Taurine 7

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Taurine 7

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Preface

Taurine (2-ethanesulfonicacid) is a unique and mysterious compound. It is present in relatively high concentrations in a wide range of cells and tissues, but exists as a free amino acid in these cells without being utilized in protein synthesis. Taurine was first isolated more than 150 years ago from ox (Taurus) bile, where it was found in conjugation with bile acids through an amide linkage. Since that time, it has been reported to exist in particularly high concentrations in the cytoplasm of excitable tissues, such as certain parts of the brain, retina, skeletal muscle, myocardium and platelets. Some of its physiological functions have already been established, for example its role as an essential nutrient during development, an osmolyte and a neuromodulator. Recently, taurine has been advanced as a cytoprotective agent against certain pathological perturbations, but the mechanisms underlying its actions are still mostly a matter of speculation. Moreover, it is possible that other putative functions of taurine remain to be discovered.

The 16th International Taurine Meeting "Taurine for Future Healthcare" was held on September 2–5, 2007, in Shimoda, Shizuoka, Japan, with the site of the meeting being the Shimoda Central Hotel. Approximately 80 individuals from 11 nations, including newcomers as well as experts in taurine research, attended the scientific meeting. A total of 79 papers were presented as either oral or poster presentations. This meeting was multidisciplinary, with participants addressing multiple areas of the biological sciences. Typhoon "Fitow", which means "beautiful fragrant flower" in a Micronesian language, hit the Shimoda region with full force at the end of the meeting, but we were able to finish the scientific sessions and enjoy an excursion prior to the onslaught. The morning after Fitow's fury, the lingering scent of flowers reminded us that we are clearly at the dawn of a new era in taurine research.

The organizers of the taurine meeting would like to thank Taisho Pharmaceutical Co., Ltd., Tokyo, Japan, for their generous financial support and assistance in the organization of the meeting. We would also like to thank Dong-A Pharmaceutical Co, Korea, for their generous financial support. In addition, we would like to thank all participants of the meeting, especially the participants from the Osaka University and Taisho Pharmaceutical Company. The staff of the Shimoda Central Hotel was extremely helpful in making sure that all participants were comfortable and for ensuring the success of their planned events. Finally, we would like to express our

appreciation for the untiring effort before, during and after the scientific sessions of Dr. Abe, who helped ensure the success of the meeting.

We are pleased to provide you with *Taurine 7*, which contains the proceedings of the 16th International Taurine Meeting consisting, of 54 original papers. This volume focuses on all aspects of taurine research, including topics of interest to today's scientists as well as future clinical applications.

Part I. Cardiovascular and Renal Effects of Taurine.
Part II. Effect of Taurine on Brain and Retina.
Part III. Effect of Taurine on Skeletal Muscle.
Part IV. Gastroenteric and Hepatic Effects of Taurine.
Part V. Effect of Taurine on Bone.
Part VI. Effect of Taurine on Diabetes and Obesity.
Part VII. Potential Therapeutic Effects of Taurine.
Part VII. Taurine as an Antioxidant; Role in Immune System and Other Tissues.
Part IX. Regulation of the Taurine Transporter.

Future interest in taurine will undoubtedly be robust. However, considerable work remains to develop and uncover key new facts regarding taurine. This book should provide insight into new avenues of investigation and help propel the field into the new era of taurine research. Finally, the organizers wish to thank all of the participants for their stimulating discussions, probing questions and written contributions that made the Shimoda taurine meeting an unmitigated success.

We are deeply thankful to all scientists who have an interest in taurine, and are looking forward to seeing the taurine family at the next Taurine meeting in Florida.

Alone we can do so little; together we can do so much.
Helen Keller

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Part I
Cardiovascular and Renal Effects of Taurine

Chapter 1
Effect of Taurine on Protein Kinase C Isoforms: Role in Taurine's Actions?

