

Protective effect of taurine against free radicals damage in the rat myocardium

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Abstract

Free radicals are highly cytotoxic to the heart and are involved in ischemia/reperfusion injury. In this study, we tested the ability of taurine to neutralize the deleterious effects of free radicals generated *ex vivo* and *in vitro*. Taurine was added at a concentration of 0.1 mM to the drinking water of experimental rats during 6 months. The animal hearts were then isolated and submitted to regional ischemia and reperfusion; ventricular fibrillation was significantly reduced as compared to a control group of non-treated animals. Moreover, at a concentration of 1 mM, taurine provided significant cardio-protection against the deleterious effect of free radicals generated by the electrolysis of Krebs–Henseleit buffer. When isolated hearts were perfused with electrolysed buffer, extensive fiber necrosis occurred, as observed by staining with nitro blue tetrazolium, a soluble dye which yields a dark blue formazan stain in the presence of reducing agents. This stain was barely detectable when taurine was added to the perfusing electrolysed buffer. To further understand the protecting mechanism of taurine, we used xanthine–xanthine-oxidase as a superoxide ($\cdot\text{O}_2^-$) generating system and monitored the $\cdot\text{O}_2^-$ through yield O_2^- -dependent cytochrome *c* reduction. We demonstrated that taurine did not affect this system, which indicated that it did not scavenge $\cdot\text{O}_2^-$ directly. On the other hand, taurine inhibited the auto-oxidation of adrenaline to adrenochrome at pH 7.8 where this auto-oxidation is $\cdot\text{O}_2^-$ -independent and superoxide dismutase insensitive. We thus conclude that taurine acts as a potent, but non-specific, scavenger of free radicals that cause heart damage and protects against reperfusion-induced ventricular fibrillation.

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Introduction

Oxygen may be toxic when it leads to the generation of free radicals (FR) such as superoxide ($\cdot\text{O}_2^-$), hydroxyl ($\cdot\text{OH}$), singlet oxygen ($^1\text{O}_2$), and other secondary reactive oxygen species, giving rise to a chain of

molecular alterations consisting, in the early stages, of lipid peroxidation products (Cadenas, 1989). The potential sources of toxic oxygen species include the myocardial electron transport system, purine catabolism by xanthine oxidase, catecholamine oxidation, prostaglandins biosynthesis and infiltration of phagocytes (McCord, 1993). Consequent alterations of the cell structure and function, mainly in cell membranes, often yield to cell death (Jacobson, 1996). Free radicals have been shown to play a significant role in myocardial injury since agents such as superoxide dismutase (SOD) and

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catalase that interact with reactive metabolites of oxygen, or agents that prevent the generation of free radical species decrease the extent of tissue destruction (Halliwell, 1994). The direct evidence of FR production comes from studies using electron paramagnetic resonance (Lecours et al., 1998).

For many years, research has focused on the identification of a protective agent against tissue damages caused by myocardial ischemia. Taurine, the 2-amino-ethane sulphonic acid, has been reported to be a potential candidate. It is present at high concentrations in the peripheral nervous system as well as in several tissues, including the cardiac tissue where it ranges between 11 and 38 $\mu\text{M/g}$ and represents 50% of the total cardiac amino-acid pool (Huxtable, 1992). It has been shown to maintain constant volume and osmolarity in cells and to regulate membrane excitability (Kramer et al., 1981). Besides, taurine is reported to have protective effects against pathological conditions like congestive heart failure and cardiomyopathy (Pion et al., 1992; Welty et al., 1982). Moreover, at relatively high doses, taurine prevents premature ventricular contraction induced by epinephrine and digoxin (Read and Welty, 1963).

