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Effect of ageing and hypertension on the expression and activity of PEPT2 in normal and hypertrophic hearts

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Abstract

Some dipeptides have been implicated in myocardial protection, but little is known about their membrane transporter PEPT2. The aim of this study was to determine whether the expression and activity of the cardiac-type PEPT2 cotransporter could be affected by ageing and/or hypertension. Sarcolemmal vesicles (SV) were isolated from the hearts of all rat groups using a standard procedure to investigate the transport activity and protein abundance by fluorescence spectroscopy and Western blot respectively. SLC15A2 "PEPT2" gene expression was relatively quantified by RT-qPCR. In the Wistar rat groups, the protein and gene expressions of PEPT2 were upregulated with ageing. These changes were accompanied by corresponding increases in the competitive inhibition and the transport rate (V_{max}) of β-Ala-Lys (AMCA) into SV isolated from middle-aged hearts. Although, the transport rate of β-Ala-Lys (AMCA) into SV isolated from old hearts was significantly the lowest compared to middle-aged and young adult hearts, the inhibition percentage of β-Ala-Lys (AMCA) transport by Gly-Gln was the highest. In the WKY and SHR rat groups, Y-SHR hypertrophied hearts showed an increase in PEPT2 gene expression accompanied by a significant decrease in the protein expression and activity. With advanced age, however, M-SHR hypertrophied hearts revealed significantly lower gene expression, but higher protein expression and activity than Y-SHR hearts. These findings suggest that increased expression of PEPT2 cotransporter in all types of middle-aged hearts could be exploited to facilitate di-and tripeptide transport by PEPT2 into these hearts, which subsequently could result in improved myocardial protection in these populations.

Key Words: Ageing; Hypertension; Myocardial protection; PEPT2 cotransporter.

Introduction

Hypertension and ageing are two of the main risk factors for cardiovascular disease (CVD), which is associated with high morbidity and mortality rates (Greiser et al., 2005). The protective role of single amino acids against ischaemia-reperfusion injury, oxidative stress, hypertension and myocardial infarction has been extensively studied (Jiang et al., 2011; Li et al., 2004; Liu et al., 2007; McCarty & DiNicolantonio, 2014; Nadtochiy et al., 2009). However, some of these amino acids such as glutamine, glycine, β-Alanine, and histidine showed poor solubility and thermostability in aqueous solution (Kohler et al., 2000; McCarty & DiNicolantonio, 2014; Zhang et al., 2013). Instead, the dipeptide, $_{L}$-glycyl-$_{L}$-glutamine, which has better stability and thermostability, showed a cardioprotective role against ischaemia-reperfusion, oxidative stress and burn injuries (Almashhadany et al., 2015; Zhang et al., 2013). Similarly, carnosine (β-Alanine-$_{L}$-histidine) is a stable dipeptide that contains an imidazole ring with a pK_a of 6.83, which can be a good physiological buffer for tissues in case of high glycolytic production of lactic acid (McCarty & DiNicolantonio, 2014). The cardioprotective role of carnosine against reactive oxygen species (ROS), hypertension, and ischemia-reperfusion injury has been also reported (Kurata et al., 2006; reviewed in McCarty & DiNicolantonio, 2014; Nagai et al., 2012).

The proton-dependent PEPT1 and PEPT2 membrane transporters have been implicated not only in the transport of a wide range of di-and tripeptides (more than 8000 substrates), but also peptidomimetic drugs such as some ACE inhibitors, β-lactam antibiotics, and selected anti-tumour compounds (Rubio-Aliaga & Daniel, 2008; Smith et al., 2013). The PEPT2 cotransporter shows wide distribution including kidney, brain, liver, intestine, and mammary gland (Boll et al., 1996; Daniel & Kottra, 2004). While the PEPT1 cotransporter was not found in the heart, the functional expression of PEPT2 has been reported in isolated cardiomyocytes

(Lin and King, 2007). Since it was reported that di-and tripeptides could account for 50% of the circulating amino acids (Frey et al., 2006; Seal & Parker, 1991), any changes to PEPT2 expression and activity due to hypertension and/or ageing could affect the intracellular pool of amino acids. In the same fashion evidence has shown that changes in the expression and activity of membrane cotransporters affects the efficacy of exogenously applied putative cardioprotective agents in the ischaemia-reperfused hypertrophic heart (King et al., 2004; 2006). Thus, any changes in the expression and activity of PEPT2 in the heart with ageing and chronic hypertension could have implications for the role of di- and tripeptides in cardiac protection. Therefore, the aim of the current study was to systematically investigate the effects of ageing and chronic hypertension on the expression and activity of PEPT2 in the heart.

Material and Methods

Materials

Primers were obtained from GeneWorks and Sigma. ISOLATE II RNA Mini Kit (BIOLINE), Tetro cDNA Synthesis Kit (BIOLINE), and SensiFAST^{TM} SYBR (Bioline) were from Australian Bioline. PEPT1, PEPT2, and β-Actin antibodies were from SANTA CRUZ . TSA-Cy 3.5 amplification kit was from PerkinElmer. Sudan Black B was from Sigma. β-ala-(L)-lys-N-7-amino-4-methylcoumarin-3-acetic acid (β-ala-lys (AMCA)) was purchased from Biotrend Chemicals (Destin, USA). All other reagents, chemicals, and consumables were of analytical grade.