Takashi Ito, Viktor Pastukh, Viktoriya Solodushko, Junichi Azuma, and Stephen W. Schaffer

Abstract Taurine is generally found to be cytoprotective, diminishing damage resulting from ischemia and from initiators of heart failure. Also linked to similar events in the heart is the protein kinase C (PKC) family, which consists of at least 12 different isoforms. Therefore, we proposed that PKC might contribute to the beneficial effects of taurine on cell viability and growth. One of the PKC isoforms that has been advanced as an important mediator of cytoprotection during ischemia is PKCε. In this study, we found that incubation of isolated cardiomyocytes with medium containing 20 mM taurine led to the translocation of PKCε into the membrane, an event commonly associated with the cardioprotective actions of the PKC isozyme. In addition, taurine promoted the upregulation of PKCα PKCβ2 and PKCζ. Because the effects of taurine and angiotensin II on PKC distribution were largely additive, PKC does not appear to contribute to the antagonism between taurine and angiotensin II. However, the upregulation of PKC by taurine is consistent with a role of taurine in normal cell growth. In the taurine deficient heart, cardiomyocyte size is reduced, an effect that is consistent with the effect of taurine on PKCε. In conclusion, the cytoprotective and pro-growth actions of taurine appears to be mediated in part by the activation of PKCε.

Abbreviations *PKC*, Protein kinase C; *Ang II*, Angiotensin II

1.1 Introduction

Taurine is the most abundant free amino acid in mammalian tissue, reaching concentrations as high as 5–20 μmol/g wet wt (Chapman et al. 1993; Chesney 1985). The relationship between intracellular taurine content and cardiac function remains unclear, largely because of the multiple functions of taurine. It is generally accepted that maintenance of intracellular taurine homeostasis is essential for normal cardiac function. Indeed, severe reductions in myocardial taurine content either through dietary taurine deficiency or genetic taurine transporter deficiency leads to the

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development of a cardiomyopathy (Novotny et al. 1991, 1994; Pion et al. 1987). In the failing heart, taurine levels rise, with the increase being directly associated with the severity of heart failure (Newman et al. 1977). The suggestion that the increase in taurine levels might represent an adaptation designed to re-establish normal function led the study of taurine therapy in various animal models of heart failure. These studies have generally shown a beneficial effect of taurine treatment. In the calcium sensitive cardiomyopathic hamster, oral taurine therapy reduced intracellular Ca^{2+} content and decreased the severity of myocardial lesions (Azari et al. 1980; McBroom and Welty 1977). Taurine therapy has also found to reduce mortality and improve contractile function in an aortic regurgitation model of congestive heart failure (Takihara et al., 1986), studies that led to clinical trials that established taurine as useful therapy in the treatment of congestive heart failure (Azuma et al. 1982). Among the factors implicated in these and other models of heart failure have been oxidative stress and calcium overload (Harada et al. 1990; Ohta et al. 1988). Significantly, taurine therapy prevented calcium overload and diminished the degree of oxidative stress in these models.

The pathophysiology of heart failure is complex, involving impaired contractile function, abnormal Ca^{2+} transport, elevations in neurohumoral agents, vascular resistance, diastolic dysfunction and ventricular remodeling. While the initial insult is a decrease in systolic function, the rise in sympathetic and angiotensin II (Ang II) activity triggers a constellation of events that lead to overt heart failure. Inhibition of the neurohumoral agents disrupts the progression of heart failure and reduces mortality, with inhibition of Ang II serving as the mainstay in the treatment of heart failure.

It has been proposed that taurine therapy may benefit the heart by preventing the actions of Ang II (Schaffer et al. 2000). This contention is largely based on the finding that incubation of isolated cardiomyocytes in medium containing 20 mM taurine prevents Ang II-mediated hypertrophy and cell death (Takahashi et al. 1997). Conversely, Ang II-mediated apoptosis is potentiated in taurine deficient cells (Schaffer et al. 2003). Since Ang II initiates signaling pathways that lead to enhanced oxidative stress, elevated $[Ca^{2+}]_i$ and cell death, taurine might act at an early step in Ang II signaling to protect the cardiomyocyte. The present study examines the effect of taurine treatment on the distribution of key protein kinase C (PKC) isoforms, enzymes involved in the pro-apoptotic and hypertrophic activities of angiotensin II.

1.2 Methods

1.2.1 Cell Culture

The care and treatment of animals were in accordance with the guidelines of the National Institute of Health and the procedures approved by the Institutional Care and Use Committee of the University of South Alabama. Rat neonatal cardiomyocytes were prepared as described previously (Pastukh et al. 2005). The cells were suspended in minimal essential medium containing 10% newborn calf serum and 0.1 mM 5-bromo-2-deoxyuridine and plated onto polystyrene treated Petri dishes at a density of 10×10^6 cells/dish (10 cm diameter). They were then placed in serum free

medium containing either 0 (control) or 20 mM taurine, for a period of 3 days. The cells were then exposed to medium supplemented with either no addition (control) or 100 nM Ang II. The concentration of Ang II was chosen that induced apoptosis (Kajstura et al. 1997). At the appropriate time, the cells were used for Western blot analysis.