We have previously reported that taurine may have a beneficial effect by promoting the synthesis of prostacyclin, as vasodilator and anti-aggregation agent, as evidenced by an increase in prostacyclin/thromboxane ratio in the myocardium (Chanh et al., 1987). We have also published two studies demonstrating that taurine may decrease noradrenaline release, via its ability to prevent a rise of intracellular calcium ion, and may prevent reperfusion arrhythmias when added to the perfusing buffer of isolated rat hearts (Chahine and Feng, 1998; Chahine et al., 1994a).

In the present study, we have added taurine to the drinking water of rats before inducing experimental ischemia/reperfusion on their isolated hearts to test the ability of taurine to protect against reperfusion arrhythmias. We have also used FR generating systems in order to investigate whether this protective effect is correlated with taurine ability to scavenge free radicals.

Materials and methods

Addition of taurine to the drinking water

Male Wistar rats, one month old, were used in this experiment. Taurine was added at a concentration of 0.1 mM to the drinking water of 15 animals while 15 others (control group) were given water alone. At the age of 6 months, all rats were anaesthetized with diethyl ether and 200 IU of heparin were injected intravenously. The hearts were promptly excised and perfused at a

constant pressure of 100 cm H_2O with a modified Krebs–Henseleit (KH) solution, continuously gassed with O_2/CO_2 (95:5), at a temperature of 37 °C, according to the Langendorff technique (Chahine and Feng, 1998; Chahine et al., 1994b). Electrophysiological parameters were recorded via two electrodes placed one in the apex of the heart and the other around the aorta. Acute myocardial ischemia was induced by ligation of the left anterior descending coronary artery with a silk ligature, 12–14 mm from its origin. A decrease in coronary flow to 50% indicated successful ligation. After 15 min of ischemia, reperfusion was induced by cutting the ligation. The incidence of arrhythmia was analysed according to the Lambet study.

Addition of taurine in the perfusion buffer of isolated hearts

Hearts of male Wistar rats were isolated according to the technique described above. They were perfused with KH buffer in the presence or absence of 1 mM taurine. To generate FR in the KH perfusing buffer, two platinum wire electrodes were placed above the heart into the inflow cannula, at 12 cm and 15 cm, respectively, from the left atrium. A constant 10 mA direct current generated by an electrical stimulator was applied for 1 min. A glass bubble trap placed above the aorta prevented gas bubble formation. In addition to the electrical parameters, left ventricular pressure was monitored by a latex balloon, inserted into the left ventricle via the left atrium and connected by a polyethylene cannula to a pressure transducer (Chahine et al., 1991). FR were detected by the addition of NBT. The oxidative stress was evaluated by the extent of formazan deposition in the myocytes in histological sections.

Five experimental groups, of six rats each, were designed. Group a used as a control and hearts were perfused with unelectrolysed buffer, in the absence of NBT. Group b received electrolysed buffer without NBT. Group c received electrolysed buffer in the presence of NBT. Group d was similar to group c, except that taurine was added to the perfusion prior to electrolysis. Group e (sham group) were perfused with unelectrolysed buffer in the presence of NBT.

To identify free radical generation, nitro blue tetrazolium (NBT) was dissolved in the buffer solution at a concentration of 3.3 mg/ml at a temperature of 37 °C, filtered through a millex-GS 0.22 μm Millipore mounted on a disposable syringe and perfused in the inflow cannula above the heart together with the perfusing buffer during electrolysis (Mateescu et al., 1995).

For histological studies, heart specimens were fixed with 20% formalin, embedded in paraffin,

counterstained with eosin or Kenechtrot, and observed using light microscopy.

Addition of taurine to free radicals generating systems in vitro

Two systems were used based on colorimetric methods.

