSW 2154).

Langendorff perfusion

Following dissection, the rat heart was rinsed in cold Krebs containing in mM: 120 NaCl, 25 $NaHCO_3$, 11 glucose, 4.8 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 1.2 $CaCl_2$, and then cannulated via the aorta. Perfusion was then started for twenty minutes with Krebs at 11 ml/min, 37 °C, and aerated with 95 % O_2, 5 % CO_2. For the measurement of functional parameters [heart rate (HR), left ventricular developed pressure (LVDP) and rate pressure product (HR × LVDP)], a water-filled balloon connected via a pressure transducer to a Powerlab was inserted into the left ventricle. A maximal end diastolic pressure of <10 mmHg was measured during baseline perfusion. To expose the heart to 40 min global normothermic ischaemia, the pump was turned off, and the heart was immersed in warm Krebs. Reperfusion was then initiated by switching the pump on and continued for 30 min. Where present, 0.5, 2, or 5 mM gly-gln was added to the perfusate 10 min into baseline perfusion, was present throughout ischaemia before wash-out after 10 min reperfusion. The length of time chosen for ischaemia and reperfusion was based on our previous work investigating the effects of single amino acids in the isolated and perfused normal rat heart (King et al. 2004; Shackebaei et al. 2015).

Reperfusion damage was assessed from the release of lactate dehydrogenase into the coronary effluent during reperfusion. This was measured using a kit from Sigma (Tox7) and was performed according to the manufacturer's instructions.

Collection and analysis of intracellular metabolites (lactate and TBARS)

In a separate series of experiments, the rat heart was perfused for 20 min baseline perfusion with/without gly-gln followed by exposure to 40 min global normothermic ischaemia as described above. These experiments were performed without insertion of the balloon. For the lactate measurements, a small biopsy sample from the left ventricle was taken as soon as the pump was switched off to initiate ischaemia. A second sample was taken from the left ventricle at the end of the 40 min ischaemic period. For the measurement of thiobarbituric acid reactive substances (TBARS), samples were taken from the right ventricle at the end of ischaemia. Different ventricles were used for these biopsies in order to maximise the amount of tissue available for the TBARS assay. All samples were snap frozen in liquid nitrogen and then stored at −80 °C until use. Lactate concentrations in neutralised extract were measured as described previously (King et al. 2004, 2006).

The amount of lipid peroxidation in the various samples after ischaemia was determined by the following process: right ventricle samples were homogenised for 30 s in 500 µl double distilled water and then centrifuged at 4000 rpm for 10 min at 4 °C. Measurements were carried out by taking a mean of two readings. Two hundred fifty µl of each of the samples was pipetted into two separate labelled Eppendorff tubes. To each tube, 250 µl of 50 mM 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was added and mixed well. Following this, 1 ml of a solution containing

15 % (w/v) trichloroacetic acid (TCA) and 0.375 % (w/v) Thiobarbituric acid (TBA) in 0.25 M HCl was added and placed into a boiling water bath for 15 min. The tubes were removed from the water bath and cooled on ice for a further 10 min. The tubes were then centrifuged at 10,000 rpm for 5 min at 4 °C. The blank comprising 250 µl AAPH mixed with 1 ml of the TCA/TBA mixture was pipetted into a cuvette; the absorbance of the supernatant at 535 nm was read against the blank. The concentration of lipid peroxidation product in the samples was measured applying the Beer/Lambert Law as described below:

$$\text{Abs} = \epsilon cl,$$

where ϵ is the molar extinction coefficient of MDA, which is $1.56 \times 10^5\ \text{M}^{-1}\ \text{cm}^{-1}$, c is concentration (M) and l is the path length (1 cm).

Data analysis

Data are expressed as mean ± SE. "Baseline" in experiments measuring functional parameters refers to measurements made at the end of the 20 min pre-ischaemic perfusion, whilst "Reperfusion" refers to measurements made at the end of the 30 min reperfusion. Statistical comparisons were carried out using GraphPad Instat version 3.05 for Windows. Statistical differences between groups were calculated using ANOVA with a Tukey or Dunn post-test as appropriate. In all comparisons, a p value of less than 0.05 was considered significant.

