Amino Acids (2002) 23: 395–400
DOI 10.1007/s00726-002-0201-3

Amino
Acids
Printed in Austria

Effect of ischemia, calcium depletion and repletion, acidosis and hypoxia on cellular taurine content

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Received November 15, 2001
Accepted January 15, 2002
Published online October 3, 2002;

Summary. Occlusion of the left main coronary artery led to a time-dependent release of taurine from the heart. Upon reperfusion, there was a second phase of taurine release, which exceeded the amount of taurine that exited the heart during the 45 min ischemic insult. To obtain information on the mechanism underlying the release of taurine, three variables were examined, acidosis, hypoxia and calcium overload. It was found that large amounts of taurine also leave the cell during the calcium paradox, a condition induced by perfusing the heart with calcium containing buffer following a period of calcium free perfusion. However, little taurine effluxes the hearts exposed to buffer whose pH was lowered to 6.6. Isolated neonatal cardiomyocytes subjected to chemical hypoxia also lost large amounts of taurine. However, the amount of taurine leaving the cells appeared to be correlated with the intracellular sodium concentration, $[Na^+]_i$. The data suggest that taurine efflux is regulated by $[Na^+]_i$ and cellular osmolality, but not by cellular pH.

Keywords: Amino acids – Taurine content – Ischemia – Calcium paradox – Acidosis – Hypoxia – Sodium concentration – Osmolality

Introduction

Taurine, an amino acid found in very high concentration in excitable tissues, serves as an important cellular osmoregulator (Hoffman and Dunham, 1995; Pasantes-Morales and Schousboe, 1997; Schaffer et al., 2000). Cells subjected to an osmotic insult respond by modifying the intracellular content of taurine, with the magnitude of the taurine change dependent upon the severity of the insult and the osmotic gradient established across the cell membrane (Pasantes-Morales and Schousboe,1997; Rasmusson et al., 1993). While a hypo-osmotic insult (either an increase in cellular osmolarity or a reduction in medium osmolality) triggers the release of taurine from the cell, a hyperosmotic insult (resulting from a decrease in cellular osmolality or an increase in medium osmolality) leads to an elevation in tissue taurine content. The movement of taurine into or out of the cell is an important process in the regulatory volume change that accompanies an osmotic insult. Rasmusson et al. (1993) have shown that taurine efflux assumes a critical role following a mild hypo-osmotic insult, but contributes less to the regulatory volume decrease triggered by a severe hypo-osmotic insult. Taurine influx occurs during the regulatory volume increase that accompanies a hyperosmotic insult, but this regulatory step is a slow process requiring an initial activation of the taurine transporter (Hoffman and Dunham, 1995; Pasantes-Morales and Schousboe, (1997)).

In the myocardium, taurine content is also regulated by the transmembrane Na^+ gradient (Chapman et al., 1993). Suleiman et al. (1992) found that an elevation in $[Na^+]_i$ above 20 mM evokes an efflux of taurine from the cell, an effect antagonized by extracellular taurine and the transport inhibitor, azaserine. Since a reciprocal relationship was observed between $[Na^+]_i$ and taurine efflux, Chapman et al. (1993) suggested that the Na^+-dependent efflux of taurine from the cell involves the reversal of the Na^+/taurine symporter.

Osmotic stress develops in several pathological conditions, triggering changes in cellular taurine transport. During myocardial ischemia, large amounts of taurine are lost from the cell (Lombardini and Crass, 1981; Kramer et al., 1981). Since both $[Na^+]_i$ and tissue osmolality increase during an ischemic insult

(Steenbergen et al., 1985; Tani and Neely, 1989), taurine efflux could proceed via volume sensitive channels, reversal of the taurine transporter or a combination of both. To provide information on the cause of taurine loss, three models were used that simulate key aspects of ischemia. A respiratory acidosis model was used to mimic the drop in pH that accompanies an ischemic insult. The Ca^{2+} paradox model, which is produced by perfusing hearts with Ca^{2+} containing buffer following a period of Ca^{2+}-free perfusion, simulates the condition of Na^+ and Ca^{2+} overload. Finally, a hypoxic model was used to test the role of oxygen deprivation on taurine movement.