- Xanthine–xanthine oxidase system (X–XO):** Super-oxide generation by XO action on X was determined by monitoring cytochrome *c* reduction at 550 nm in the absence or presence of different concentration of taurine or SOD. A typical assay included 50 μ M X, 0.005 U/ml XO, and 0.05 mM cytochrome *c* in potassium phosphate buffer (50 mM, pH 7.8) containing 0.1 mM EDTA (Atanasiu et al., 1995).
- Adrenaline auto-oxidation:** Auto-oxidation of adrenaline to adrenochrome was quantified by using 10 μ l of stock adrenaline 0.01 M to initiate the reaction in buffer (0.1 M EDTA/50 mM potassium carbonate buffer, pH 7.8) (Sun and Zigman, 1978) in the absence or presence of different concentration of taurine or SOD. The rate of adrenaline oxidation was monitored at 320 nm up to 10 min.

Reagents

All reagents used were purchased from Sigma Chemical Co, St Louis, MO, USA. The enzymes were dissolved in the KH solution.

Statistics

Results are expressed as either means \pm SEM or percentage of incidence. Concerning the biochemical assays, significance of the results was assessed by the mean of Student's *t*-test for unpaired data. Binomially distributed data (arrhythmia incidence) were compared, using the χ^2 test and the Fisher exact test. $p < 0.05$ was considered to be the limit of statistical significance.

Results

Preventive effect of dietary taurine against reperfusion arrhythmias

Isolated hearts of rats treated with taurine in the drinking water and of untreated rats were submitted to 15 min ischemia followed by 10 min reperfusion. Several parameters of arrhythmia were monitored, including total and irreversible ventricular fibrillation, ventricular tachycardia, premature ventricular beats and time of

sinus rhythm. Results are shown in Table 1. According to all parameters, a significant reduction in arrhythmia incidence during reperfusion is observed in the treated group.

Protective effect of taurine against FR generation in heart perfusing buffer

A marked decrease in the left ventricular pressure was observed during and after perfusion with electrolysed buffer in the untreated hearts but not in hearts perfused with taurine (data not shown). Fig. 1a shows a micrograph of a control heart (group a). In Fig. 1b, representing the heart of a rat perfused with electrolysed buffer (group b), several areas of acute necrosis, expressed by hyalinisation and loss of striation, were observed, which present a pattern of wavy disrupted myofibers and an increase in interstitial spaces. The areas of necrosis were dispersed in the subendocardium as well as in more peripheral areas. In Fig. 1c, the same pattern of necrosis can be seen. Additionally, formazan deposition was not uniform in all cells, myocardial sections present an extensive formazan deposition, mostly in the areas of fiber necrosis (dark areas in Fig. 1c). Interestingly, pre-treatment with taurine (Fig. 1d) decreased to a large extent formazan deposition in hearts perfused with electrolysed buffer. In hearts perfused with unelectrolysed buffer, there was a good diffusion of NBT, but there were only very faint areas of deposition of formazan (group e, not illustrated).

Free radical scavenging effect of taurine in vitro

The ability of taurine to scavenge $\cdot\text{O}_2^-$ radical was examined in vitro in comparison with SOD. Using the X–XO as a $\cdot\text{O}_2^-$ generating system, taurine was seen not to scavenge $\cdot\text{O}_2^-$ directly, because it did not inhibit $\cdot\text{O}_2^-$ -dependent cytochrome *c* reduction (Fig. 2). However,

Table 1. Effect of taurine (0.1 mM in drinking water) on reperfusion-induced arrhythmias in the isolated heart

	Control group	Treated group
Ventricular fibrillation (%)	87	13 ^a
Irreversible ventricular fibrillation (%)	87	7 ^a
Ventricular tachycardia (%)	100	42 ^a
Premature ventricular beats (%)	44 \pm 9	28 \pm 7 ^a
Time of sinus rhythm (s)	60 \pm 15	455 \pm 82 ^a

^a $p < 0.005$ versus control, $n = 15$.