Results

The first series of experiments carried out was proof of concept experiments regarding the potential of gly-gln as a cardioprotective reagent. These experiments were performed on young adult rat hearts (8-week-old), where there is an abundance of literature investigating different modes of cardioprotection with which to compare the current findings. Figure 1 shows the rate pressure product measured during baseline perfusion (before ischaemia) and at the end of reperfusion following 40 min global normothermic ischaemia. Three gly-gln concentrations were tested namely 0.5, 2 and 5 mM. The presence of 5 mM gly-gln led to a significant improvement in the post-ischaemia rate perfusion product compared to control.

In addition to measuring the functional performance of the rat hearts, the degree of reperfusion damage was also measured. This was assessed from the release of lactate dehydrogenase into the coronary effluent during reperfusion following the 40 min global normothermic ischaemia. Lactate dehydrogenase release vs. time was plotted and then the area under the curve calculated as an indication of the total time dependent release. Figure 2 shows that the area under the curve was significantly reduced compared to control in the presence of either 2 or 5 mM gly-gln. A

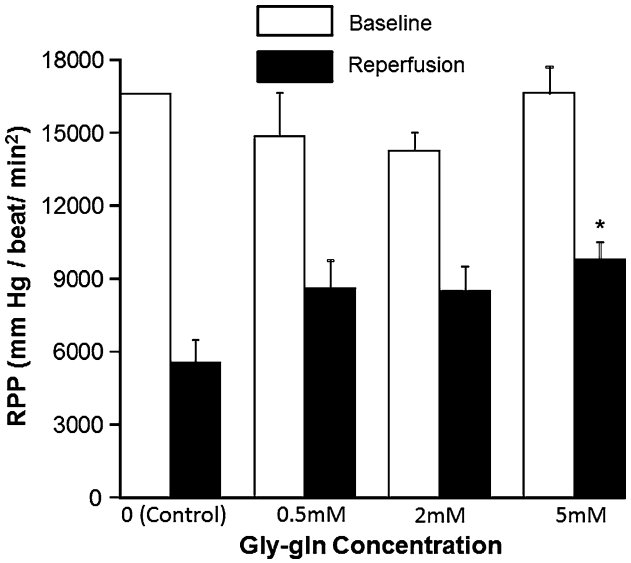

Fig. 1 Effect of gly-gln on the rate pressure product (RPP) before and after 40 min global normothermic ischaemia in 8-week-old rat hearts perfused with or without (control) 0.5, 2 or 5 mM gly-gln. *$p < 0.05$ vs. reperfusion in control. Data shown are mean ± SE of $n = 6$–7

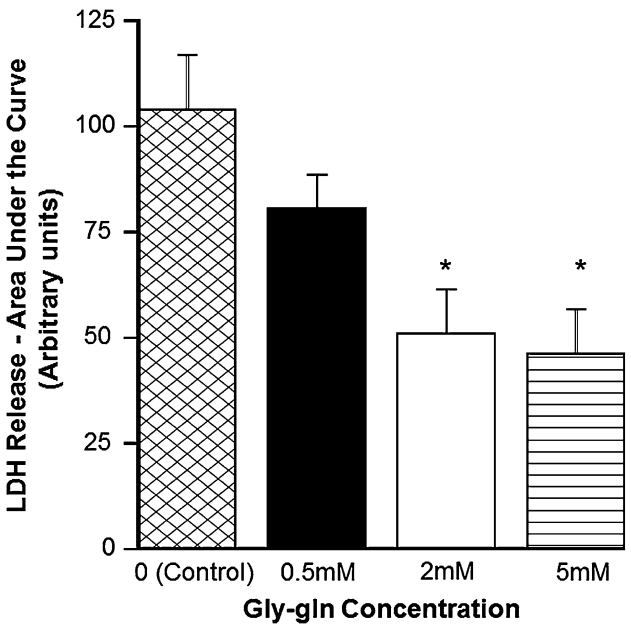

Fig. 2 Effect of gly-gln on the extent of reperfusion damage in 8-week-old rat hearts. Graph of the mean area under the curve when the release of lactate dehydrogenase into the coronary effluent during reperfusion was measured. *$p < 0.01$ vs. control. Data shown are mean ± SE of $n = 6$–7