Animal groups

Hearts were obtained from six male rat groups, three of which were Wistar rats at three different ages (6, 18, and 23 months) to investigate the effect of ageing. The other three groups were normotensive young adult Wistar Kyoto (Y-WKY), spontaneously hypertensive young adult (Y-SHR), and middle-aged (M-SHR) male rat groups. The Y-WKY and Y-SHR groups were age-matched at 5-6 months, while the M-SHR group was 12 months old. Mean arterial pressure were 134.1 ± 1.7 for Y-WKY, 185.2 ± 4.8 for Y-SHR and 228.2 ± 4.4 mmHg for M-SHR ($P=6x10^{-5}$ and 0.001 respectively). The age categories of the animals were chosen based on previous studies (Boengler et al., 2009; Sengupta, 2013). The hearts of Wistar rat groups did not develop myocardial hypertrophy with ageing, whilst hypertensive rat groups did develop myocardial hypertrophy compared to normotensive rat groups, as well as with ageing when comparing Y-SHR with M-SHR (for bodies and hearts weights see tables 1 and 2), in agreement with the study conducted by King et al. (2004). Wistar rat groups were raised in the animal house of the University of New England, while WKY and SHR rat groups were purchased from Animal Resources Centre (ARC) Australia.

All animals were sacrificed by stunning and cervical dislocation, followed by the removal of the hearts which were snap frozen in liquid nitrogen prior to transferring to -80 °C for later use. This study was approved by the Animal Ethics committee of the University of New England and complies with the *Guide for the care and use of laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Isolation of Cardiac Sarcolemmal vesicles (SV)

Cardiac SV were isolated using homogenisation and differential centrifugation as described previously (King et al., 2001). In order to prepare for the transport studies, the final pellet representing the purified SV was suspended in 10ml of a solution containing in mM: 100 KH_2PO_4, 100 mannitol, and 10 HEPES (pH 7.4 with TRIS) and centrifuged at 48,000 x g and 4°C for 30 minutes. The resulting pellet was then re-suspended in 500 µl of the same solution, followed by immediate freezing in liquid nitrogen and transfer to a -80°C freezer for later use.

Uptake of β-Ala-Lys (AMCA) by SV

Transporter activity in the SV was measured by the uptake of the fluorophore dipeptide derivative β-Ala-Lys (AMCA), which is a known substrate of PEPT2 (Alghamdi et al., 2017; 2018). Briefly, β-Ala-Lys (AMCA) was prepared in its maximum soluble concentration of 2.32 mM with the extra-vesicular buffer containing in mM: 100 KH_2PO_4, 100 mannitol, and 2-(N-Morpholino) ethanesulfonic acid (MES)-Tris (pH 6.6). A constant protein concentration (60µg) of SV isolated from the hearts of all rat groups was incubated with different concentrations (5-2000µM) of β-Ala-Lys (AMCA). This was adjusted with extra-vesicular buffer to a total volume of 250µl and incubated for 20 min at 20°C. Following incubation, SV were pipetted into centrifuge tubes containing 5ml of ice-cold extra-vesicular buffer prior to centrifugation at 31,000×g for 20min at 4°C. The resulting pellets were washed 3 times with ice-cold extra-vesicular buffer, followed by suspension of these SV in 500µl of the same buffer using a 1ml syringe and 21G x 1/5 gauge needle. After that, they were transferred to 96-well black microplates in triplicates, followed by fluorescence measurements at 455nm using an excitation wavelength of 350nm in a microplate reader (SpectraMax-M2^e). Each plate was loaded with

SV originating from three rat groups either Wistar rats (three different age groups) or WKY or SHR rat groups. These experiments were repeated at least 4 times using different SV isolated from different rat groups.

Substrate inhibition experiment

Vesicles containing 60 µg of protein from the same SV used for β-Ala-Lys (AMCA) uptake were added to Eppendorf tubes containing an appropriate volume of extra-vesicular buffer, 100 µM β-Ala-Lys (AMCA), and either 10 mM Gly-Gln or carnosine. These experiments were run concurrently with their corresponding concentration-dependent uptake experiments and read on the same plates.

Immunoblotting

Western blotting was used to relatively quantify the protein expression of PEPT2 versus β-actin proteins in SV. The same SV as were used for the functional assays were loaded at a constant concentration of 50µg onto Mini-Protean gels (Bio-Rad). The loading buffer, the running of the gels, and the transfer of the proteins onto nitrocellulose membranes were as described previously (Alghamdi et al 2017; 2018). Identical conditions as described previously (Alghamdi et al 2017; 2018) were used to probe the membranes with goat polycloncal anti-PEPT2, followed by exposure to the secondary antibody and visualisation. Following stripping of the membranes, the membranes were re-probed for β-actin as described previously (Alghamdi et al 2017; 2018).

Real-Time RT-qPCR

RT-qPCR was used to quantify the expression of SLC15A2 (PEPT2) RNA in the hearts of all rat groups. The ISOLATE II RNA Mini Kit was used to isolate total RNA from the heart ventricle biopsies (18 mg), followed by three steps to check RNA integrity. Subsequently, the Tetro cDNA Synthesis Kit was used to convert all RNA samples to cDNA in a 96-well plate using T 100^{TM} Thermal Cycler (Bio-Rad). The qPCR reactions were started by adding 10ng cDNA to 5µl SensiFAST^{TM} SYBR and 500 nM forward and reverse primers (Table 3) in a 384-well plate and in duplicates. This plate was run in QRT-PCR Bio-Rad CFX machine under the conditions described in Alghamdi et al (2018). The amplification of single and specific products was confirmed by (1) the single peak resulting from melt curve and visualisation of the product bands on 2% agarose gel (not shown). Three reference genes (β-Actin, Tbp, and SDHA) were used as a baseline and to normalise the expression of SLC15A using Bio-Rad CFX Manager 3.1 with ΔΔ Cq method. All samples were loaded in one 384-well plate. MIQE guideline for the minimum information of RT-qPCR experiments was the followed in this study (Bustin et al., 2009).

