

Beneficial effect of taurine depletion on osmotic sodium and calcium loading during chemical hypoxia

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Schaffer, Stephen W., Viktoriya Solodushko, and David Kakhniashvili. Beneficial effect of taurine depletion on osmotic sodium and calcium loading during chemical hypoxia. *Am J Physiol Cell Physiol* 282: C1113–C1120, 2002; 10.1152/ajpcell.00485.2001.—Cellular sodium excess is cytotoxic because it increases both the intracellular osmotic load and intracellular calcium concentration ($[Ca^{2+}]_i$). Because sodium levels rise during hypoxia, it is thought to contribute to hypoxic injury. Thus the present study tested the hypothesis that taurine-linked reductions in $[Na^+]_i$ reduce hypoxia-induced cell injury. Taurine depletion was achieved by exposing isolated neonatal cardiomyocytes to medium containing the taurine analog β -Alanine. As predicted, the β -Alanine-treated cell exhibited less hypoxia-induced necrosis and apoptosis than the control, as evidenced by less swelling, shrinkage, TdT-mediated dUTP nick end labeling staining, and accumulation of trypan blue. After 1 h of chemical hypoxia, $[Na^+]_i$ was 3.5-fold greater in the control than the taurine-deficient cell. Although more taurine was lost from the control cell than from the β -Alanine-treated cell during hypoxia, the combined taurine and sodium osmotic load was lower in the β -Alanine-treated cell. Taurine deficiency also reduced the degree of hypoxia-induced calcium overload. Thus the observed resistance against hypoxia-induced necrosis and apoptosis is probably related to an improvement in sodium and calcium handling.

cardiomyocytes; metabolic inhibition; osmolality; osmotic stress; necrosis

ACCORDING TO THE OSMOTIC STRESS HYPOTHESIS advanced by several investigators (18, 26), a major cause of ischemia-induced sarcolemmal damage is cell stretching linked to osmotic swelling. Three factors are thought to contribute to osmotic-induced cell swelling during ischemia (4). First, the concentration of several metabolic end products increases, causing a rise in intracellular osmolality. Although these metabolic end products contribute to the cell's osmotic load during the initial phase of ischemia, with time these intermediates leak out of the cell and their contribution to the osmotic load diminishes. Second, cellular edema arises in part from ischemia-induced interstitial edema. Third, disruption of cellular transporters leads to a net gain in intracellular ions, of which sodium and Cl^- are the most important. A good correlation has been found

between the ischemia-induced increase in cellular water and the rise in intracellular sodium ($[Na^+]_i$) and Cl^- concentration ($[Cl^-]_i$) (18).

Opposing the unfavorable influence of the three hyperosmotic events is the release of organic osmolytes from the hypoxic myocyte (9, 11, 26). One of the primary organic osmolytes in the heart is the amino acid taurine (6). Extremely high intracellular levels of taurine are maintained by a specific β -amino acid transporter. In accordance with taurine's role as an osmolyte, the activity of the taurine transporter in the myocyte is increased following exposure of the cell to hyperosmotic medium (2). On the other hand, large amounts of taurine are rapidly lost from the myocyte during hyposmotic shock (17). Interestingly, there is also evidence that the movement of taurine in and out of the cell is closely tied to changes in the intracellular and extracellular concentration of sodium (29). Therefore, changes in the intracellular content of taurine can influence the osmotic balance of the cell through alterations in the intracellular levels of both taurine and sodium.

On the basis of putative importance of osmotic imbalances in the pathology of ischemia-reperfusion injury, one would predict that the movement of taurine and sodium should influence the outcome of an ischemic event. Indeed, we recently found that the drug-induced taurine-deficient heart is extremely resistant to ischemic injury (1). In that study, we proposed that taurine depletion might act by reducing the osmotic load of the ischemic heart. We also suggested that the beneficial effects of taurine depletion could be related in part to the regulation of $[Na^+]_i$, an effect thought to be linked to changes in tissue osmolality, intracellular pH (pH_i), and/or the cotransport of taurine and sodium. In this regard, it is significant that $[Na^+]_i$ contributes both to the osmotic load of the cell and to calcium influx via the sodium-calcium exchanger (10, 24). In addition, taurine exerts multiple effects on the heart, including alterations in both tissue osmolality and calcium movement (20). The direct and indirect effects of taurine on calcium movement could be of particular relevance because calcium overload is a major contributor to

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myocardial cell necrosis during ischemia (16, 24, 25). Thus taurine depletion could affect the outcome of a hypoxic or ischemic insult through a mechanism involving improved cellular calcium levels, a reduction in the osmotic load, or a combination of the two factors. To provide more information on these possibilities, we examined the effect of taurine deficiency on hypoxia-induced necrosis and apoptosis. We also examined the influence of taurine deficiency on hypoxia-induced changes in pH_i , $[\text{Na}^+]_i$, $[\text{Ca}^{2+}]_i$, and taurine content.