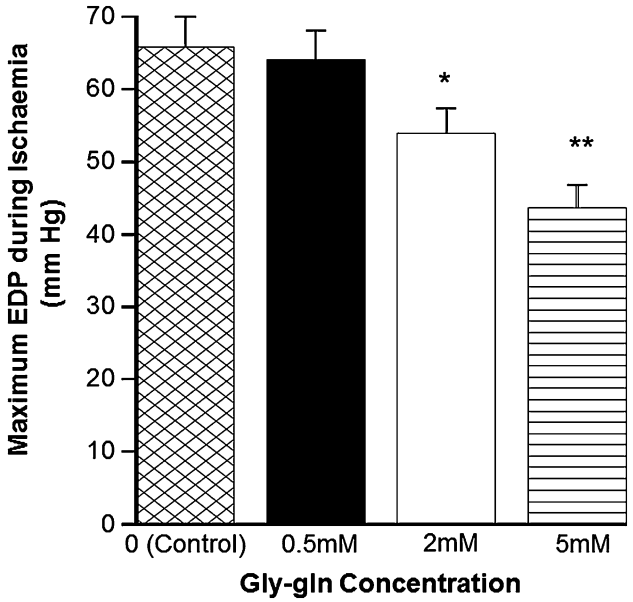

Fig. 3 Effect of gly-gln on the maximum end diastolic pressure (EDP) developed during ischaemia in 36-week-old rat hearts perfused with or without (control) 0.5, 2 or 5 mM gly-gln. $*p < 0.05$ vs. control. $**p < 0.01$ vs. control. Data shown are mean ± SE of $n = 6$

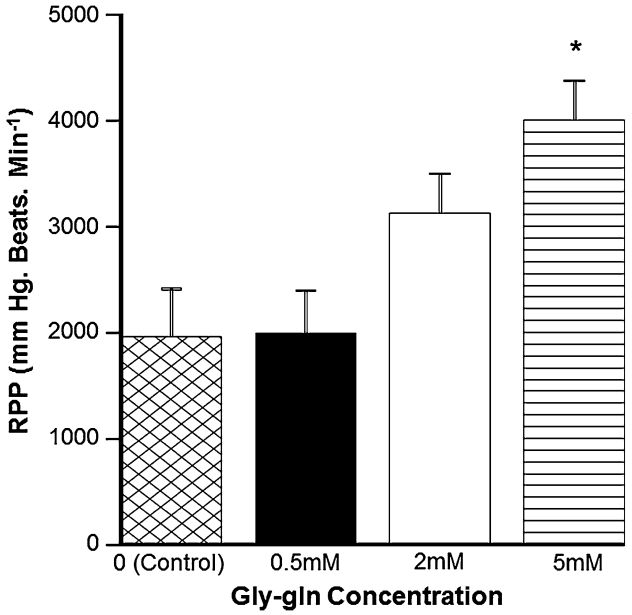

Fig. 4 Effect of gly-gln on the rate pressure product at the end of reperfusion following 40 min global normothermic ischaemia in 36-week-old rat hearts. $*p < 0.01$ vs. control and +0.5 mM. Data shown are means ± SE of $n = 6$

similar story was also apparent in the change in the maximal end diastolic pressure (EDP) during ischaemia (Fig. 3) with 2 and 5 mM gly-gln both leading to a smaller rise in EDP compared to 0.5 mM and control. Taken together, the findings in Figs. 1, 2, 3 suggest gly-gln has potential as a cardioprotective agent in young adult rat hearts.

An important aim of these experiments was to investigate the cardioprotective potential of gly-gln in older rats as being more representative of the corresponding age when humans are first diagnosed with coronary artery disease. Figure 4 shows the effect of different gly-gln concentrations on the rate pressure product during reperfusion following 40 min global normothermic ischaemia in 36-week-old rat hearts. There was a significantly greater rate pressure product in the presence of 5 mM gly-gln compared both to control and to 0.5 mM gly-gln. A similar pattern was seen when the time to ischaemic contracture was measured (Fig. 5). The presence of 5 mM gly-gln significantly lengthening the time taken to undergo contracture compared to both controls and the 0.5 mM gly-gln group. When it came to reperfusion damage, the area under the curve for lactate dehydrogenase release was significantly lower in the presence of both 2 and 5 mM compared to control (Fig. 6).