Results

Measurement of β-Ala-Lys (AMCA) uptake as a function of concentration

The kinetics of β-Ala-Lys (AMCA) transport was measured in SV isolated from all rat groups. Least squares analysis was then used to fit each dataset to the Michaelis-Menten equation. The results for each age group of Wistar rats is shown in Figure 1, whilst Figure 2 shows the results for young WKY, young SHR and middle-aged SHR. Table 3 summarises the parameters measured. In the Wistar rat groups, SV isolated from middle-aged hearts showed significantly higher capacity than young and old hearts (P=0.02 and P=0.001 respectively). SV isolated from old hearts had a significantly lower affinity compared to middle-aged and young hearts (P=0.02 and P=0.03 respectively). In addition, SV isolated from young adult SHR group showed significantly lower capacity than WKY and middle-aged SHR (P=0.001 and P=0.004 respectively). There were no significant differences in the affinity of β-Ala-Lys uptake into WKY and SHR SV.

Substrate competition experiment

Gly-Gln and carnosine 10 mM were used to inhibit the uptake of 100 μM β-Ala-Lys (AMCA) by SV isolated from the hearts of all rat groups. These experiments were concurrently run with the concentration-dependent uptake experiments (i.e. using the same vesicles) in the same plates. The uptake of β-Ala-Lys (AMCA) into SV isolated from the hearts of young adult, middle-aged, and old rats was reduced by Gly-Gln by 71.4, 77.8, and 81.1% respectively. There was significantly lower inhibition in young compared to middle-aged and old (P=0.03 and P=0.003 respectively), and again significantly lower inhibition for middle-aged when

compared to old rats (P=0.01). Similarly, carnosine significantly inhibited the uptake of β-Ala-Lys (AMCA) into the same SV by 64.7, 69.4, and 73.5 % respectively. There was a significant difference between young adult compared to middle-aged and old rats (P=0.004 and 0.03 respectively), but not between middle-aged and old (Figure 3A).

Similarly, the transport of β-Ala-Lys (AMCA) into SV isolated from the hearts of young WKY, young SHR, and middle-aged SHR was significantly inhibited by Gly-Gln by 64.4, 47.1, and 71.1 % respectively. Statistical analysis of the inhibition between these groups shows significant differences between Y-WKY and Y-SHR (P=0.04) and significantly lower inhibition in Y-SHR compared to M-SHR (P=0.002). Likewise, carnosine significantly inhibited the uptake of β-Ala-Lys (AMCA) into the same SV by 59.2, 38.9, and 65.6 %, respectively, with significant difference between Y-WKY and Y-SHR (P=0.01) and Y-SHR compared to M-SHR rats (P=0.0001) (Figure 3B).

Expression of PEPT2 protein in the heart of different rat groups

Western blotting was used to relatively quantify PEPT2 protein expression in the different hearts in order to investigate the effect of ageing and hypertension. The band intensities of PEPT2 versus their corresponding β-actin bands on Hyperfilm ECL (GE Healthcare) were analysed using the BIO-RAD GEL DOC EQ system and its associated Quantity One software (Figures 4&5). Statistical analysis showed significant differences in the expression of cardiac-type PEPT2 protein with ageing in Wistar rat groups and with hypertension in WKY and SHR rat groups (ANOVA, P=0.015 and P=0.0005 respectively). This was followed by post-hoc t-test to compare between groups individually. In Wistar rats, the expression of cardiac-type

PEPT2 protein in the middle-aged and old groups was significantly higher compared to young adult group (P=0.01) (Figure 4&5). Other groups WKY and SHR showed that the expression of cardiac-type PEPT2 in young adult SHR group was significantly lower compared to young adult WKY and middle-aged SHR groups (P=0.02 and P=0.004 respectively) (Figure 5).

Expression of PEPT2 mRNA in the hearts of different rat groups

The values of the relative normalised expression of SLC15A2 gene were obtained using Bio-Rad CFX Manager 3.1, which were statistically analysed and plotted as shown in Figure 6. ANOVA test showed significant differences in the expression of SLC15A2 gene in Wistar rat groups, and WKY and SHR rat groups (P= 0.02 and P=0.002 respectively). In the Wistar rat groups, middle-aged and old hearts showed significantly higher (1.8 fold) SLC15A2 gene expression than the young adult hearts (P=0.04 and P=0.036 respectively). In addition, SLC15A2 gene expression was significantly higher (3 fold) in the heart ventricles of Y-SHR group than that of the heart ventricles of Y-WKY and 1.7 fold higher than in the heart ventricles of M- SHR groups (P=0.01).

Discussion

This study was carried out to investigate the effect of ageing and hypertrophy on PEPT2 expression and the kinetics of β-Ala-Lys (AMCA, a known substrate of PEPT2) uptake in the heart. This could have implications for myocardial protection and the treatment of bacterial endocarditis. The results showed that the capacity of β-Ala-Lys (AMCA) uptake into sarcolemmal vesicles increased from young to middle aged, but then decreased in old age. There was also a change in affinity in old age. Protein and gene expression both progressively

increased from young to old. In young SHR SV the capacity was decreased compared to the WKY controls and then increased in the middle aged SHR SV. Protein expression was lowered in young SHR SV, whilst gene expression was highest in young SHR hearts. These results show that increased expression and activity in middle-aged hearts could be exploited for myocardial protection, whilst decreased activity in the older population has implications for nutrient handling and the search for suitable protective agents. Decreased activity and protein expression in the young hypertrophied heart may suggest that the addition of exogenous dipeptides may not be the best cardioprotective agent in this population, whilst there is greater hope for the middle-aged hypertrophied heart.