METHODS

Cell preparation and incubation conditions. Rat neonatal cardiomyocytes were isolated according to the method of McDermott and Morgan (12). The cells were suspended in minimal essential medium containing 10% newborn calf serum and 0.1 mM 5'-bromo-2'-deoxyuridine and plated on either glass coverslips or glass petri dishes precoated with 0.1% gelatin. They were then placed in standard serum-free medium containing 56 U/l insulin and 10 $\mu\text{g}/\text{ml}$ transferrin. To induce taurine deficiency, some of the cells were incubated in standard serum-free medium supplemented with 5 mM β -Alanine. All experiments were initiated after a 2-day incubation at 37°C under a 5% CO_2 -20% O_2 environment. To induce chemical hypoxia, the cells were placed in Krebs-Henseleit buffer containing the metabolic inhibitors 10 mM deoxyglucose and 3 mM Amytal. The duration of the metabolic insult was 60 min, unless otherwise stated. The control cells (normoxic and hypoxic) were defined as those cells that were incubated for 2 days with serum-free medium lacking β -Alanine.

Cellular taurine content. Taurine content of the β -Alanine-treated and control cells was determined both before and 60 min after onset of chemical hypoxia. After a 60-min incubation with medium lacking or containing metabolic inhibitors, the cells were scraped from the surface of the petri dishes and an aliquot was removed to determine protein content. The remaining cells were treated with 2% perchloric acid, and after neutralization, taurine content of the extracts was determined according to the procedure described by Mozaffari et al. (15).

Cell volume analysis. Before chemical hypoxia, some β -Alanine-treated and control cells were incubated with medium containing 5 μM calcein-acetoxymethyl ester (AM) for 30 min. The calcein-loaded cells were then washed three times with calcein-free medium and placed in standard serum-free medium. The volume of a series of cells was determined before and after 60 min of chemical hypoxia. To measure cell volume, a confocal microscope was used to scan calcein fluorescence for each 1- μm confocal slice, proceeding from the top of the cell to the bottom (~10 slices). From the surface area of the scan and pixel thickness of each confocal slice, the cell volume was calculated (21). Because hypoxia does not influence the distribution of calcein in the cell, the only condition interfering with the measurement of cell volume is the complete loss of calcein. This occurred in only a few cells and was associated with the rupture of the cell membrane. No data were obtained from these cells. The average cell volume of the β -Alanine-treated and control cells before the chemical hypoxic insult was $5.2 \pm 0.2 \times 10^3$ and $6.5 \pm 0.2 \times 10^3 \mu\text{m}^3$, respectively. The confocal microscope procedure was accurate within 2%. Therefore, a deviation of >5% from the prehypoxic value was required to classify a cell as swollen (necrotic) or shrunken (apoptotic). Each cell served as its own control.

Cellular H^+ , sodium, and calcium content. To determine $[\text{Na}^+]_i$ of the normal and β -Alanine-treated cells, the normoxic cells were first loaded with sodium-binding benzofuran isophthalate (SBFI) by incubating the myocytes for 1.5 h at room temperature with serum-free medium containing 10 μM SBFI-AM. They were then washed three times with dye-free medium and kept in the dye-free medium for at least 45 min to facilitate the hydrolysis of the ester. The cells were then placed in either normoxic or chemical hypoxic Krebs-Henseleit buffer. Sodium content of the normoxic and chemically hypoxic cells was determined fluorometrically by use of an Olympus (IMT-2) microscope, with emission fluorescence (>420 nm) examined at two excitation wavelengths, 340 and 380 nm. The background emission was corrected at the start of the experiment. During the course of the chemical hypoxic insult, the ratio of the fluorescence signals generated at the two excitation wavelengths was determined. $[\text{Na}^+]_i$ was calculated from the fluorescence ratio (340 nm/380 nm) after calibration curves were generated using the procedure of Harootunian et al. (5).

To determine the calcium content of the control and β -Alanine-treated cells, some cardiomyocytes were loaded with 2 μM indo 1 for 20 min at 37°C. The loading and subsequent rinsing steps took place before the onset of chemical hypoxia. To prevent cell movement due to cell beating, some of the cells were treated with 20 mM 2,3-butanedione monoxime, an agent effective in blocking the interaction of the muscle proteins. indo 1 fluorescence at 410 and 490 nm was measured using the point scan mode of the confocal microscope. Data obtained before and 60 min after onset of chemical hypoxia were expressed as the fluorescence ratio F_{410}/F_{490} . $[\text{Ca}^{2+}]_i$ was calculated from the fluorescence ratio after a calibration curve was generated. Before chemical hypoxia, indo 1 fluorescence of the β -Alanine-treated and control cells was not significantly different. Each cell served as its own control.

Intracellular pH was measured by using a modification of the method of Blank et al. (3). Cells were loaded with 20 μM carboxysemaphthorhodofluor (SNARF) by incubation for 15 min at 37°C in medium supplemented with 10 mM HEPES (pH 7.4). The cells were then rinsed three times and resuspended in the standard Krebs-Henseleit buffer. When pH_i was measured, the cells were excited at 488 nm and the fluorescence was monitored at both 580 and 640 nm; the proton-bound and unbound forms of SNARF emit at 580 and 640 nm, respectively. The pH_i fluorescence ratio (F_{580}/F_{640}) was first determined after a 15-min incubation under standard conditions (normoxia). The cells were then transferred to Krebs-Henseleit buffer containing the metabolic inhibitors 10 mM deoxyglucose and 3 mM Amytal. After 60 min, fluorescence at 580 and 640 nm was measured. An in situ pH calibration curve was then generated, which allowed the conversion of the pH fluorescence ratio data into pH_i values. This was accomplished through the dissipation of the transmembrane pH gradient. Initially, the cells were incubated for 10 min at 37°C in a buffer containing 140 mM KCl, 1 mM EGTA, and 10 mM HEPES (pH 7.4) to prevent calcium overload. The initial buffer was then removed and replaced by a calibration solution [140 mM KCl, 1.2 mM magnesium sulfate, 1.2 mM potassium phosphate, 10 mM HEPES or 10 mM PIPES, 20 μM nigericin, 1 μM valinomycin, and 1 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)] whose pH ranged from 6.6 to 7.4. After a 10-min incubation in the calibration solution, the SNARF fluorescence ratio was determined. A standard pH calibration curve was generated (medium pH vs. fluorescence ratio) to allow the determination of actual pH_i values.