In order to investigate the possible mechanisms underlying the protection afforded 36-week-old hearts by gly-gln, lactate accumulation during ischaemia as an indicator of glycolytic activity and the accumulation of thiobarbituric acid reactive substances (TBARS) during ischaemia as an

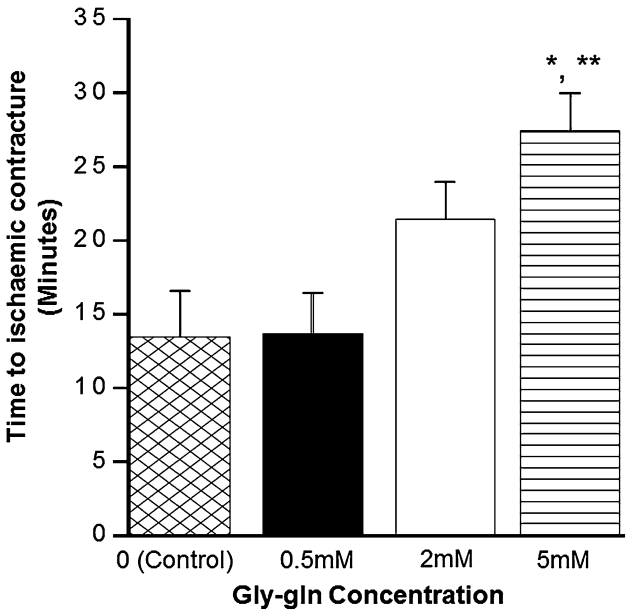

Fig. 5 Effect of gly-gln on the time to ischaemic contracture during ischaemia in 36-week-old rat hearts perfused with or without (control) 0.5, 2 or 5 gly-gln. $*p < 0.05$ vs. 0.5 mM gly-gln. $**p < 0.01$ vs. control. Data shown are means ± SE of $n = 6$

indication of lipid peroxidation were measured. Figure 7 shows the results for lactate. At the end of ischaemia, the lactate concentration in the control was significantly greater compared to the value at the beginning of ischaemia in the

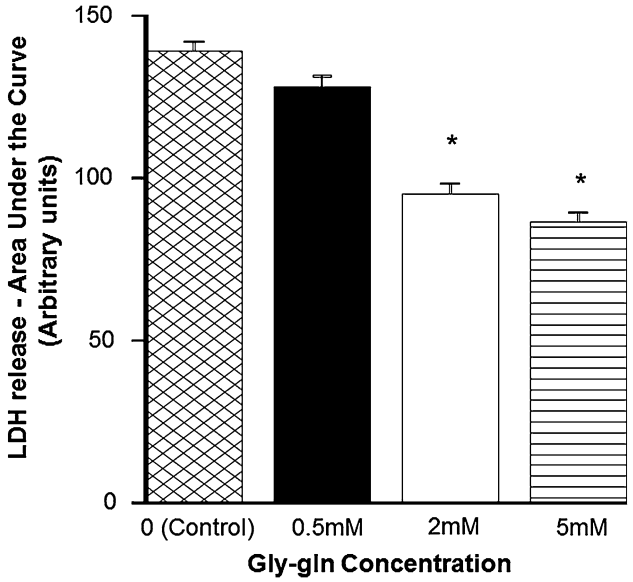

Fig. 6 Effect of gly-gln on reperfusion damage in 36-week-old rat hearts. Mean area under the curve calculated from the release of lactate dehydrogenase into the coronary effluent during reperfusion. *$p < 0.05$ vs. control. Data shown are means ± SE of $n = 6$