Materials and methods

Perfusion techniques

Hearts from male Wistar rats (220–260 g) were perfused in the Langendorff mode, with the coronary arteries fed from a reservoir placed 100 cm above the aortic cannula. The standard perfusate was a 37°C Krebs-Henseleit buffer supplemented with 5 mM glucose and 2.5 U/l insulin and gassed with 95% O_2–5% CO_2 to yield a final pH of 7.4. To induce the Ca^{2+} paradox, hearts were perfused for 20 min with the standard Krebs-Henseleit buffer and then rapidly switched to Krebs-Henseleit buffer lacking Ca^{2+}. After 10 min of Ca^{2+} depletion, the hearts were again perfused with the standard Krebs-Henseleit buffer. Following 15 min of Ca^{2+} repletion, the hearts were frozen with tongs precooled in liquid nitrogen. Other hearts were frozen following the Ca^{2+} depletion phase or after 45 min of control perfusion.

Hearts subjected to respiratory acidosis were initially perfused for 20 min with standard Krebs-Henseleit buffer equilibrated with 95% O_2–5% CO_2 (pH 7.4) and then 30 min with standard Krebs-Henseleit buffer equilibrated with 65% O_2–35% CO_2 (pH 6.6). The hearts were then frozen with tongs precooled in liquid nitrogen. Control hearts were frozen after 50 min of perfusion with standard Krebs-Henseleit buffer (pH 7.4).

Hearts subjected to a regional ischemic insult were perfused with standard Krebs-Henseleit buffer supplemented with 11 mM glucose and equilibrated with 95% O_2–5% CO_2. To initiate the experiment, a 2–1 silk suture was loosely placed around the left main coronary artery and then passed through a vinyl tubing to form a snare. After a 20 min control perfusion, the left main coronary artery was occluded by pulling the suture through the snare and clamping the snare with a hemostat. The occlusion was maintained for 45 min, at which point flow was restored to the coronary artery. Coronary effluent was collected at 5 min intervals and assayed for taurine content.

Taurine assay

Myocardial and perfusate taurine content was assayed by the method of Shaffer and Kocsis (1981). Frozen heart samples were freeze dried, weighed and then homogenized with 2% perchloric acid. After neutralization with K_2CO_3, the supernatant was used for taurine determination. The perfusate was directly assayed for taurine content without treatment with perchloric acid. Samples were passed through small Dowex 50 columns and eluted with 2.0 ml deionized water. An aliquot was then reacted with 0.1 ml of 2,4 dinitrofluoro-1-benzene (DNFB) in the presence of 0.1 ml of 1 M NaOH and 0.5 ml of dimethyl sulfoxide. Deionized water was added to raise the final volume to 5 ml. The samples were then extracted with ethyl acetate (20 ml) to remove the derivatized carboxylic amino acids and unreacted DNFB. The content of 2,4 dinitrophenyltaurine was determined by absorbance at 355 nm.

Cardiomyocyte preparation

Isolated neonatal cardiomyocytes were prepared from 2 day-old Wistar rats using the procedure of McDermott and Morgan (1989). Dissociated cells were pre-plated on plastic culture flasks for 90 min at 37°C to allow the attachment of non-myocyte cells. The myocytes were then resuspended in MEM supplemented with vitamins, antibiotics, amino acids, 10% newborn calf serum and 0.1 mM 5-bromo-2-deoxyuridine. After plating the myocytes on glass chamber slides or polystyrene treated dishes, the cells were incubated overnight to allow attachment. The cells were then cultured in serum-free medium containing MEM supplemented with vitamins, antibiotics, amino acids and 0.1 mM 5-bromo-2-deoxyuridine. After 3 days of control culture, the cells were transferred to medium lacking glucose but containing 10 mM deoxyglucose and 3 mM Amytal. At either 25 min or 60 min some of the samples were scraped from the Petri dishes and an aliquot removed to determine protein content. The remaining cells were treated with 2% perchloric acid and after neutralization, taurine content of the extracts was determined.

Cellular Na^+ content

Following 3 days of control incubation, the cells were loaded with sodium binding benzofuran isophthalate (SBFI) by incubating the cells for 1.5 hrs at room temperature with serum- free medium containing 10 λM SBFI/acetoxymethyl (AM) ester. After washing the cells, the myocytes were kept in dye free medium for 45 min to facilitate the hydrolysis of the ester. The cells were then placed in Krebs-Henseleit buffer and sodium content was determined fluorometrically (emission wavelength > 429 nm) using an Olympus (IMT-2) microscope, with excitation wavelengths being 340 nm and 380 nm. The cells were then transferred into Krebs-Henseleit containing 10 mM deoxyglucose and 3 mM Amytal. After 25 min and 60 min of chemical hypoxia, the emission fluorescence signal was again determined at two excitation wavelengths, 340 nm and 380 nm. $[Na^+]_i$, was calculated from the fluorescence ratio (F_{340}/F_{380}) after generating a calibration curve according to the method of Harootunian et al. (1989).