Trypan blue staining and TdT-mediated DUTP nick end labeling procedures. To measure cell viability, hypoxic and normoxic cells were incubated for 5 min at room temperature with medium containing 0.4% trypan blue. After 5 min of incubation, the cells were fixed in Krebs-Henseleit buffer containing 2% glutaraldehyde. Four separate fields in the light microscope were used to assess trypan blue staining.

End-labeled DNA fragments of apoptotic nuclei were monitored by the Klenow Frag EL DNA fragmentation detection kit (catalog no. Q1A21; Calbiochem). After either the 3-day control incubation or 1 h of chemical hypoxia, glass slides were fixed with 4% formaldehyde for 15 min and then resuspended in 80% ethanol for 20 min. After rehydration, the samples were permeabilized with proteinase K (20 $\mu\text{g}/\text{ml}$). Endogenous peroxidases were inactivated by exposing the samples to 3% H_2O_2 for 5 min. After the samples were rinsed, the slides were placed in Klenow buffer for 20 min. The Klenow-labeling reaction mixture was allowed to proceed for 1.5 h at 37°C. Termination of the reaction was accomplished by a 5-min incubation with buffer containing 0.5 mM EDTA (pH 8.0) followed by a 10-min exposure to blocking buffer. The samples were then exposed for 30 min to a peroxidase-streptavidin conjugate. After a 15-min incubation with 3,3'-deaminobenzidine (0.7 mg) and H_2O_2 -urea (0.6 mg), the samples were rinsed and then counterstained with hematoxylin. The slides were briefly immersed in 100% ethanol and then in xylene. Four fields in the light microscope were counted for dark brown (apoptosis) and purple (normal) nuclei. The data were expressed as percentage of apoptotic cells.

DNA ladder analysis. Cells were first scraped from the incubation dishes. After cell lysis, DNA was isolated according to the method described in the DNA apoptosis ladder kit (Boehringer-Mannheim). Samples of DNA (10 μg , amount determined by absorbance at 260 nm) were subjected to agarose gel (2%) electrophoresis. The gels were stained with ethidium bromide (2 $\mu\text{g}/\mu\text{l}$) and then destained for 20 min. The bands were visualized under ultraviolet light.

Statistical analysis. The statistical significance of the data was determined by using the Student's *t*-test for comparison within groups and analysis of variance (ANOVA) combined with Tukey's post hoc test for comparison between groups. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Previously, it was shown that rats fed a diet containing high concentrations of the taurine analog β -Alanine slowly lose a significant fraction of their intracellular taurine store (15). As shown in Fig. 1, incubation of isolated rat neonatal cardiomyocytes with β -Alanine containing medium for a period of 2 days also induced a dramatic drop (40%) in the size of the intracellular taurine pool. β -Alanine treatment was also associated with a 30% decrease in $[\text{Na}^+]_i$ from 11.0 ± 1.4 to 7.12 ± 0.9 mM (Fig. 2). Resting $[\text{Ca}^{2+}]_i$ was unaffected by taurine depletion, but the calcium transient was prolonged, as evidenced by a 70% increase in the time from peak calcium to 90% relaxation (TR_{90} was 0.7 ± 0.03 s in the control vs. 1.2 ± 0.11 s in the β -Alanine-treated group). As a result of the osmotic imbalance created by the loss of both taurine and sodium, the β -Alanine-treated cell also underwent a reduction in cell volume from $6.5 \pm 0.2 \times 10^3$ to $5.2 \pm 0.2 \times 10^3$ μm^3 .

The diminished sodium and osmotic load of the taurine-deficient cell was maintained even after 60 min of