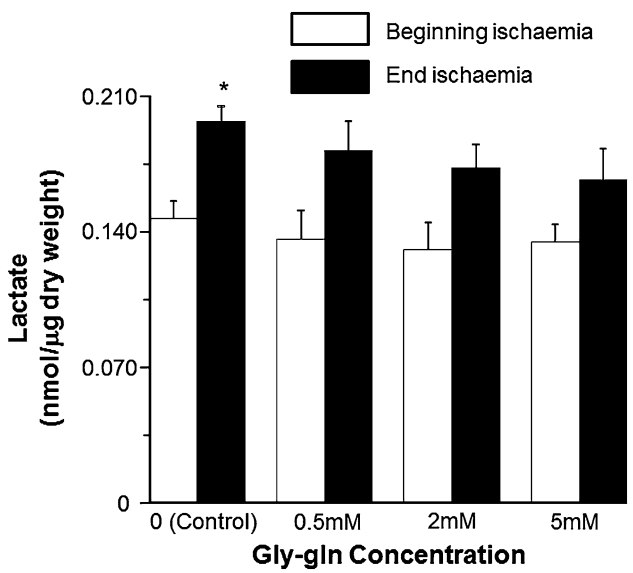

Fig. 7 Effect of gly-gln on glycolytic activity in samples of left ventricle from 36-week-old rat hearts. Lactate concentrations measured at the beginning and end of 40 min global normothermic ischaemia. *$p < 0.05$ vs. control at the beginning of ischaemia. Data shown are means ± SE of $n = 8$–9

control. This was not the case in the presence of any of the different gly-gln concentrations where the values at the beginning and end of ischaemia were not significantly different from each other. As shown in Fig. 8, large numeric differences indicative of a protective trend were evident in

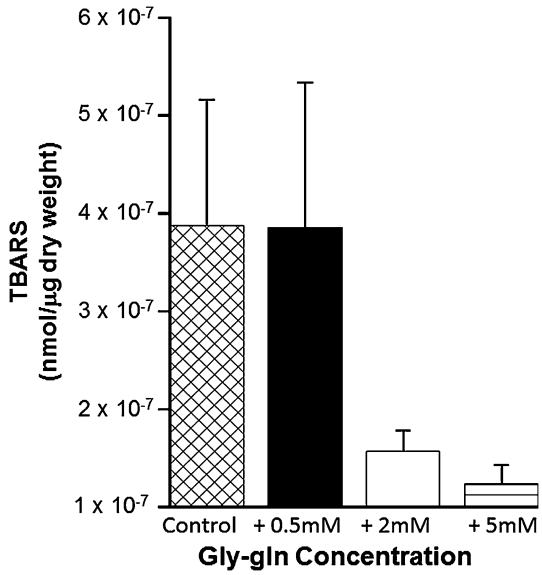

Fig. 8 Effect of gly-gln on lipid peroxidation in samples of right ventricle from 36-week-old rat hearts collected at the end of 40 min global normothermic ischaemia. *TBARS* thiobarbituric acid reactive substances. Data shown are means ± SE of $n = 5$–9

the TBARS data but these did not reach statistical significance ($p = 0.07$).

Discussion

This study aimed to determine whether there is a place for gly-gln in myocardial protection against ischaemia–reperfusion injury in the young adult and middle-aged heart. Three gly-gln concentrations were tested namely 0.5, 2 and 5 mM. There was a significant improvement in post-ischaemic function in the presence of 5 mM gly-gln in the 8-week-old hearts (Fig. 1) with both 2 and 5 mM gly-gln lowering reperfusion damage (Fig. 2) and decreasing the maximum end diastolic pressure developed during ischaemia (Fig. 3) in this age group. 5 mM gly-gln continued to offer protection in the older age group both in improving the post-ischaemic rate pressure product (Fig. 4), and lengthening the time to ischaemic contracture (Fig. 5). The presence of either 2 or 5 mM gly-gln decreased reperfusion damage (Fig. 6). In the older age group, gly-gln protected against the ischaemia-induced rise in lactate (Fig. 7). Taken together, these results suggest that gly-gln shows good potential as a protective agent against ischaemia–reperfusion injury in both young and middle-aged hearts.

This is the first study to investigate the effects of different gly-gln concentrations in two different age groups. It was considered important to study the older aged rats because of the increasing difficulty in protecting the older heart