This is the first study to investigate the effect of ageing on the cardiac-type PEPT2 cotransporters at the gene and protein expression and function levels. The different ages of hearts used in this study represent the typical ages (young adult, middle-aged, and old) of human hearts (Boengler et al., 2009; Sengupta, 2013). Some studies have shown the advantage of using dipeptide over single amino acids in protecting hearts from myocardium damage (Almashhadany et al., 2015; McCarty & DiNicolantonio, 2014; Zhang et al., 2013). Since the interrelation between membrane transporters and their substrates play a crucial role in the efficacy of cardiac protection (King et al., 2004, 2006), it was considered important to investigate the effect of ageing on the dipeptide PEPT2 cotransporter. This is also because of the increasing need to better understand age-induced alterations in gene and protein expression and function, since several studies have shown that with advanced ageing, it is increasingly difficult to protect older hearts (Boengler et al., 2009; Jahangir et al., 2007). In our previous study, Almashhadany et al. (2015) we showed that the dipeptide L-glycyl-L-glutamine could be used as a good cardioprotective molecule against ischaemia-reperfusion injury. It was found that 5mM Gly-Gln could improve the post-ischaemic rate pressure product and decrease

reperfusion damage, which was evaluated from the release of lactate dehydrogenase. Interestingly, the middle-aged hearts showed higher sensitivity to protection by Gly-Gln than young hearts. This could be explained by the current results as the PEPT2 cotransporter, by which Gly-Gln is mediated, was significantly upregulated at both gene and protein level in middle-aged hearts compared to young adult hearts. This resulted in significantly higher inhibition percentage and significantly faster rate (V_{max}) of β-Ala-Lys (AMCA) transport by SV isolated from the middle-aged group compared to the young SV.

Although there were no significant changes in the gene and protein expression between middle-aged and old hearts, the latter ones demonstrated a significantly lower V_{max} and a higher K_m. There could be several explanations for this apparent discrepancy. Possibilities include post-translational modification of the PEPT2 protein by either ubiquitination or glycosylation. The latter has previously been shown to affect EAAT1 expression in SV also isolated from male Wistar rat hearts (King et al., 2001). It is also possible that other transporter(s) may be involved as suggested by the change in K_m. If this is the case the identity of these other transporter(s) is unknown but is unlikely to be PEPT1 as this was shown to be absent from the guinea pig heart (Lin and King., 2007). However, the conserved inhibition efficiency by Gly-Gln in the old hearts still suggested its potential use as a competent cardioprotective agent at this age.

This is also the first study to examine the effects of chronic hypertension on the expression of PEPT2 and activity of dipeptide transporters in the heart. The increased V_{max} and increased protein expression in the middle aged SHR compared to young SHR supports the idea that dipeptides could be effective myocardial protective agents in all types of middle aged hearts. This possibility is further supported by previous work showing that changes in the expression

and activity of EAAT2 and EAAT3 in the hypertrophic heart are accompanied by increased protection against ischaemia reperfusion when either exogenous aspartate or glutamate are applied (King et al., 2004; King et al., 2006). The apparent discrepancy between the gene and protein levels might possibility be explained by the different samples used, i.e. SV for protein expression and transporter activity experiments and whole heart for gene expression.

In conclusion, it is obvious that ageing and hypertension differentially affect the cardiac-type PEPT2. These findings suggest that increased expression and activity of the PEPT2 cotransporter in the hearts of all types of middle rats could be employed for facilitating the entry of potential cardioprotective di-and tripeptides and some β-lactam antibiotic, which are used, for example, in treating individuals with bacterial infective endocarditis.

Declarations

Funding: This work was supported by the University of Jeddah and the University of New England.

Conflicts of interest/competing interests: The authors have nothing to declare.

Ethical Approval: This study was approved by the Animal Ethics committee of the University of New England and complies with the *Guide for the care and use of laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Consent to participate: NA

Consent for publication: NA

Availability of data and material: available on request.

Code availability: NA

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Figure legends

Figure 1: Increases in fluorescence as a function of β-Ala-Lys (AMCA) concentration incubated at 20°C for 20 min with SV (60 μg protein) isolated from the hearts of young (squares, dotted line) middle-aged (circles, dashed line) and old (triangles, solid line) Wistar rat groups. Least squares analysis was used to fit the curves to the Michaelis-Menten equation (R^2 >0.95). Data shown are means ± SE, where n= 6 (in triplicate).

Figure 2: Increases in fluorescence as a function of β-Ala-Lys (AMCA) concentration incubated at 20°C for 20 min with of SV (60 μg protein) isolated from the hearts of Y-WKY (squares, dotted line) Y-SHR (circles, dashed line) and M-SHR (triangles, solid line) rat groups. Least squares analysis was used to fit the curves to the Michaelis-Menten equation (R^2 >0.95). Data shown are means ± SE, where n= 6 (in triplicate).

Figure 3: Inhibition of 100μM β-Ala-Lys (AMCA) transport into (A) SV (Wistar rat) and (B) SV (WKY&SHR rats) by 10mM Gly-Gln and Carnosine (sample intensity/ mean control intensity X 100 for each reading) at pH 6.6 for 20 min. (A) * $p<0.05$ vs gly-gln in young; ** $p<0.05$ vs gly-gln in middle-aged; and, *** $p<0.05$ vs carnosine in young. (B) * $p<0.05$ vs gly-gln in Y-SHR; and, ** $p<0.05$ vs carnosine in Y-SHR. Data shown are means ± SE, where n= 6 (in triplicate).