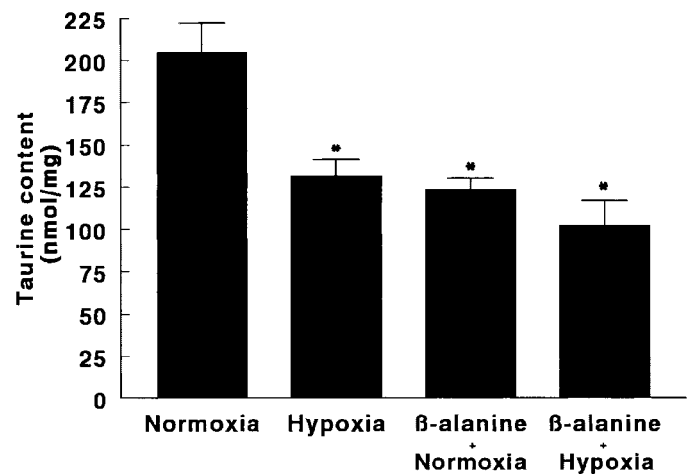


Fig. 1. Effect of chemical hypoxia on cellular taurine content of the untreated and β -Alanine-treated myocyte. Isolated rat neonatal cardiomyocytes were incubated for 2 days with medium containing or lacking 5 mM β -Alanine and then exposed for 60 min to either normal Krebs-Henseleit buffer (normoxic groups) or Krebs-Henseleit buffer containing 10 mM deoxyglucose and 3 mM Amytal (hypoxic groups). Cells were then assayed for taurine levels as described in METHODS. Values represent means \pm SE of 4 different preparations. *Significant difference from the normoxic control group ($P < 0.05$).

chemical hypoxia. As shown in Fig. 2, $[\text{Na}^+]_i$ increased in a nearly linear fashion during the first 30 min of chemical hypoxia. Thereafter, the rate of sodium accumulation accelerated in the control cell, but it remained at a depressed level in the taurine-deficient cell. This led to a pattern in which the $[\text{Na}^+]_i$ of the two groups of cells showed a greater divergence with time. After 60 min of chemical hypoxia, the $[\text{Na}^+]_i$ of the control myocyte was 3.5 times greater than its concentration in the β -Alanine-treated cell.

Organic osmolytes, of which taurine is the most important (1, 6, 9, 11), serve a very crucial physiological function. By exiting the osmotically stressed cell, organic osmolytes act as safety valves to minimize the degree of osmotic stress. Interestingly, control cells treated with β -Alanine contain a reduced intracellular osmotic load. Although this favorable osmotic condition would be expected to benefit the metabolically inhibited cell, it was felt that competing influences could diminish the beneficial effect. For example, the advantage accrued through the reduction in hypoxia-induced sodium loading could be lost by limitations in the amount of taurine available to exit the β -Alanine-treated cell during chemical hypoxia. To determine the importance of these competing events, taurine content was measured both before and after 60 min of chemical hypoxia. As expected, the taurine content of the control myocyte fell 37% after 60 min of chemical hypoxia (Fig. 1). By comparison, the β -Alanine-treated cell experienced no significant decline in taurine content during chemical hypoxia. Despite the greater loss of taurine in the control myocyte during chemical hypoxia, the dual contribution of sodium and taurine toward the tissue's osmolality was higher in the control cell. This confirmed that the dominant osmotic effect in both the

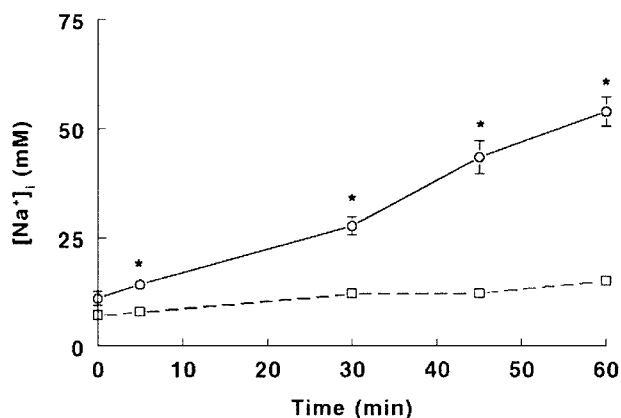


Fig. 2. Effect of taurine deficiency on intracellular sodium concentration ($[Na^+]_i$) before and after chemical hypoxia. Rat neonatal cardiomyocytes were incubated for 2 days with medium containing (□) or lacking (○) 5 mM β -Alanine. They were then loaded with the sodium probe, sodium-binding benzofuran isophthalate, before being subjected to a chemical hypoxic insult in medium containing 10 mM deoxyglucose and 3 mM Amytal. Fluorescence emission (>420 nm) was examined at 2 excitation wavelengths, 340 and 380 nm. $[Na^+]_i$ was calculated from the fluorescence ratio at the 2 wavelengths. Values represent means \pm SE of 4–5 different preparations, with 10–15 cells examined per preparation. *Significant difference between the control and the β -Alanine-treated group ($P < 0.05$).

taurine-deficient and control myocyte was the change in $[Na^+]_i$. As shown in Table 1, hypoxia led to only a 6 mosmol/l elevation in the combined sodium-plus-taurine osmotic load of the β -Alanine-treated cell, whereas the combined load in the control cell was increased 33 mosmol/l after 60 min of chemical hypoxia, with the difference caused by the change in sodium loading.

Generally, a reduction in the osmotic load of the hypoxic cardiomyocyte would be expected to reduce the degree of hypoxia-induced cell swelling. However, only a fraction of both the control and the β -Alanine-treated cells swelled during chemical hypoxia, with the amount of swelling averaging 12% in both groups of cells (Fig. 3). Although the degree of cell swelling was identical in those cells that showed an increase in volume, there was a dramatic reduction in the number of β -Alanine-treated cells that swelled during chemical hypoxia (Fig. 3).