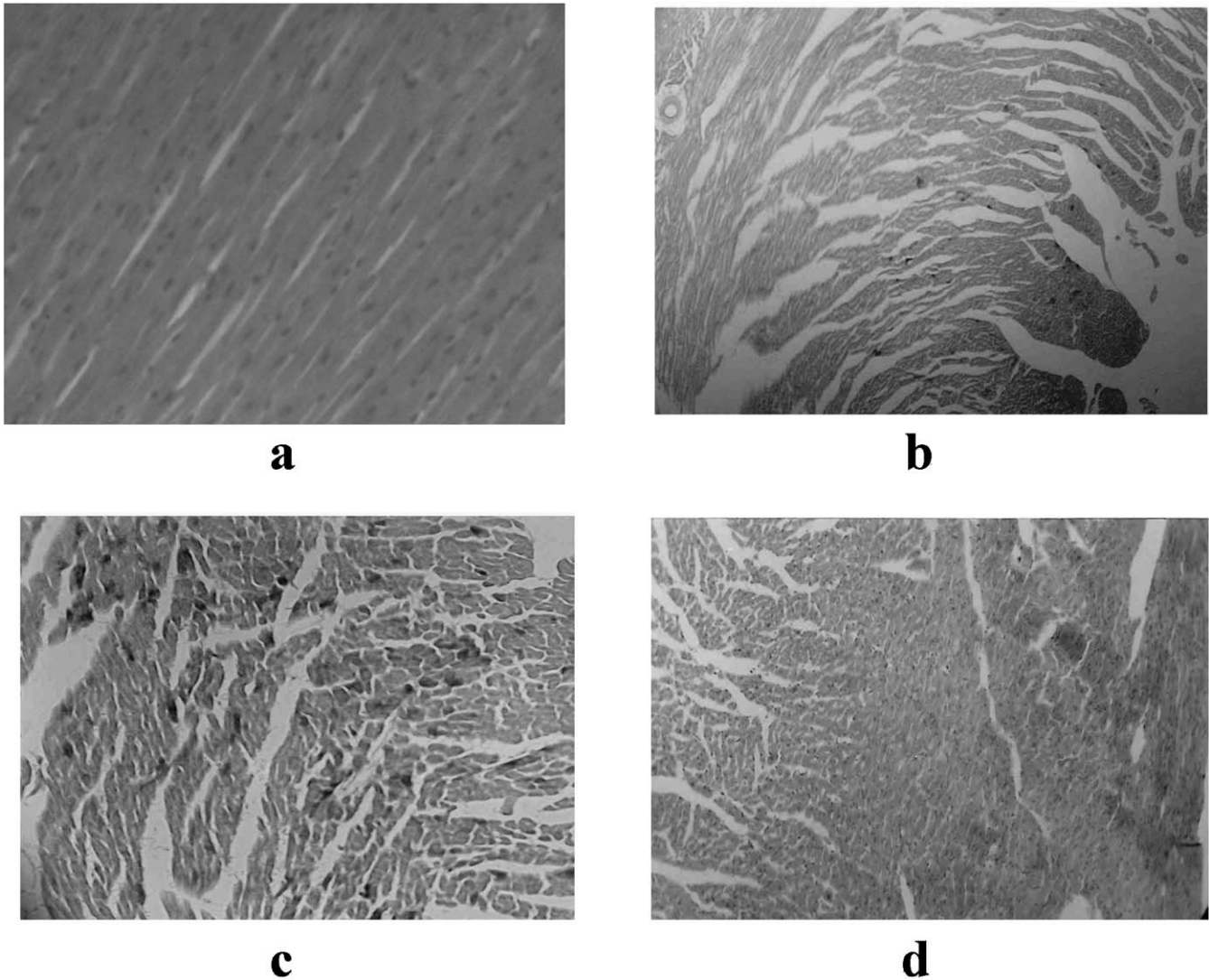


Fig. 1. Light micrographs of specimens from the myocardium of the isolated rat heart ($\times 300$): (a) Control hearts; (b) Hearts perfused with electrolysed buffer for 1 min. Histological alterations (loss of striation and increased in interstitial spaces) are apparent. (c) Hearts perfused with electrolysed buffer and nitroblue tetrazolium (NBT) showing formazan deposition (dark areas) in the myocytes. (d) Hearts perfused with taurine, with electrolysed buffer and NBT.

taurine inhibited the auto-oxidation of adrenaline to adrenochrome at pH 7.8 where this auto-oxidation is $\cdot\text{O}_2^-$ -independent and SOD insensitive (Fig. 3a), but not at pH 10.2 (data not shown).