Figure 4: Western blotting bands for PEPT2 proteins with their corresponding β-actin protein bands. (A) Shows the bands of PEPT2 proteins in SV isolated from the hearts of young adult, middle-aged, and old Wistar rats, and (B) shows the bands of PEPT2 proteins in SV isolated from Y-WKY, Y-SHR, and M-SHR. Scanning densitometry was used to measure the density ratio (PEPT1 or PEPT2/ corresponding β-actin).

Figure 5: Immunoblot analysis of PEPT2 proteins in SV isolated from the hearts of (A) Wistar group (young adult, middle-aged, and old) and (B) young adult WKY, young adult SHR and

middle-aged SHR. The protein concentration loaded in each lane of the gel was constant at 60µg. Anti-PEPT2 goat polyclonal and anti-β actin mouse monoclonal were used against PEPT2 and β-actin respectively. The relative protein abundance was measured by scanning densitometry. This is followed by calculating the ratio of protein abundance for PEPT2 to β-actin (as PEPT/ β-actin ×100). (A) * $p<0.05$ vs young. (B) * $p<0.05$ vs Y-SHR. Data shown are means ± SE, where n=4-6.

Figure 6: Relative normalised expression of SLC15A2 "PEPT2" gene (against three reference genes SDHA, TBP and β-Actin) measured with ΔΔ Cq method using Bio-Rad CFX manager 3.1. These genes are quantified in the hearts of two main groups: (A) Wistar rats and (B) WKY and SHR rats as indicated in the graph. (A) * $p<0.05$ vs young. (B) * $p<0.05$ vs Y-WKY and M-SHR. Data shown are means ± SE, where n=5 (all samples were run in one 384 well plate and in duplicate).

Table 1: Body and ventricle weight for the Wistar rats used in this study. * $p < 0.05$ vs. middle aged rats, ** $p < 0.05$ vs. Young adult rats. Data shown are mean ± SE of n=8-16.

Table 2: Body and ventricle weight for the WKY and SHR rats used in this study. * $p<0.05$ vs. young adult WKY, ** $p<0.05$ vs young adult SHR. Data shown are the mean ± SE of n=8-16.

Table 3: Details of the primers used in this study.

Table 4: Comparison of kinetic parameters (Km and Vmax) for the uptake of β-Ala-Lys (AMCA) in SV isolated from the hearts of Wistar, WKY and SHR rat groups. * $p<0.05$ vs young Wistar or WKY; ** $p<0.05$ vs middle-aged or young SHR; *** $p<0.05$ vs young SHR. Data shown are means ± SE of n=6 (measured in triplicate).