Cell swelling is a characteristic feature of both hyposmotic stress and the latter stages of cell necrosis. However, the two modes of swelling differ. Whereas swelling caused by hyposmotic stress promotes volume expansion in both the injured and uninjured cells, only

Table 1. Contribution of sodium and taurine to cellular osmolality

Group	Taurine	Sodium	Total
Control	27.6	11.0	38.6
Hypoxia	17.5	53.9	71.4
β -Alanine treated	16.5	7.2	23.7
β -Alanine treated + hypoxia	13.7	15.8	29.5

Values shown are based on data in Figs. 1 and 2 and assume constant cell water content of 7.45 μ l/mg protein. All data are expressed as mosmol/l.

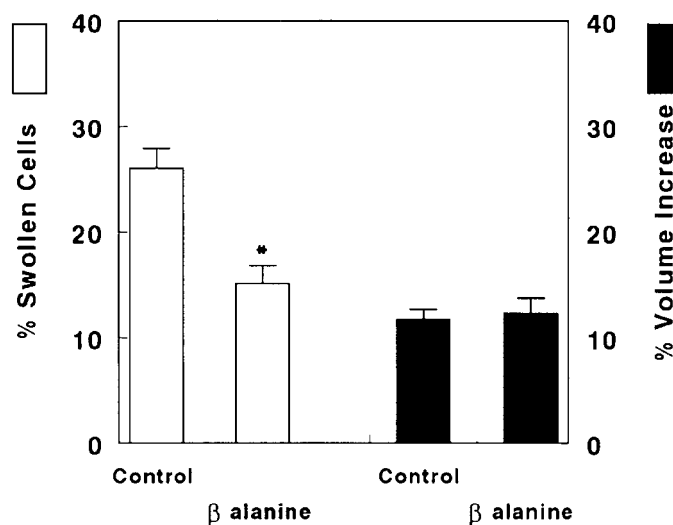


Fig. 3. Effect of taurine deficiency on hypoxia-induced cell swelling. Control and β -Alanine-treated cells were preloaded with the fluorescent dye calcein. With the confocal microscope, cell volume was determined both before and after 60 min of chemical hypoxia. The % of cells that underwent at least a 5% increase in cell volume during the chemical hypoxic insult is termed %swollen cells. The degree of swelling experienced by the swollen cells is referred to as %volume increase. Values represent means \pm SE of 4–5 preparations, with 10–20 cells examined per preparation. *Significant difference between the control and the β -Alanine-treated group ($P < 0.05$).

the damaged cells will undergo necrosis-linked swelling. Thus the observation that taurine deficiency only affects hypoxia-induced cell swelling in a fraction of the metabolically inhibited cells appears to be more consistent with an effect of taurine deficiency on an early reversible step in the necrotic pathway. To test this idea, cell viability of the taurine-deficient and control cells was examined after 60 min of chemical hypoxia. As shown in Fig. 4, taurine deficiency rendered the

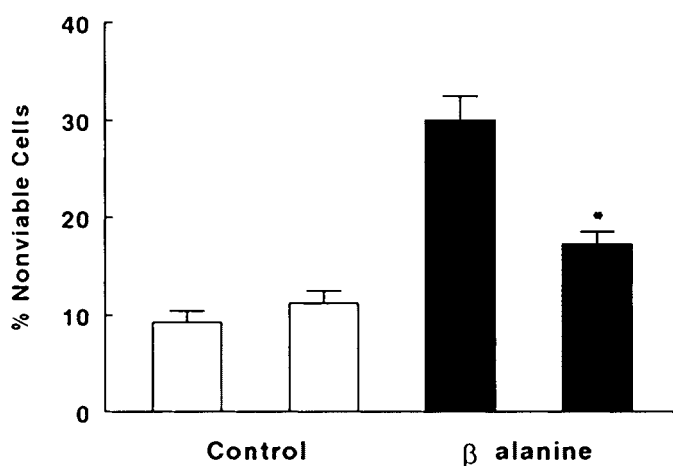


Fig. 4. Effect of taurine deficiency on cell viability following chemical hypoxia. Control and β -Alanine-treated cells were transferred to medium containing 10 mM deoxyglucose and 3 mM Amytal. After a 60-min incubation, cell viability was evaluated by the ability of the cell to exclude trypan blue. Each value represents the mean \pm SE of 4 preparations, with 10–15 cells examined per preparation. *Significant difference between the control and β -Alanine-treated cell ($P < 0.05$).

β -Alanine-treated cell resistant to hypoxia-induced necrosis. Whereas 30% of the control cells were unable to exclude trypan blue following 60 min of chemical hypoxia, β -Alanine treatment reduced the number of non-viable cells to about 17%. Similar values were obtained by using the propidium iodide assay of cell death.

Besides contributing to the osmotic load of the cell, sodium excess can also lead to calcium overload, a major cause of hypoxia-induced cell necrosis (16, 24, 25). Because of the importance of $[Ca^{2+}]_i$ in cardiac pathology, the effects of chemical hypoxia and taurine depletion on $[Ca^{2+}]_i$ were examined. As shown in Fig. 5, $[Ca^{2+}]_i$ of the control cell increased 250% from 0.15 ± 0.02 to $0.53 \pm 0.06 \mu\text{M}$ after 60 min of chemical hypoxia. However, the β -Alanine-treated cell was extremely resistant to a change in calcium content during the hypoxic insult, increasing only 20% after 60 min of chemical hypoxia (Fig. 5).