Discussion

In this study, we clearly demonstrate that taurine, when added as a dietary supplement to the drinking water of experimental rats for several months, provides strong protection against subsequent post-ischemic reperfusion arrhythmias.

In order to investigate whether this protection is correlated with its capacity to neutralize free radicals

causing reperfusion arrhythmias, we first studied the direct toxic effects of FR on the heart, in the absence of ischemia. FR and their metabolites were generated by electrolysis of the KH buffer perfusing isolated rat hearts. Although FR scavengers have been effective against electrolysis (Chahine et al., 1991; Mateescu et al., 1995), we used NBT reduction as a marker in the present study to show that this blood-free and enzyme-free procedure produces a milieu containing several reactive oxygen species of free radicals and derivatives, leading to alterations in myocardial function. Therefore, electrolysis, a well-known exogenous system for FR generation, may be considered as a primary source of ischemia-inducing FR production in the rat myocardium and may transfer electrons to NBT via a complex reaction including univalent reduction of the molecular

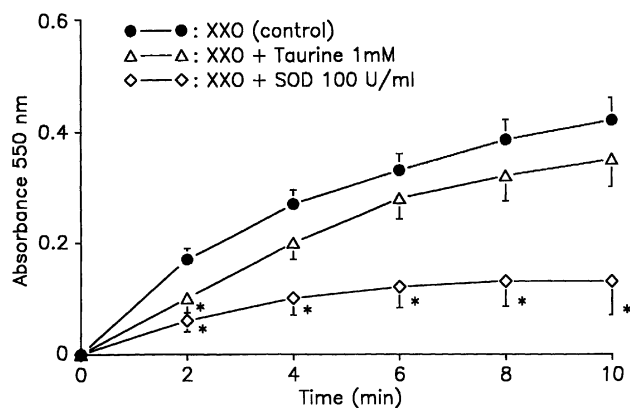


Fig. 2. Inability of taurine to scavenge superoxide generated from xanthine oxidase action on xanthine as compared to superoxide dismutase (SOD). * $p < 0.05$ versus control.

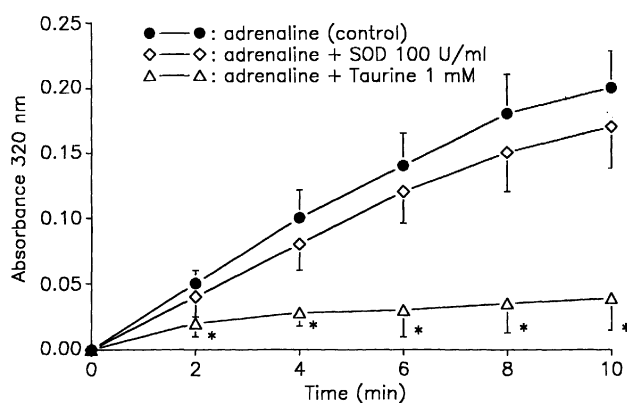


Fig. 3. Inhibitory effect of taurine on adrenaline oxidation as compared to SOD. * $p < 0.05$ versus control.

oxygen, giving rise to a tetrazonyl radical and then to formazan (Amano et al., 1975).