Figure 1

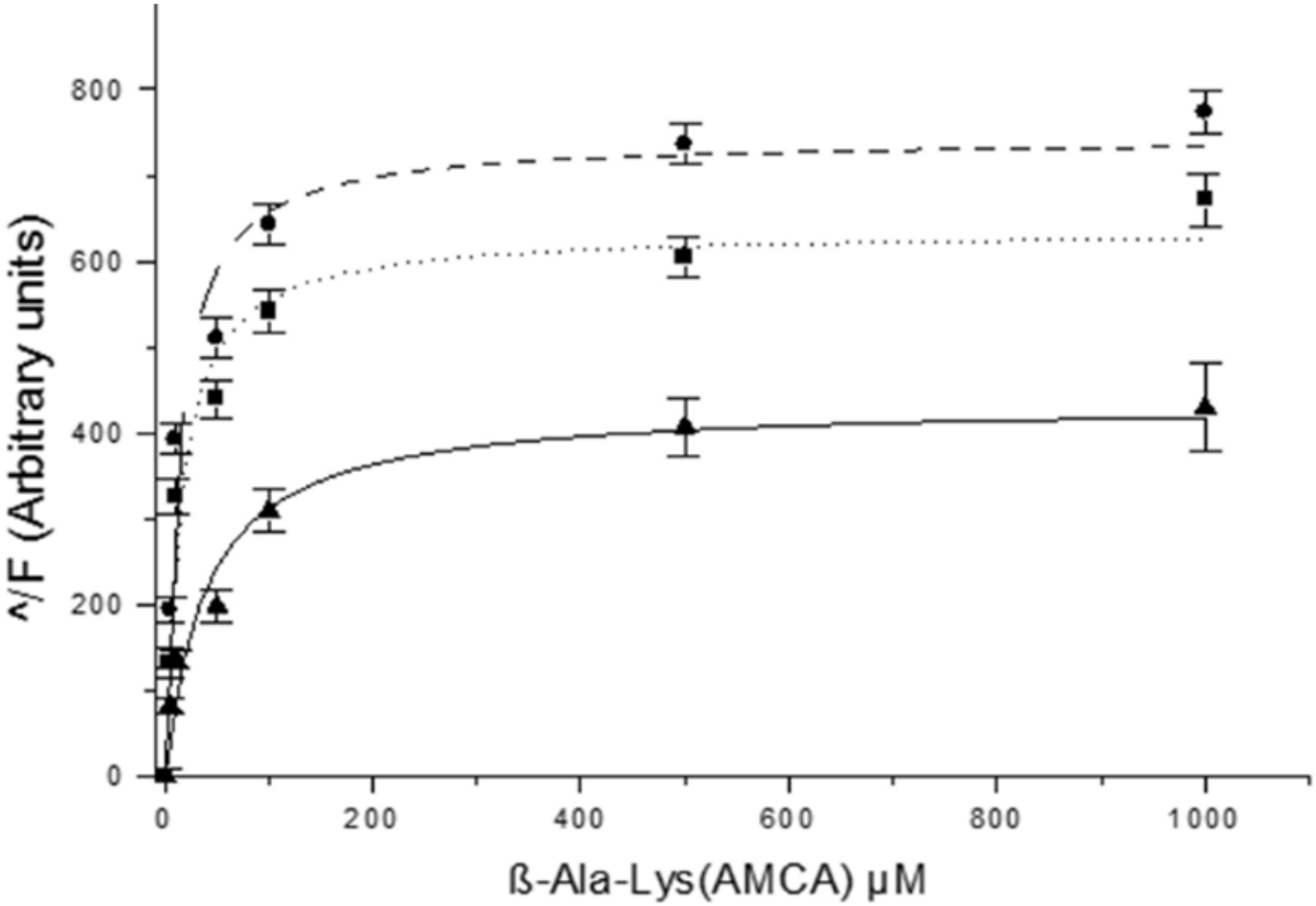

800
600
400
200
0
^/F (Arbitrary units)
0
200
400
600
800
1000
ß-Ala-Lys(AMCA) µM

Figure 2

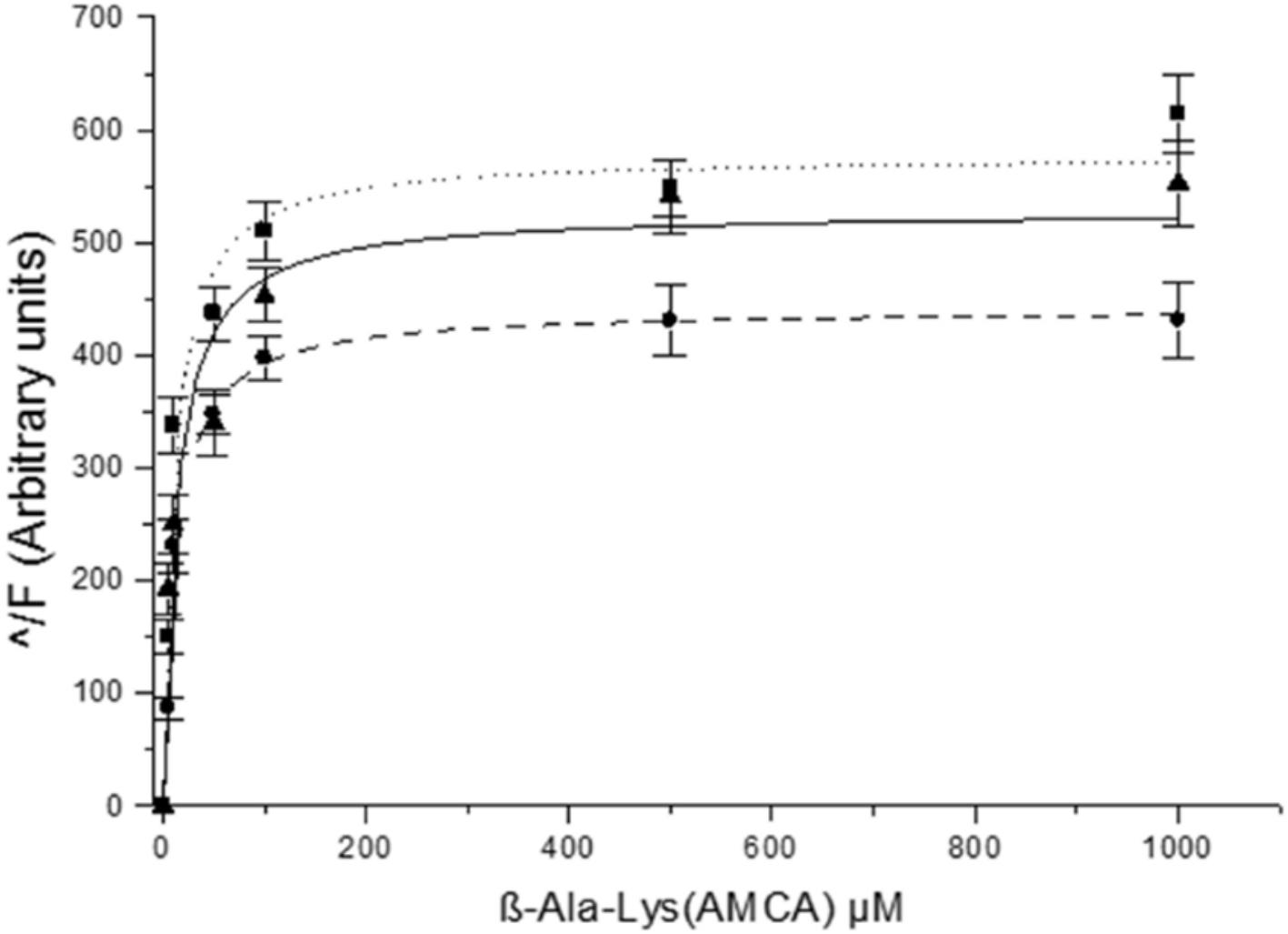

700
600
500
400
300
200
100
0
^/F (Arbitrary units)
0
200
400
600
800
1000
ß-Ala-Lys(AMCA) µM