In the ischemic heart, sodium and calcium accumulation has been attributed to enhanced proton (H^+) production. Some of the protons that are generated leave the cell via the sodium- H^+ exchanger, leading to an accumulation of sodium. When coupled with the sodium-calcium exchanger, sodium excess also leads to calcium overload, which can then damage the cell (7, 10). To determine whether taurine depletion disrupts this detrimental sequence of events, pH_i was determined both before and after 60 min of chemical hypoxia. Under normoxic conditions, the pH_i of the control and β -Alanine-treated cell was 7.16 and 7.32, respectively (Fig. 6). Chemical hypoxia reduced the pH_i to 7.03 and 7.15 in the control and β -Alanine-treated cells, respectively.

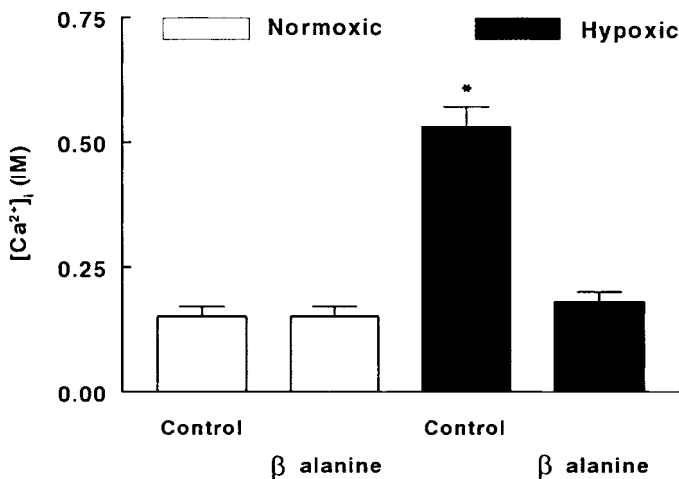


Fig. 5. Effect of taurine deficiency on intracellular free calcium concentration $[Ca^{2+}]_i$ before and after chemical hypoxia. Control and β -Alanine-treated cells were loaded with the calcium indicator dye indo 1. After a short reincubation under normoxic conditions, the fluorescence of specific cells at 410 and 490 nm was measured using the point-scan mode of the confocal microscope. The fluorescence of the same cells was measured after 60 min of chemical hypoxia. The data are expressed as $[Ca^{2+}]_i$, which was calculated from the fluorescence ratio, F_{410}/F_{490} . Each value represents the mean \pm SE of 3–4 preparations, with 10 cells examined per preparation. *Significant difference between the control and the β -Alanine-treated cell following the hypoxic insult ($P < 0.05$).

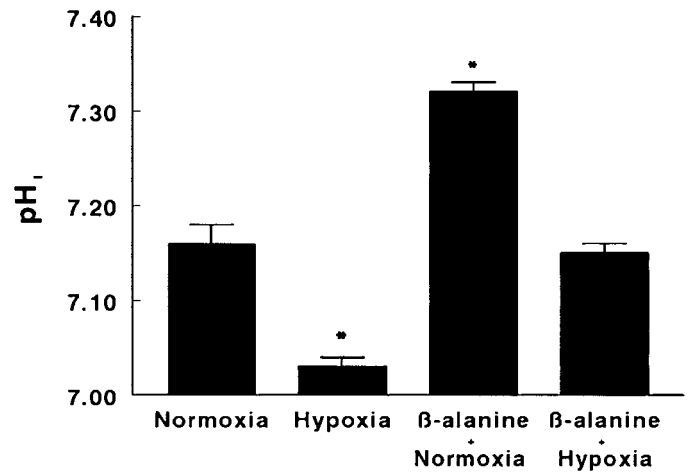


Fig. 6. Effect of taurine deficiency on intracellular pH (pH_i) before and after chemical hypoxia. Control and β -Alanine-treated cells were loaded with the pH indicator, carboxysemaphorhodofluor. After a 15-min incubation under control conditions, the fluorescence at 580 and 640 nm were measured using the point scan mode of the confocal microscope. The fluorescence of the same cells was again measured after 60 min of chemical hypoxia. pH_i was calculated after the generation of a standard curve. Each value represents the mean \pm SE of 4 preparations, with 10 cells examined per preparation. *Significant difference compared with the control normoxic cell ($P < 0.05$).

Hypoxia also led to an increase in the number of apoptotic cells, a process also diminished by β -Alanine treatment (Fig. 7). Whereas 29% of the control cells were TdT-mediated dUTP nick end labeling (TUNEL) positive after 1 h of hypoxia, 19% of the β -Alanine-treated cells were TUNEL positive. One of the characteristic features of apoptotic cells is a reduction in cell size. Among the cardiomyocytes that underwent apoptosis, the average reduction in cell size was 16% for the