1.2.2 Western Blot Analyses

After the cells were detached from the dish with trypsin, they were washed in phosphate-buffered saline and then centrifuged for 5 min at 500 g at room temperature. Membrane and cytosolic fractions were prepared according to previous reports. Each sample was homogenized in ice-cold lysis buffer (pH 7.4) consisting of the following: 25 mM Tris-HCl; 2 mM EDTA; 5 mM EGTA; 100 mM NaF; protease inhibitors [a 1/100 dilution of protease inhibitor cocktail set III (Calbiochem) and 1% solutions of leupeptin and PMSF], 1 mM orthovanadate and 5 mM dithiothreitol. The samples were then centrifuged at 100,000 g for 60 min. The pellet represents the membrane-particulate fraction and particulate-free supernatant fraction was defined as the cytosolic fraction. The particulate fraction is resuspended in homogenizing buffer containing 0.5% Triton-X100 and centrifuged at 100,000 g for 60 min. The resulting detergent-treated supernatant was used in the Western blot analyses. The protein concentration of each sample was determined by the Bradford or Lowry assay. Cytosolic and membrane proteins were analyzed for PKC isoform content by electrophoresis using 8% SDS-polyacrylamide gels. Following electrophoresis the proteins were transferred to nitrocellulose membranes, where they were blocked. After incubation with the appropriate antibody, the membranes were washed and then incubated with a secondary antibody, goat anti-rabbit IgG. The Western blots were detected by the enhanced chemiluminescence reaction. All data were analyzed by densitometry using ChemiImage 4400 (Alpha Innotech).

1.2.3 Statistical Analysis

The statistical significance of the data was determined using either the Student's test for comparison with groups or ANOVA combined with Tukey's post hoc test for comparison between groups. Values of $P<0.05$ were considered statistically significant.

1.3 Results

1.3.1 Effect of Taurine Exposure on the Status of PKC Isoforms

One of the steps involved in the activation of specific PKC isoforms is their translocation within the cell (Churchill et al. 2008). As a measure of the activation state

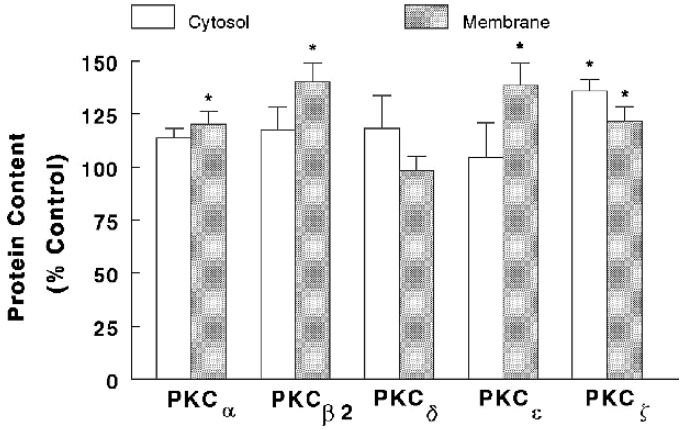

Fig. 1.1 Influence of taurine on the distribution of protein kinase C isoforms. Isolated cardiomyocytes were incubated for 3 days with medium containing either 0 or 20 mM taurine. After harvesting the cells, particulate and cytosolic fractions were obtained from the cell extract. Western blot analyses were performed using antibodies specific for the appropriate PKC isoform. The data are expressed as percent of the control, which is represented as 100%. All data denote means ± SEM of 5–6 different cellular preparations. *: $p<0.01$ vs. control

of the enzyme, the distribution of PKC in particulate and cytosolic fractions was determined. Figure 1.1 shows the protein levels of six PKC isoforms (α, β2, γ, δ, ε, ζ) in the cytosolic and membrane fractions of cardiomyocytes cultured in medium containing either 0 or 20 mM taurine for 3 days. Chronic taurine exposure led to a shift in the membrane/cytosol ratio for three PKC isoforms (α, β2 and ε) in favor of the membrane fraction. While the levels of PKCα and PKCζ in the membrane fraction were increased by taurine exposure, there was no shift in the membrane/cytosolic ratio associated with the activation event. Rather, taurine treatment led to a net increase in the content of the two isoforms in both the cytosolic and membrane fractions. Taurine exerted no influence on either the level or distribution of PKCδ.