Figure 3

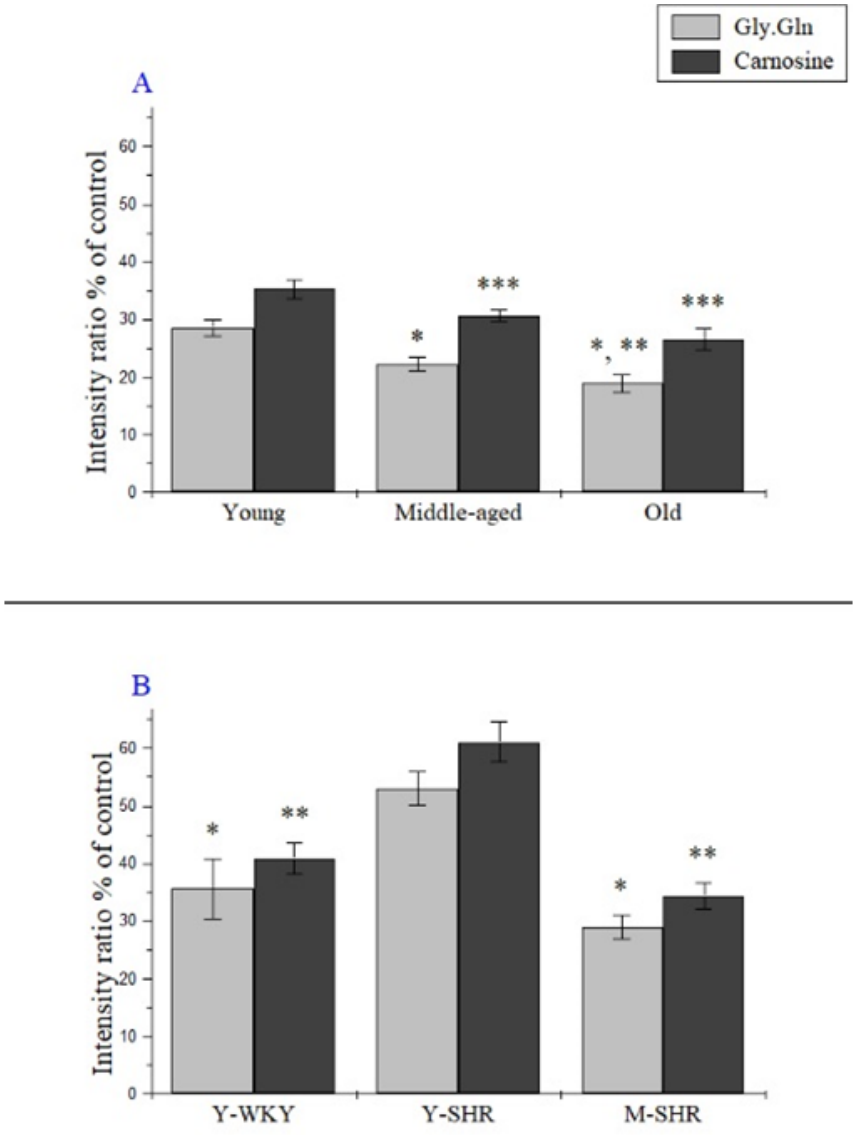

Gly.Gln
Carnosine
A
Intensity ratio % of control
0
10
20
30
40
50
60
*
***
*, **
***
Young
Middle-aged
Old
B
Intensity ratio % of control
0
10
20
30
40
50
60
*
**
*
**
Y-WKY
Y-SHR
M-SHR