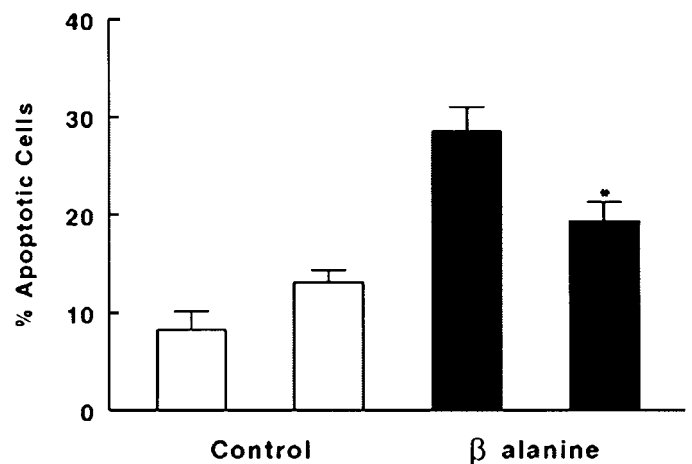


Fig. 7. Effect of chemical hypoxia on cellular apoptosis. Control and β -Alanine-treated cells were transferred to medium containing 10 mM deoxyglucose and 3 mM Amytal. After 60 min, the presence of nuclear end-labeled DNA fragments was detected by the TdT-mediated dUTP nick end labeling (TUNEL) technique. Data are expressed as percentages of cells exhibiting positive TUNEL staining. Each value represents the mean \pm SE of 4 preparations, with 10–15 cells examined per preparation. *Significant difference between the control and β -Alanine treated cells ($P < 0.05$).

control cells and 19% for the β -Alanine-treated cells. Therefore, β -Alanine treatment did not protect the cell by reducing the degree of cell shrinkage. Rather, β -Alanine treatment reduced from 24% to 8% the number of cells that shrank during the hypoxic insult (Fig. 8). Figure 9 reveals that β -Alanine treatment also reduced the intensity of the DNA ladder, which indicates the extent of DNA damage that occurred during the hypoxic insult.

DISCUSSION

Previous reports have shown that large amounts of taurine are lost from the ischemic myocardium (9, 11). Similarly, taurine is lost in large quantities from cardiomyocytes during chemical hypoxia (Fig. 1). It has been proposed that this loss of taurine might benefit the cell by protecting against the development of a severe osmotic imbalance (1). In the present study, we found that the contribution of sodium to the hypoxia-induced increase in cellular osmolality was 43 mosmol/l in the control cell. Taking into account the loss of taurine, the imbalance was reduced to 33 mosmol/l. A further reduction in tissue osmolality was observed in the taurine-deficient cell.

Although taurine loss clearly limits the osmotic imbalance that develops across the cell membrane of the hypoxic cell, three lines of evidence suggest that the acute efflux of taurine from the compromised cell is not a major cause of cardioprotection during chemical hypoxia. First, the β -Alanine-treated and control cells differ in their combined $[Na^+]_i$ and taurine osmotic contributions by 42 mosmol/l. About 90% of this difference can be attributed to the lower $[Na^+]_i$ in the β -Alanine-

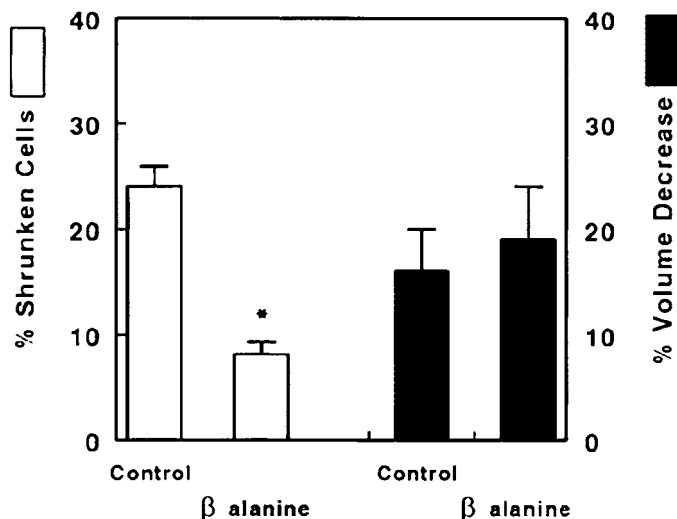
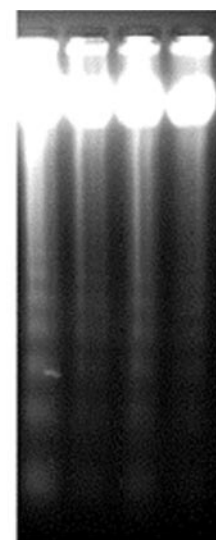


Fig. 8. Effect of taurine deficiency on hypoxia-induced cell shrinkage. Control and β -Alanine-treated cells were preloaded with the fluorescent dye calcein. Cell volume before and after 60 min of hypoxia was determined using the confocal microscope. The percentage of cells that underwent at least a 5% decrease in cell volume during the hypoxic insult is termed %shrunken cells. The degree of cell shrinkage experienced by the shrunken cells is referred to as %volume decrease. Values represent means \pm SE of 4–5 preparations, with 10–20 cells examined per preparation. *Significant difference between the control and β -Alanine-treated cells ($P < 0.05$).