1.3.2 Effect of Taurine on the Translocation of PKC by Angiotensin II

We have previously demonstrated that taurine exposure prevents Ang II-mediated hypertrophy of neonatal cardiomyocytes in culture (Azuma et al. 2000, Takahashi et al. 1997). One of the early events in the signaling pathway initiated by Ang II is the activation of PKC leading to the stimulation of NADPH oxidase (Ricci et al. 2008). Pastukh et al. (2005) previously showed that acute exposure of isolated cardiomyocytes to medium containing 1 nM Ang II led to an increase in the membrane/cytosol content ratio of PKCε and PKCδ without affecting that of PKCα,

PKCβ2 and PKCζ. Figure 1.2 shows that Ang II also increases the levels of PKCε and PKCδ in the membranes of cells exposed for 3 days to medium containing 20 mM taurine. Indeed, the effects of taurine and Ang II appear to be additive. While separate addition of Ang II and taurine to the medium elevated membrane levels of PKCε by 37% and 36%, respectively, addition of both Ang II and taurine to the medium increased membrane levels of PKCε by 76%. As expected, the levels of PKCδ were elevated to a similar degree in the presence of Ang II irrespective of taurine content; taurine alone had no effect on the distribution of PKCδ. Moreover, while taurine increased membrane levels of PKCα by 20%, Ang II reduced it by 17%. The combination of both effectors to the medium caused an inconsequential 4% increase in PKCα content.

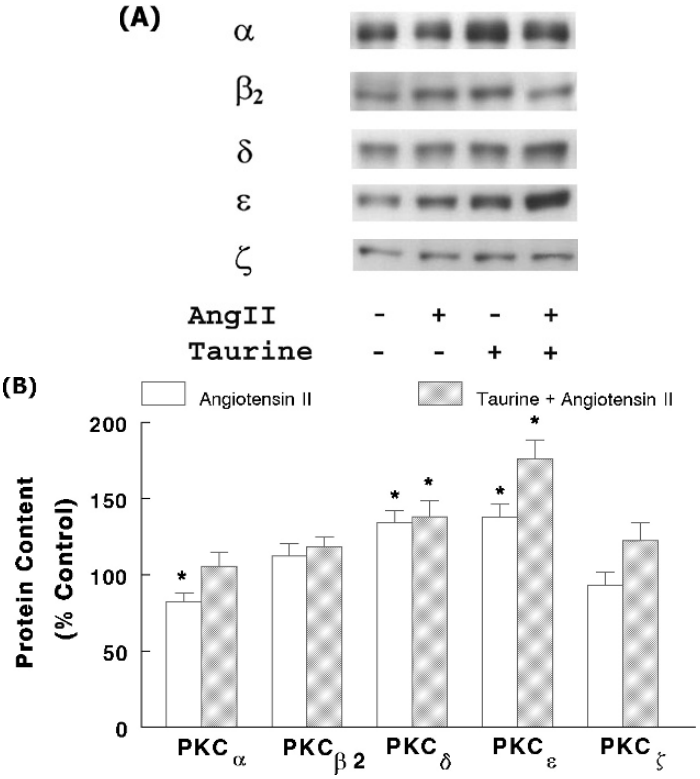

Fig. 1.2 Effect of taurine on AngII-mediated PKC translocation. Isolated cardiomyocytes incubated for 3 days with medium containing either 0 or 20 mM taurine were exposed for 5 min to 100 nM angiotensin II. The cellular particulate fraction was subjected to Western blot analyses using antibodies specific for the appropriate PKC isoform. (**A**) A representative gel showing membrane content of individual PKC isoforms of cells exposed to Ang II, taurine or the combination of Ang II and taurine. (**B**) The data are expressed as % control, with control = 100%. Data represent means ± SEM of 5–6 different cellular preparations. *: $p<0.01$ vs. control

1.4 Discussion

Upon activation individual PKC isoforms are translocated to a distinct subcellular site (Churchill et al. 2008). Mochly-Rosen and coworkers (2008) maintain that this site of translocation is defined by the location of the selective anchor protein, referred to as a RACK, to which the PKC isoform binds. The translocation of PKCδ and PKCε from the cytosol to the membrane fraction is a key step in the signaling pathway initiated by Ang II (Churchill et al. 2008). The involvement of the two PKC isoforms in Ang II signaling is a characteristic feature of pathways initiated by Gq proteins (Churchill et al. 2008).