Severe injury to the myocyte membranes was induced by electrolysis, as could be detected in the micrographs. The membrane damage and consequent permeation may explain why formazan was deposited exclusively in disrupted cells. Superoxide anion generated by electrolysis is a potent nitric oxide inhibitor and/or may function as an endothelium-derived contracting factor that may also affect myocardial cells (Katusic and Vanhoutte, 1989). However, during electrolysis, superoxide and hydroxyl radicals (very short half-lives species) probably reacted together before reaching the heart to generate more stable species (H_2O_2 , HOCl , OCl^-) responsible for cardiotoxicity (Chahine et al., 1991). In addition, H_2O_2 may generate $\cdot\text{OH}$ (via the Fenton reaction and in the presence of iron) which is extremely reactive and subsequently very toxic. This hypothesis is supported by the potent inhibitor effect of mannitol and by the protective effect of ceruloplasmin against damage caused by electrolysis via the Fenton

reaction inhibition previously reported (Chahine et al., 1991).

Nitroblue tetrazolium is a test widely used to measure superoxide anion release by polymorphs and macrophages during the oxidative burst. Moreover, in the liver and jejunum, reduction of NBT into formazan, attributed to XO activity, has been described by Sackler et al. (Sackler, 1966). It has been also reported that NBT can be a useful tool for estimating the extent of oxidative stress in hepatocytes (Mochida et al., 1992).

In vitro, NBT reduction to formazan, which is caused by the collision of radicals generated photolytically with NBT, was monitored and quantified by kinetic spectrophotometry by (Green and Fellman, 1994). In this test, the difference between reduction of NBT to formazan in the presence SOD or its absence was taken as an indicator of superoxide production.

Although NBT quantification is very difficult in vivo, Powell et al. (1995) have described a test to quantify NBT reduction by spectrophotometry after inducing intestine ischemia for different lengths of time and incubating the tissue with NBT. In our study, we have generated FR exogenously and did not incubate the heart tissue with NBT. It is therefore difficult to quantify FR formation for at least 3 reasons: (i) part of the formazan may be washed out of the myocardium, (ii) besides the exogenous source of free radicals, NBT could also react with different electrons produced intracellularly depending on the mitochondrial oxidative chain status, which could account for background NBT reduction, and (iii) much less overall reduction of NBT may be observed at high O_2 pressure due to reoxidation by O_2 of both the tetrazonyl radical and the formazan, which could lead to false interpretation of the results.

The experiments conducted in vitro may appear somehow paradoxical: How could taurine, which is not an $\cdot\text{O}_2^-$ radical scavenger, prevent NBT reduction, knowing that this reduction occurs when NBT reacts with this radical? Two explanations are possible: (i) since the half-life of $\cdot\text{O}_2^-$ radical is very short, other radicals generated by electrolysis could participate in NBT reduction, and these radicals may be scavenged by taurine (ii) taurine was perfused before electrolysis and stabilized the myocardial cell membranes, thus playing an indirect protective role against FR. In support of this hypothesis, we have previously reported that electrolysis injured the sympathetic nerve endings in isolated hearts, thereby releasing noradrenaline. We also reported that taurine prevented this release by blocking calcium influx in the pre-synaptic neuron leading to a calcium overload (Chahine et al., 1994a, b). In the post-synaptic cell, calcium overload (Kaminishi et al., 1989) and the transformation of noradrenaline into adrenochrome may cause cardiac arrhythmia. Since taurine blocks calcium influx, it could prevent adrenochrome formation.

Our results corroborate the study of Kaplan et al. (1993) in which taurine decreased malondialdehyde levels in anoxic guinea pig hearts. Taurine also protects liposome membranes against damage caused by FR (Koyama et al., 1992). More recently two studies reported an effect of taurine against oxidative stress in cerebellar granule cells (Boldyrev et al., 1999; Saransaari and Oja, 2000). Taurine has been also recognised as a useful antioxidant against radiation induced-FR release (Fang et al., 2002).

In conclusion, taurine is a potent protective substance against ischemia reperfusion injury. It acts both by scavenging a variety of free radical species and by reinforcing cardio-myocytes membranes and sympathetic nerve endings in isolated perfused rat hearts.

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