Results

Figure 1 shows the time course of taurine efflux from the ischemic and reperfused myocardium. In agreement with previous studies (Lombardini and Crass, 1981; Kramer et al., 1981), it was found that large amounts of taurine leave the ischemic heart. However, in contrast to the immediate response to a hypoosmotic insult, taurine efflux from the regionally ischemic heart was delayed several minutes and only reached a peak after 30 minutes. Reperfusion led to a second phase of taurine release, with the peak rate of taurine loss being about 1.6 μmol/min.

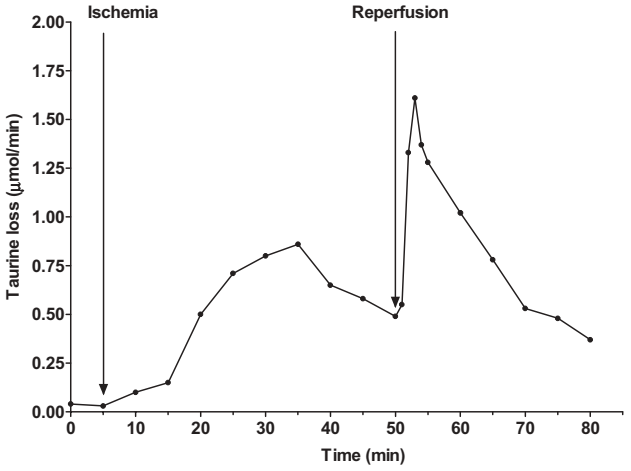

Fig. 1. Efflux of taurine from the regionally ischemic heart. Hearts were perfused for 20 min under controlled normoxic conditions, at which point two 5 min perfusate samples were collected (Times 0 and 5). The hearts were then subjected to an ischemic insult and perfusate samples were collected at 5 min intervals for the next 45 min. Coronary flow was then restored and perfusate samples were collected at either 1 min or 5 min intervals for the next 30 min. After determining the rate of coronary flow, all samples were assayed for taurine content. The data, which were taken from a representive heart, were expressed as μmol/min

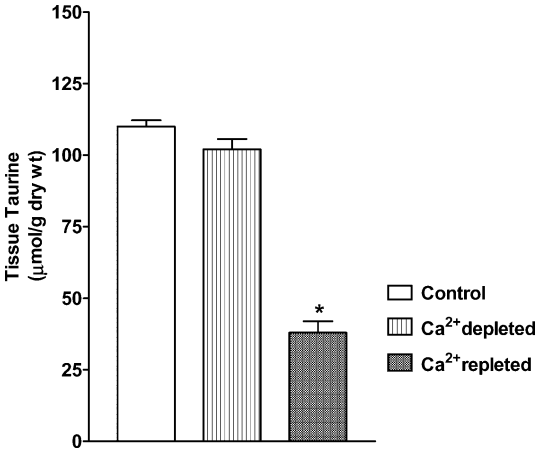

Fig. 2. Effect of calcium depletion and repletion on tissue taurine content. Hearts were perfused for 20 min with standard Krebs-Henseleit buffer containing 1.25 mM calcium (Control group). The hearts were then subjected to a 10 min perfusion with buffer lacking calcium (calcium depletion group) followed by a 15 min perfusion with standard calcium containing buffer (calcium repletion group). Hearts were frozen after 10 min of calcium depletion, 15 min of calcium repletion or 45 min of controlled perfusion with the standard calcium containing buffer. Taurine content of all hearts were then examined. Data shown represent means $\pm$ S.E.M. of 7 hearts. Asterisks denote significant differences from the control ($p < 0.05$)