Taurine also activates a number of PKC isoforms. In this study we showed that exposure of isolated cardiomyocytes to medium containing 20 mM taurine resulted in an increase in the membrane/cytosol ratio for PKCβ2 and PKCε. While the membrane levels of PKCα were significantly elevated in the taurine treated cells, cytosolic content of PKCα also tended to be elevated, leaving the membrane/cytosolic ratio unaffected. Taurine increased the cytosolic and membrane content of PKCζ, but the upregulation of PKCδ was not accompanied by a preferential association of PKCζ with the particulate fraction, ruling out a translocation event in the actions of taurine. In contrast to the other PKC isoforms, taurine had no effect on the levels of PKCδ associated with the membrane and cytosolic fractions.

The factor responsible for taurine-mediated modulation of PKC isoform status is a matter of conjecture. One means by which taurine could influence protein kinase C activity is through its osmoregulatory activity. Addition of 20 mM taurine to the extracellular medium has been shown to trigger cell shrinkage (unpublished data). In NIH/3T3 cells, hyperosmotic stress leads to an increase in diacylglycerol levels, resulting in the translocation of PKCα, PKCδ and PKCε from the cytosol to the membrane (Zhuang et al. 2000). Although membranes levels of PKCα, PKCδ and PKCε increase following exposure of the cardiomyocytes to medium containing 20 mM taurine (Fig. 1.1), the only protein kinase C isoform experiencing an elevation in the membrane/cytosol ratio, which is indicative of a translocation event, is PKCε. Together, these data suggest that either hyperosmotic stress is not responsible for the activation of protein kinase C in the taurine treated cells or that the osmotic stress experienced by the taurine treated cells is too mild to trigger the translocation of a significant number of protein kinase C isoforms. An alternative possibility is that taurine modulates the structure of the membrane, resulting in a change in the RACK-mediated translocation step. Hamaguchi et al. (1991) found that taurine interferes with phospholipid methyltransferase activity, thereby affecting the phosphatidylethanolamine/phosphatidylcholine ratio. This alteration is likely to affect the activity of membrane bound proteins, such as those associated with PKC translocation.

An important conclusion of the present study is that taurine-mediated modulation of PKC does not appear to play a role in the reversal of the myocardial actions of Ang II. Our initial hypothesis was that taurine should prevent Ang II-mediated activation of PKCδ and PKCε. However, Fig. 1.1 shows that taurine has no effect on PKCδ while increasing the translocation and activation of PKCε. Hence, combined

treatment with Ang II and taurine leads to an elevation in both PKCδ and PKCε, in line with our conclusion that the two effects are additive. The other means by which taurine might reverse Ang II's actions is through a step downstream from the PKC activation step. In hyperosmotically stressed rat hepatocytes, a PKCζ activation step lies upstream from the activation of NADPH oxidase (Reinehr et al. 2006). Since Ang II signaling also leads to PKC-mediated activation of NADPH oxidase (Ricci et al. 2008), it is possible that taurine might influence Ang II signaling at the NADPH oxidase step. This possibility is worthy of consideration, as taurine serves as an indirect antioxidant. Moreover, NADPH oxidase-mediated generation of superoxide is a key step in Ang II-mediated apoptosis and cell hypertrophy (Ricci et al. 2008), events that are inhibited by taurine (Takahashi et al. 1997).