Figure 4

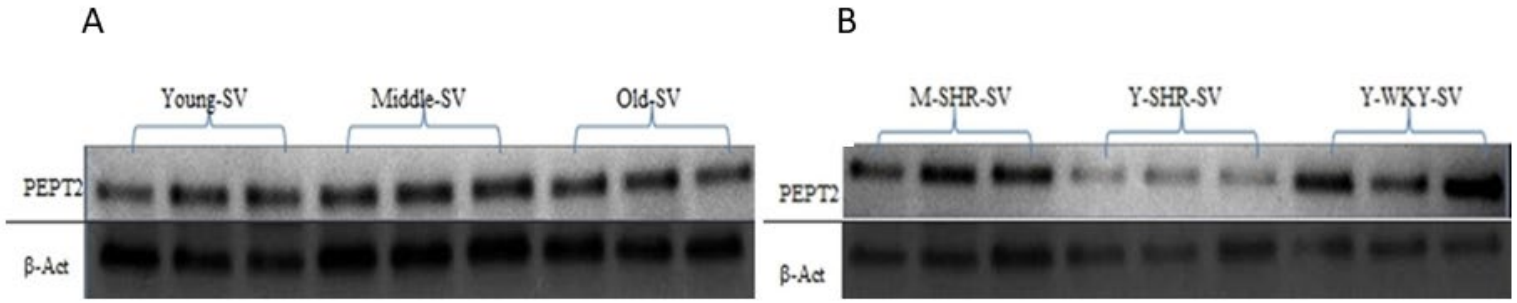

A
Young-SV
Middle-SV
Old-SV
PEPT2
β-Act
B
M-SHR-SV
Y-SHR-SV
Y-WKY-SV
PEPT2
β-Act

Figure 5

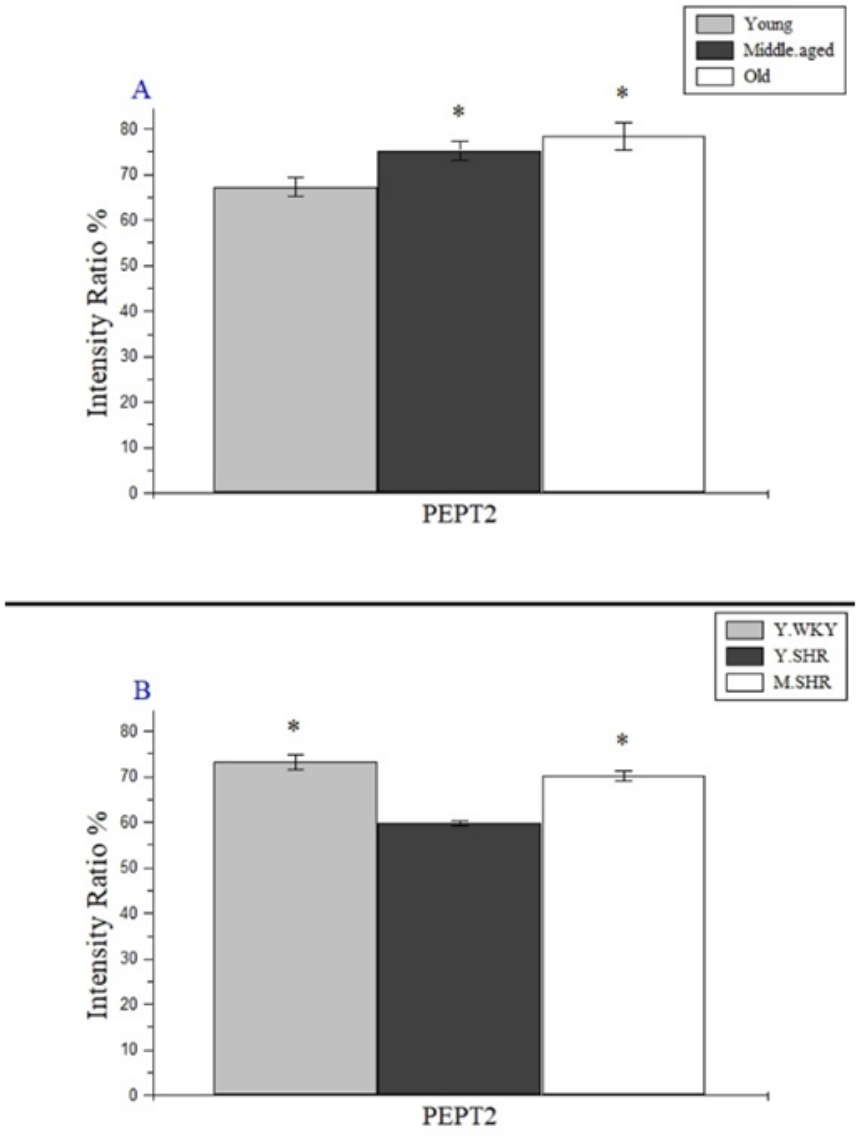
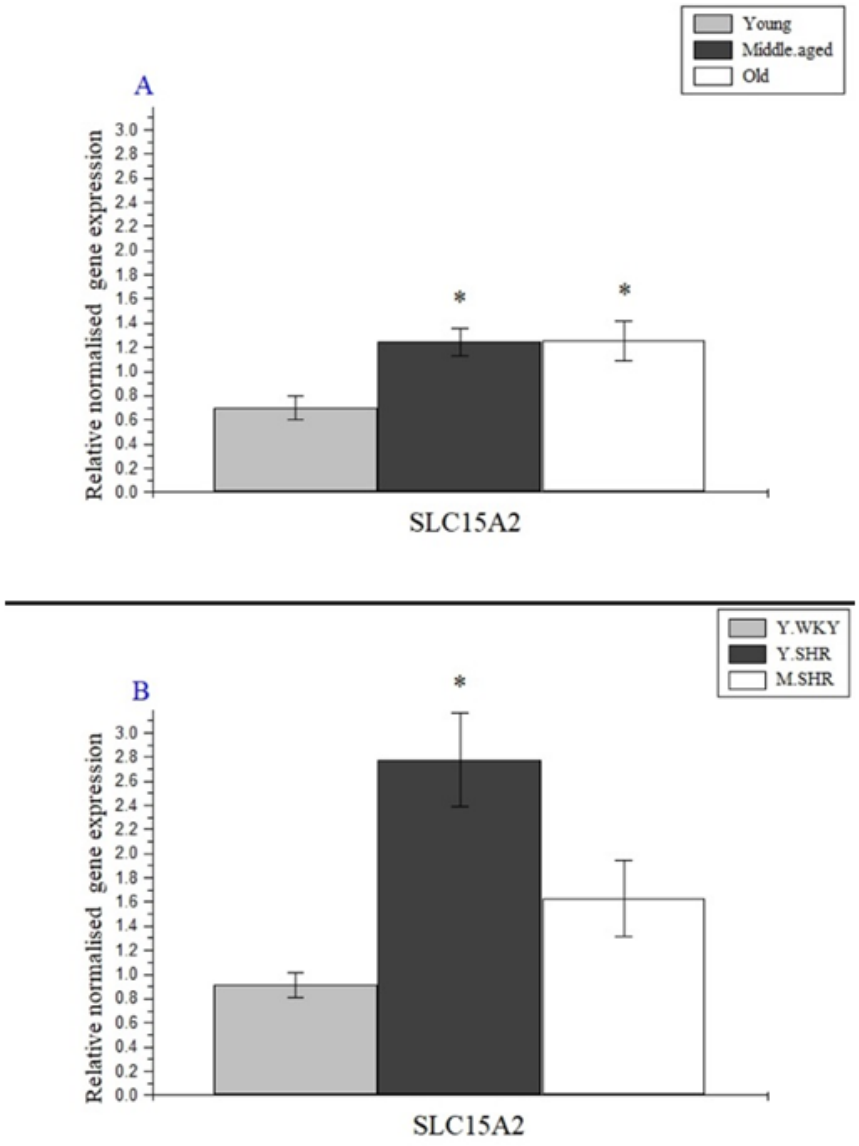

A
Young
Middle.aged
Old
Intensity Ratio %
PEPT2
B
Y.WKY
Y.SHR
M.SHR
Intensity Ratio %
PEPT2

Figure 6


A
Young
Middle.aged
Old
Relative normalised gene expression
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
SLC15A2
B
Y.WKY
Y.SHR
M.SHR
Relative normalised gene expression
*
SLC15A2