While ischemia is associated with the development of both an osmotic imbalance and Na^+ overload, reperfusion is characterized by a transient elevation in $[Na^+]_i$, an accumulation of Ca^{2+} and a restoration of osmotic homeostasis (Steenbergen et al., 1990; Tani and Neely, 1989). One model that focuses on the consequences of Na^+ and Ca^{2+} overload is the Ca^{2+} paradox, which consists of a Ca^{2+} depletion and a Ca^{2+} repletion phase. The initial period of Ca^{2+} depletion is associated with a significant increase in $[Na^+]_i$ but little change in tissue osmolality (Schaffer and Tan, 1985; Wagenknecht et al., 1994). By contrast, the Ca^{2+} repletion phase is associated with a rise in $[Ca^{2+}]_i$, a return of $[Na^+]_i$ to the normal range and the accumulation of key osmolytes, such as lactate and inorganic phosphate (Schaffer and Tan, 1985; Wagenknecht et al., 1994). Despite the rise in $[Na^+]_i$ during the Ca^{2+} repletion phase, taurine levels were unaffected by exposure of the heart to Ca^{2+} free buffer (Fig. 2). However, Ca^{2+} repletion was associated with a dramatic drop in myocardial taurine content (Fig. 2).

A model associated with cellular Na^+ accumulation without an elevation in lactate and inorganic phosphate is respiratory acidosis (Harrison et al., 1992; Williamson et al., 1976). To examine the effect of respiratory acidosis on myocardial taurine content, hearts were initially perfused with Krebs-Henseleit buffer equilibrated with 95% O_2, 5% CO_2 (pH 7.4) and then transferred to buffer equilibrated with 65% O_2, 35% CO_2 (pH 6.6). Although an abrupt change in perfusate pH from 7.4 to 6.6 is known to elevate $[Na^+]_i$ (Nakanishi et al., 1990), no significant alteration in myocardial taurine content was observed during the pH transition (Fig. 3).

Figures 2 and 3 suggest that no clear-cut relationship exists between $[Na^+]_i$ and taurine efflux in hearts undergoing the calcium paradox or subjected to a pH transition. Nonetheless, further testing of this relationship seemed warranted, particularly since key differences in cation transport exist between the ischemic heart and the two simulated models. To provide additional information on this relationship, $[Na^+]_i$ and taurine content were measured in isolated cardiomyocytes subjected to an hypoxic insult. Figures 4 and 5 show that although $[Na^+]_i$ increased 80% during the initial 25 min of hypoxia, the intracellular taurine pool was only reduced by 12%. By comparison, nearly half of the cellular taurine pool was lost after 60 min of chemical hypoxia, a time in which $[Na^+]_i$ increased to 87.5 mM (Figs. 4 and 5).

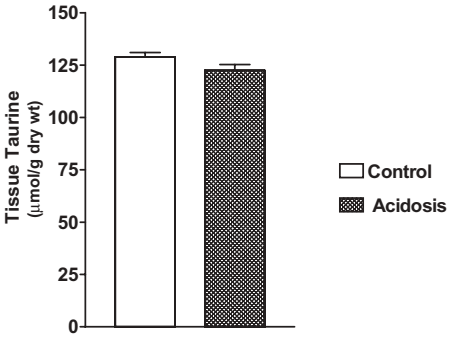

Fig. 3. Effect of acidosis on tissue taurine content. Hearts were perfused for 20 min with standard Krebs-Henseleit buffer (pH 7.4) before being subjected to a pH 7.4 to pH 6.6 transition. After a 30 min perfusion with Krebs-Henseleit buffer equilibrated with high pCO_2 (pH 6.6), hearts were frozen and assayed for taurine content (Acidosis group). Control hearts were perfused for 50 min with standard Krebs-Henseleit buffer (pH 7.4). Data shown represent means ± S.E.M. of 4–6 hearts

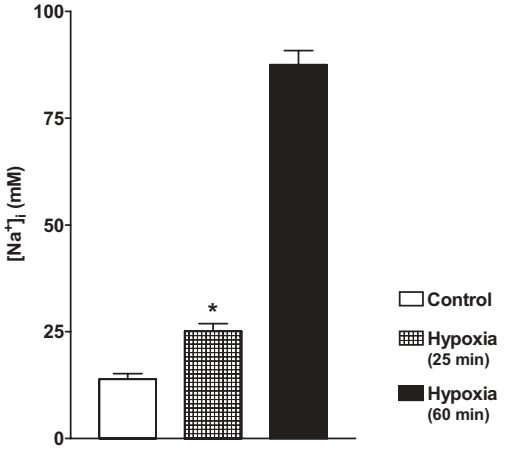

Fig. 4. Elevation of $[Na^+]_i$, during a chemical hypoxic insult. Neonatal rat cardiomyocytes were incubated for 3 days with standard serum-free medium. After loading with the Na^+ indicator dye, SBFI, the SBFI fluorescence ratio was obtained (F_{340}/F_{380}). The cells were then transferred to medium containing 10 mM deoxyglucose and 3 mM Amytal. After either 25 min or 60 min of chemical hypoxia, the SBFI fluorescence ratio was obtained. $[Na^+]_i$, was calculated from a standard curve. Values shown represent means ± S.E.M. of 4 different preparations. Asterisks denote a significant difference between the hypoxic and control groups ($p < 0.05$)