Although the present data rules out a role for PKC in the reversal of Ang II's actions by taurine, it raises the possibility that PKC might contribute to one of the other actions of taurine. Especially intriguing is the effect of taurine on PKCε. Because taurine promotes the translocation of PKCε from the cytosol to the membrane, it appears to initiate PKC signaling (Ping et al. 2001). PKCε has been the most widely studied PKC isoform in the heart. One of its recognized actions is as a mediator of ischemic preconditioning, a powerful strategy for protecting various tissues against ischemic injury. Ping et al. (1997) reported that ischemic preconditioning triggers a translocation of PKCε into the particulate fraction. This initiates a complex signaling response in which PKCε becomes associated with a large number of proteins (Ping et al. 2001). Selective inhibition of the interaction of PKCε with RACK abolishes the cardioprotection arising from ischemic or hypoxic preconditioning (Gray et al. 1997; Liu et al. 1999). Similarly, genetic PKCε deficiency has no effect on infarct size of the preconditioned heart (Saurin et al. 2002). Like ischemic preconditioning, taurine therapy has also been shown to protect the heart against an ischemic insult (Takahashi et al. 2003).

PKCε has also been implicated in hypertrophic growth (Ito et al. 2008). Not only is PKCε activated in response to hypertrophic stimuli but overexpression and activation of PKCε leads to myocardial hypertrophy (Churchill et al. 2008; Dorn and Force 2005). Although there are questions on the type of hypertrophy (maladaptive as in heart failure vs. adaptive as in development) mediated by PKCε, there is little question that it is a major component in the development of cardiac hypertrophy. Because taurine deficiency in the taurine transport knockout heart is associated with a reduction in cardiomyocyte size (Ito et al. 2008), taurine's ability to promote cell growth might be linked to its activation of PKCε.

1.5 Conclusion

The present data suggest that taurine promotes the translocation of PKCβ2 and PKCε into the particulate fraction. It also elevates the levels of PKCα and PKCζ. While these effects are not responsible for the interaction between angiotensin II and taurine, they may account for some of the cardioprotective effects of taurine. It may also explain the effect of taurine deficiency on cardiomyocyte size.

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Chapter 2
Taurine as the Nutritional Factor for the Longevity of the Japanese Revealed by a World-Wide Epidemiological Survey

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Abstract The initial observation that taurine (T) prevented stroke in stroke-prone spontaneously hypertensive rats (SHRSP) led us to study the effects of T on cardiovascular diseases (CVD), as well as the epidemiological association of T and mortality rates, by using the data from WHO-coordinated Cardiovascular Disease and Alimentary Comparison Study, which covered 61 populations in 25 countries. In this study, 24 hour urine (24-U) samples were examined along with biomarkers of CVD risk. The mortality rate from ischemic heart disease (IHD), which was lowest among the Japanese compared to the populations of other developed countries, was positively related to total serum cholesterol (TC) and inversely related to 24-U taurine excretion (24-UT), as well as the n-3 fatty acid to total phospholipids ratio of the plasma membrane, both biomarkers of seafood intake. Analysis of 5 diet-related factors revealed that TC and BMI were positively associated with IHD mortality in both genders while Mg and T were negatively associated with IHD mortality. TC and sodium (Na) were negatively and positively associated with stroke mortality, respectively. 24-UT was negatively associated with stroke mortality. These five diet-related factors explained 61 and 49% of IHD and stroke variances in male, 63 and 36% of IHD and stroke variances in female, respectively.

Abbreviations *T*, taurine; *Na*, sodium; *Mg*, magnesium; *CVD*, cardiovascular diseases; *IHD*, ischemic heart diseases

2.1 Introduction

Taurine (T) is abundant in the seafood consumed in large quantities by the Japanese, who are presently enjoy the longest life expectancy in the world, with a life expectancy of 86 among females and 79 among males, the latter which rates

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second in the world. Since various experimental studies, as well as epidemiological evidence from our world-wide cooperative study on nutrition and cardiovascular disease (CVD), suggest the importance of T in reducing the risk of CVD, it is possible that T is a food factor that contributes to Japanese longevity.

2.2 Basic Studies of T Effect on CVD Risks

2.2.1 T Effect on Hypertensive Models

Basic research on hypertension and stroke has made remarkable progress since the establishment of rat models of hypertension and stroke, the spontaneously hypertensive rat (SHR) (Okamoto and Aoki 1963) and the stroke-prone SHR (SMRSP) (Okamoto et al. 1974; Yamori 1984). These animal models develop severe hypertension and die from hemorrhagic and ischemic stroke, making these useful in studying the pathogenesis, prevention and treatment of hypertension and stroke (Yamori 1981; Yamori et al. 1987).