(Jahangir et al. 2007) and because this is more representative of the typical age when humans begin to show symptoms. In a study that focussed on functional parameters, Qi et al. (2009) found that 2.5 mM gly-gln led to a significant improvement in post-ischaemic left ventricular developed pressure alongside a reduction in reperfusion damage in young adult rats. In combination with the current results, this may suggest a dose dependence in gly-gln's protection that begins at 2–2.5 mM. In the current study, 2 mM gly-gln did not afford greater functional recovery compared to the control (Figs. 1, 4), although it did reduce reperfusion damage (Figs. 2, 6) and reduce the rise in end diastolic pressure (Fig. 3). Slight differences in the effects of 2 mM gly-gln in this study and 2.5 mM gly-gln in the study by Qi et al. (2009) could be explained by differences in experimental protocol, e.g. their study used 30 min ischaemia, whereas the ischaemic period was 40 min in the current study. The findings of both of these studies are consistent with other investigations where glycine and glutamine have been individually used to enhance myocardial protection against ischaemia–reperfusion injury (Bolotin et al. 2007; Khogali et al. 1998; Liu et al. 2007; McGuinness et al. 2009; Støttrup et al. 2006) without the benefits of their combined protective effects as afforded by gly-gln.

This is also the first study to investigate the effect of different gly-gln concentrations in the isolated and perfused young and middle-aged rat. There was no effect compared to control when using 0.5 mM gly-gln (Figs. 1, 4). This contrasts with work on the single amino acids aspartate and glutamate in the hypertrophic heart where protection was seen at 0.5 mM (King et al. 2004, 2006). Hypertrophic hearts are however a different population, which show greater vulnerability to ischaemia–reperfusion injury than the normal heart (King et al. 2004, 2006). Although there were some signs that 2 mM gly-gln was exerting some protective effects, e.g. against reperfusion damage (Figs. 2, 6), only 5 mM gly-gln improved post-ischaemic functional recovery in both the young and middle-aged hearts (Figs. 1, 4). In their work, Khogali et al. (1998) saw protection with glutamine at concentrations at or above 1.25 mM. Though the concentration required is greater, gly-gln has the advantage over glutamine that it is more stable (Zhang et al. 2013) and already in use clinically (Song et al. 2004).

In addition to enhancing post-ischaemic functional recovery, gly-gln increased the time to undergo ischaemic contracture (Fig. 5) and protected against the rise in lactate that occurred during ischaemia (Fig. 7). Though the major sources of cardiomyocyte energy are fatty acids and glucose, studies performed by Khogali et al. (1998), McGuinness et al. (2009), Støttrup et al. (2006), Bolotin et al. (2007) and Zhang et al. (2013) have shown that glutamine can increase energy synthesis in the pathological state. Gly-gln is an efficient source of glutamine and glycine. This indicates that gly-gln may be involved in energy production in the oxygen-starved state and may therefore, to an extent, mitigate against the pathological damaging anaerobic accumulation of lactate.

One of the damaging pathologies accompanying ischaemia–reperfusion is the production of reactive oxygen species (ROS) (Andreadou et al. 2009). The consequences of this include lipid peroxidation, which can be estimated by measuring TBARS (Fig. 8) and membrane damage as indicated by lactate dehydrogenase release (Figs. 2, 6). One of the indicators of ROS production is opening of the mitochondrial permeability transition pore (Halestrap and Pasdois 2009), in which the presence of glycine is protective in isolated mitochondria and intact cardiomyocytes (Ruiz-Meana et al. 2004). In the current experiments, gly-gln was able to protect against membrane damage as evidenced in the decreased release of lactate dehydrogenase (Figs. 2, 6). This is also suggested by the trend towards a lower TBARS concentration in the presence of gly-gln (Fig. 8), despite this not reaching significance.

In conclusion, these results suggest that L-glycyl-L-glutamine has potential as a cardioprotective agent in both young adult and middle-aged hearts. This could have important implications for surgical strategies targeting increased protection of the middle-aged heart against ischaemia–reperfusion injury.

Acknowledgments This work was supported by the University of New England. We would also like to thank the technical staff for their excellent assistance.

Conflict of interest The authors declare that they have no conflict of interest.