	1	2	3	4
β -alanine	-	-	+	+
Hypoxia	+	-	+	-

Fig. 9. Effect of taurine deficiency on DNA ladder. DNA was isolated from control and β -Alanine-treated cells both before and after 1 h of chemical hypoxia. After the DNA was subjected to agarose gel electrophoresis and treatment with ethidium bromide, the DNA bands were visualized by ultraviolet light. Lanes 2 and 4, control and β -Alanine-treated normoxic cells, respectively; lanes 1 and 3, control hypoxic and β -Alanine-treated hypoxic cells, respectively. A typical DNA ladder was observed for the DNA sample obtained from control hypoxic cells; however, the intensity of the DNA ladder was diminished in the β -Alanine-treated cells.

treated cell (Table 1). Second, the small amount of taurine that effluxes the taurine-deficient cell during chemical hypoxia (<3 mosmol/l) is incapable of significantly impacting the cell's total osmolality. Third, taurine depletion reduces the number of cells that shrink during the hypoxic insult but not the overall degree of shrinkage. Fourth, Fig. 3 reveals that taurine depletion reduces the number of cells that swell during chemical hypoxia but has no effect on the overall degree of swelling. If the taurine effect were caused solely by its contribution to an acute change in osmolality, the osmotic load of all of the taurine-deficient cells should have improved and the degree of cell swelling should have been affected. Because this did not occur, it suggests that taurine influences the necrotic and apoptotic pathways, presumably at a reaction that precedes the osmotic swelling step. A likely candidate for this action is the regulation of sodium transport. By reducing the hypoxia-induced sodium load, the taurine-deficient cell minimizes the degree of cellular calcium loading and thus the number of cells that succumb to necrosis and apoptosis (16, 24, 25) (Fig. 5).

Although the mechanism underlying the modulation of $[Na^+]_i$ by taurine has not been firmly established, three mechanisms deserve consideration. First, taurine is transported via a sodium-taurine symporter (22, 29). According to Suleiman et al. (29), as $[Na^+]_i$ increases to levels exceeding 20 mM, taurine efflux is

enhanced. Figure 2 shows that chemical hypoxia raises $[Na^+]_i$ sufficiently to promote taurine efflux via the symporter. However, another pathway capable of promoting taurine efflux during sodium loading utilizes phospholemman (8, 14).

Cellular taurine content is also linked to $[Na^+]_i$ through changes in the pH_i . Figure 6 shows that the pH_i of the taurine-deficient cell is elevated in relation to the control cell. This relative alkalinization of the taurine-deficient cell is also apparent during chemical hypoxia. It remains to be determined whether taurine affects pH_i by modulating the activity of the sodium- H^+ exchanger or by altering H^+ generation.

Another factor contributing to altered sodium transport in the taurine-deficient cell is the reduction in cellular osmolality. The cell adapts to hypertonic or hypotonic stresses by activating a series of events designed to restore volume and normalize the osmotic balance across the cell. In response to hypertonic stress, a regulatory volume increase is initiated, in which several transporters are activated that promote the cellular accumulation of both sodium and taurine (2, 13, 23). By contrast, a regulatory volume decrease occurs in response to hyposmotic stress and is associated with the loss of cellular osmolytes (23). Rasmussen et al. (17) found that the combined loss of taurine and sodium from the hyposmotically stressed chick myocyte accounts for nearly half of the rapid intracellular osmotic change that accompanies a 50% decrease in the osmolality of cell medium.

Ischemia and hypoxia lead to the accumulation of several metabolic end products that raise the cell's osmolality (18, 26). Consequently, a regulatory volume decrease is triggered, leading to the efflux of taurine from the cell. Although the regulatory volume decrease would also be expected to trigger the efflux of sodium from the cell, this effect is countered by the inhibition of the sodium pump and the promotion of flux through the sodium- H^+ exchanger, leading to a net increase in $[Na^+]_i$. When taurine levels are reduced before the chemical hypoxic insult, the degree of sodium accumulation by the metabolically inhibited cell is attenuated (Fig. 2). This effect is unlikely to be caused by a change in sodium- K^+ ATPase activity, because the effect of taurine on ATP generation is minimal (15). Rather, taurine deficiency presumably reduces the influx of sodium via the sodium- H^+ exchanger and promotes the efflux of sodium from the cell during the regulatory volume decrease. In the control myocyte, chemical hypoxia leads to a sodium gain equivalent to 43 mosmol/l (Table 1). By contrast, the sodium gain in the β -Alanine-treated cell adds only 8.5 mosmol/l to tissue osmolality. Although a reduction in sodium influx via the sodium- H^+ exchanger may account for the decrease in sodium accumulation in the β -Alanine-treated cell, the possibility that more sodium exits the β -Alanine-treated cell is also a viable option. During the chemical hypoxic insult, 10.1 mosmol/l equivalents of taurine leave the control cell, whereas only 2.5 mosmol/l equivalents of taurine leave the β -Alanine-treated cell. It is

known that the taurine-deficient cell has a propensity to retain cellular taurine (27). Therefore, the taurine-deficient cell might limit the amount of taurine that is lost at the expense of other osmolytes. Consequently, the metabolically inhibited cell might preferentially extrude sodium and retain taurine. This interpretation would be consistent with the finding that the rise in $[Na^+]_i$ in the β -Alanine-treated cell during chemical hypoxia is less than one would have predicted. It would be attractive to suggest that the improvement in sodium handling is a major cause for the cardioprotection seen in the taurine-deficient cardiomyocyte.

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