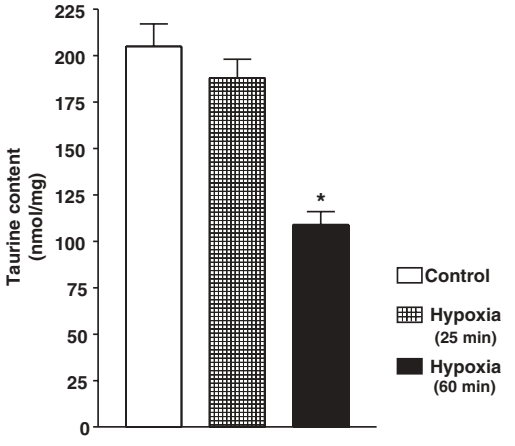

Fig. 5. Effect of chemical hypoxia on cellular taurine content. Rat neonatal cardiomyocytes were incubated for 3 days with standard serum-free medium. Some of the control cells were used in the determination of cellular taurine. Other cells were subjected to either 25 min or 60 min of chemical hypoxia and then used in the analysis of cellular taurine. Values shown represent the means ± S.E.M. of 4 different preparations. The asterisk denotes a significant difference from the normoxic controls ($p < 0.05$)

Discussion

Taurine efflux is a characteristic feature of most models of ischemia and simulated ischemia (Lombardini and Crass, 1981; Saransaari and Oja, 1998; Kramer et al., 1981), one exception being the isolated skate hepatocyte, which remains resistant to either basal or volume sensitive taurine release during KCN poisoning (Ballatori and Boyer, 1992). The mechanism underlying the release of taurine has not received much attention. In the ischemic cortex and hypoxic hippocampal slice, the efflux of taurine appears to involve both osmotic stress and a Na^+-dependent reversal of the taurine transporter (Phillis et al., 1999, Saransaari and Oja, 1992; Saransaari and Oja, 1998).

The myocardium is characterized by very active pathways for both osmotic-induced and Na^+-dependent release of taurine (Kowdley et al, 1997; Rasmusson et al., 1993). Nonetheless, in the Ca^{2+} depleted and acidotic hearts, Na^+ overload is not accompanied by taurine efflux (Figs. 2 and 3). Based on the work of Suleiman et al. (1992) this is not surprising. While taurine is lost in large amounts from hearts perfused with buffer lacking both Ca^{2+} and Mg^{2+}, addition of 0.5 mM Mg^{2+} to the buffer significantly reduces the degree of taurine loss (Suleiman et al., 1992). In the present study, 1.2 mM Mg^{2+} was included in the perfusion medium, a concentration that completely prevented the decline in taurine. One of the effects of elevated Mg^{2+} is to limit the extent of Na^+ overload (Suleiman et al., 1992), however, other factors contribute to the reversal of Ca^{2+} dependent taurine efflux by Mg^{2+}. Saransaari and Oja (1992) found that taurine efflux from cerebral cortical slices is accelerated following treatment with the Ca^{2+} chelator, EDTA, an effect diminished by addition of high Mg^{2+} to the incu-

bation medium. Presumably, the substitution of Mg^{2+} for Ca^{2+} minimizes taurine efflux in the Ca^{2+} depleted cell because the cations share similar properties (Saransaari and Oja, 1992).

It is generally accepted that intracellular acidosis leads to Na^+ overload (Harrison et al., 1992). Nonetheless, taurine efflux does not occur during respiratory acidosis. One logical explanation for this phenomenon is that the taurine transporter remains relatively inactive at an extracellular pH of 6.6. Although the pH dependency of the myocardial taurine transporter has not been studied, Kramhoft et al. (1997) have shown that the taurine transporter of the protozoan, *Tetrahymena pyriformis*, is inactive at pH values below 7.0.