References

Andreadou I, Iliodromitis EK, Farmakis D, Kremastinos DT (2009) To prevent, protect and save the ischemic heart: antioxidants revisited. Expert Opin Ther Targets 13:945–956

Bolotin G, Raman J, Williams U, Bacha E, Kocherginsky M, Jeevanandam V (2007) Glutamine improves myocardial function following ischemia-reperfusion injury. Asian Cardiovasc Thorac Ann 15:463–467

Broer S (2014) The SLC38 family of sodium-amino acid transporters. Pflügers Arch 466:155–172

Halestrap AP, Pasdois P (2009) The role of the mitochondrial permeability transition pore in heart disease. Biochim Biophys Acta Bioenerg 1787:1402–1415

Jahangir A, Sagar S, Terzic A (2007) Aging and cardioprotection. J Appl Physiol 103:2120–2128

Jiang L, Qin X, Zhong X, Liu L, Jiang L, Lu Y, Fan L, He Z, Chen Q (2011) Glycine-induced cytoprotection is mediated by ERK1/2 and AKT in renal cells with ATP depletion. Eur J Cell Biol 90:333–341

Khogali SE, Harper AA, Lyall JA, Rennie MJ (1998) Effects of L-glutamine on post-ischaemic cardiac function: protection and rescue. J Mol Cell Cardiol 30(4):819–827

King N, Lin H, McGivan JD, Suleiman M-S (2004) Aspartate transporter activity in hypertrophic rat heart and ischaemia-reperfusion injury. J Physiol 556:849–858

King N, Lin H, McGivan JD, Suleiman M-S (2006) Glutamate transporter activity in hypertrophic rat heart and ischaemia-reperfusion injury. Pflügers Arch 452:674–682

King N, Lin H, Suleiman M-S (2011) Oxidative stress increases SNAT1 expression and stimulates cysteine uptake in freshly isolated rat cardiomyocytes. Amino Acids 40:517–526

Lin H, King N (2007) Demonstration of functional dipeptide uptake into freshly isolated guinea-pig cardiomyocytes. Pflügers Arch 453:915–922

Liu J, Marchase RB, Chatham JC (2007) Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein *O*-GlcNAc levels. J Mol Cell Cardiol 42:177–185

McGuinness J, Neilan TG, Cummins R, Sharkasi A, Bouchier-Hayes D, Redmond JM (2009) Intravenous glutamine enhances COX-2 activity giving cardioprotection. J Surg Res 152:140–147

Nadtochiy SM, Burwell LS, Ingraham CA, Spencer CM, Friedman AE, Pinkert CA, Brookes PS (2009) In vivo cardioprotection by S-nitroso-2-mercaptopropionyl glycine. J Mol Cell Cardiol 46(6):960–968

Pantos C, Mourouzis I, Cokkinos DV (2007) Protection of the abnormal heart. Heart Fail Rev 12:319–330

Qi R-B, Xiong Y, Lu D-X, Wang Y-P, Fu Y-M (2009) Protection of glycyl-L-glutamine against myocardial ischemia reperfusion injury in the isolated rat heart. Chin J Pathophysiol 25:853–858

Ruiz-Meana M, Pina P, Garcia-Dorado D, Rodriguez-Sinovas A, Barba I, Miro-Casas E, Mirabet M, Soler-Soler J (2004) Glycine protects cardiomyocytes against lethal reoxygenation injury by inhibiting mitochondrial permeability transition. J Physiol 558:873–882

Schaffer SW, Jong CJ, Ito T, Azuma J (2014) Effect of taurine on ischemia-reperfusion injury. Amino Acids 46:21–30

Shackebaei D, King N, Shukla B, Suleiman M-S (2015) Mechanisms underlying the cardioprotective effects of L-cysteine. Mol Cell Biochem 177:27–31

Smith DE, Clemencou B, Hediger MA (2013) Proton-coupled oligopeptide transporter family SLC15: physiological pharmacological and pathological implications. Mol Aspects Med 34:323–336

Song JX, Tu XH, Wang L, Li CJ (2004) Glutamine dipeptide-supplemented parenteral nutrition in patients with colorectal cancer. Clin Nutr 1:49–53

Støttrup NB, Kristiansen SB, Løfgren B, Hansen BF, Kimose HH, Bøtker HE, Nielsen TT (2006) L-Glutamate and Glutamine improve haemodynamic function and restore myocardial Glycogen content during postischaemic reperfusion: a radioactive tracer study in the rat isolated heart. Clin Exp Pharmacol Physiol 33:1099–1103

Zhang Y, Yan H, Lv S-G, Wang L, Liang G-P, Wan Q-X, Peng X (2013) Effects of glycyl-L-glutamine dipeptide supplementation on myocardial damage and cardiac function in rats after severe burn injury. Int J Clin Exp Pathol 6:821–830