The effect of fish protein-rich diet on stroke prevention was first demonstrated by Yamori et al. (Yamori 1981; Yamori et al. 1987). When SHRSP were fed a normal or low protein diet maintained on drinking water containing 1% salt, they quickly developed severe hypertension and all died of stroke within a short period. Without excess salt, 80% of them died of stroke. In contrast, when fed a high protein fish diet with excess salt, the incidence of stroke was markedly reduced. And in the group fed a high protein fish diet without excess salt intake, the development of severe hypertension was attenuated and no stroke was observed. Thus, it can be concluded that a high protein fish diet attenuates the development of severe hypertension and counteracts the adverse effect of salt.

Of the amino acids in fish and protein that could attenuate the development of severe hypertension and counteract the adverse effect of salt in SHR and SHRSP were the sulfur amino acids, T and methionine (Yamori 1981; Yamori et al. 1987; Nara et al. 1978). T supplementation also prevented the development of hypertension in DOCA-salt hypertensive rats (Sato et al. 1991) and suppressed the elevation in plasma epinephrine and norepinephrine levels, which is likely one of the possible mechanisms underlying the anti-hypertensive actions of T.

2.2.2 Physiological Effect and Distribution of T

Although a simple sulfur amino acid, T has been experimentally found to exert various effects (Huxtable 1992, 2000), such as an antihypertensive effect through central suppression of sympathetic tone, a hypocholesterolemic effect through activation of hepatic 7α-hydroxylase activity to accelerate cholesterol excretion into bile acids and an antiatherogenic effect possibly through the scavenging of hypochlorous acid and formation of T chloramines (Jerlich et al. 2000).

There are species differences in T synthesis with synthesis being particularly poor in humans and cats (Huxtable 1992). In newborn humans, T is considered to be an essential amino acid, since the potential to synthesize T is limited (Huxtable 2000).

In men, T in mainly obtained from fish and seafood, which contain large amounts of T compared to meat (Tsuji and Yano 1984) and are eaten customarily by the Japanese.

2.3 Epidemiological Survey of T Effect on CVD Risks and Mortality

2.3.1 Food Culture and Lifespan in Various Populations

In order to prove whether or not dietary components, such as T, are important in preventing hypertension and atherosclerosis in humans, as well as in animal models, Yamori introduced the idea of performing a world-wide epidemiological study to WHO in 1982. The CARDIAC Study is the acronym of *Car*diovascular *Di*seases and *A*limentary *C*omparison Study and the study was designed to investigate the relation of biological markers of diet with hypertension in"Core Study"and with CVD mortalities in "Complete Study" (WHO and WHO Collaborating Centers 1986; Yamori, 1981, 1989, 2006; Yamori et al. 1990, 2006).

This epidemiological survey has been carried out over the past 20 years in 61 populations in 25 countries (Fig. 2.1). About 100 males and 100 females in ages ranging from 48 to 56 were randomly selected, with the number of participants being over 14,000 in all. Some study sites were revisited at 10 year intervals for a follow-up health survey (MONALISA study; *Mon*eo *Ali*mentationis *Sa*nae = Reminding healthy food), and we noted the populations marked with large clear dots in Fig. 2.1

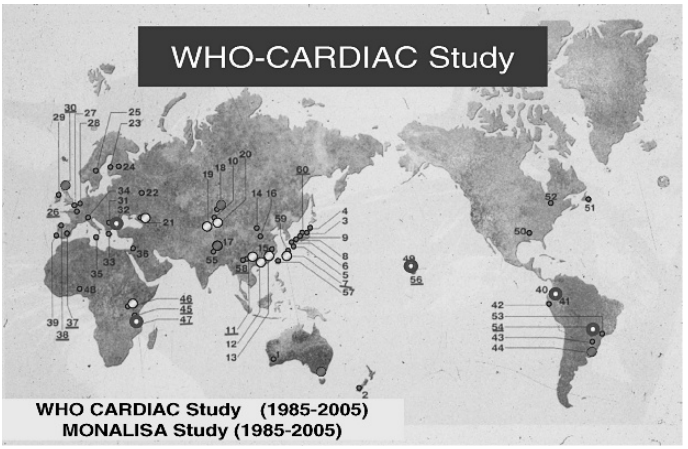

Fig. 2.1 Distribution of study sites in the world, 61 in total, for the WHO-coordinated Cardiovascular Diseases and Alimentary Comparison (WHO-CARDIAC) Study