While acidosis and Ca^{2+} overload are important consequences of ischemia, many other events contribute to the pathology of ischemia-reperfusion injury. One of these events is the increase in intracellular osmolality (Steenbergen et al., 1985), which promotes the accumulation of water by the cell. This is potentially damaging to the cell because water uptake leads to cell swelling, a stretching of the cell membrane and some degree of membrane disruption (Steenbergen et al., 1985). Efflux of taurine from the ischemic myocyte reduces the degree of osmotic-induced cell swelling, protecting the cell against injury (Allo et al., 1997). Suleiman et al. (1992) have pointed out that the efflux of taurine via the taurine transporter would have the added benefit that it would be accompanied by the efflux of Na^+, thereby minimizing the influx of Ca^{2+} via the Na^+/Ca^{2+} exchanger.

The loss of taurine following a hyperosmotic insult is only one of many regulatory events designed to reestablish osmotic homeostasis. Nonetheless, the loss of taurine is a major contributor to volume regulation following a mild hypo-osmotic insult (Rasmusson et al., 1993). It also contributes to the restoration of osmotic balance following a severe hypo-osmotic shock, although it is quantitatively less important than other volume regulators (Rasmusson et al., 1993).

Figure 1 shows that taurine begins to efflux the heart 10 min following the cessation of coronary flow. This is not surprising since tissue osmolality increases slowly during ischemia (Steenbergen et al., 1985). By comparison, reperfusion is associated with a rise in tissue water and an instantaneous release of taurine from the heart (Figure 1; Garcia-Dorado et al., 1992). No doubt, "washing out" from the vasculature contributes to the spike in taurine efflux. However, substantial amounts of taurine also leave the cell through volume sensitive channels. The rise in $[Na^+]_i$ also contributes to taurine efflux during the initial phases of reperfusion, a time in which Na^+ enters the cell in exchange for H^+.

In contrast to reperfusion, reoxygenation of the isolated cardiomyocyte leads to a rapid decrease in $[Na^+]_i$ (Piper et al., 1993). However, the presence of a high $[Na^+]_i$ during the hypoxic period results in an accumulation of tissue water upon reoxgenation (Inserte et al., 1997). In accordance with the increase in osmotic stress, cellular taurine levels are modestly reduced during the reoxygenation phase (data not shown). The pattern of osmotic and cation changes are quite different between the ischemic and hypoxic myocyte, primarily because of the accumulation of toxic metabolic end products, such as H^+, by the ischemic cell. The severe decline in pH_i during the ischemic insult explains the rapid exchange of H^+ for Na^+ upon reperfusion, resulting in a transient elevation in $[Na^+]_i$ (Tani and Neely, 1989). By contrast, hypoxia is not associated with an impairment in the efflux of metabolic end products from the cell. Therefore, H^+ exchange for Na^+ during the course of the hypoxic insult, resulting in a progressive rise in $[Na^+]_i$. Figure 5 shows that 25 minutes of hypoxia leads to an 80% increase in $[Na^+]_i$ while 60 min of hypoxia is associated with a 6 fold increase in $[Na^+]_i$. According to Chapman et al. (1992) very little taurine effluxes the cell when the $[Na^+]_i$ is below 20 mM. Since $[Na^+]_i$ ~25 mM after 25 min of hypoxia, it is not surprising that the amount of taurine efflux is quite modest (~12%). However, by 60 min of hypoxia, $[Na^+]_i$ rises to about 85 mM, dramatically elevating Na^+-dependent taurine efflux and contributing to the two fold decrease in intracellular taurine content.

While acute taurine loss may benefit the ischemic heart by minimizing the degree of hyperosmotic shock, chronic taurine loss has adverse effects. Novotny et al. (1991) found that cats fed a taurine deficient diet develop a cardiomyopathy characterized by both systolic and diastolic dysfunction; taurine administration reverses these effects (Pion et al., 1992). Moreover, rabbits suffering from congestive heart failure exhibit an increased mortality rate when treated with a taurine depleting agent but prolonged life expectancy when treated with taurine (Takihara et al., 1986). These beneficial effects of taurine relate in part to its anti-angiotensin II activity (Azuma et al., 2000). Therefore, the best outcome following a myocardial

infarction may be achieved by initiating taurine therapy when angiotensin II levels become significantly elevated.

Acknowledgements

This study was supported with a grant from the Taisho Pharmaceutical Company.

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