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Inhibition of Rat Vascular Smooth Muscle Cell Proliferation by Taurine and Taurine Analogues

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ABSTRACT. The growth of rat aorta vascular smooth muscle cells (VSMCs) was measured in the presence and absence of taurine. Concentrations of taurine as low as 0.3 mM in the culture medium inhibited the proliferation of the cells, as monitored by measuring cell count, and also inhibited the rate of DNA synthesis, as examined by measuring [^{3}H]thymidine incorporation into DNA. However, even at the highest concentration of taurine (30 mM), the doubling time of the VSMCs was only increased by 38%. Protein content of the VSMCs was decreased by 30 mM taurine. [^{3}H]Leucine incorporation into newly synthesized protein was not affected by the highest concentration of taurine tested (30 mM), indicating that taurine did not inhibit protein synthesis but rather decreased total protein content by inhibiting cellular proliferation. The effects of other amino acids such as alanine, glycine, and serine and of various taurine analogues such as β-alanine, guanidinoethanesulfonic acid (GES), and isethionic acid also were tested at a concentration of 20 mM for their effects on the growth of the VSMCs. Alanine, glycine, and serine had only a minimal effect or no effect on cell count, quantity of protein, and incorporation of [^{3}H]thymidine into DNA. GES, β-alanine, and isethionic acid had a significant effect on cell count, protein content, and incorporation of [^{3}H]thymidine into DNA. β-Alanine was the only analogue tested that significantly depressed [^{3}H]leucine incorporation into newly synthesized protein. It is concluded that taurine, GES, and isethionic acid inhibited proliferation of VSMCs but did not alter normal protein synthesis or survivability of VSMCs. In contrast, other amino acids, alanine, glycine and serine, had minimal effects on VSMC proliferation and protein synthesis, whereas β-alanine appeared to be toxic, inhibiting both VSMC synthesis and *de novo* protein synthesis. BIOCHEM PHARMACOL **57**;11:1331–1339, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. taurine; taurine analogues; vascular smooth muscle cells; rat aorta

Taurine (2-aminoethanesulfonic acid) is present in high concentrations in all mammalian tissues [1]. However, while there has been considerable effort in recent years in pursuing a function for taurine in various tissues due to its reported physiologic effects, there is presently no well-defined mechanism of action for taurine at the molecular level. Taurine is reported to have numerous physiologic and pharmacologic roles such as osmoregulatory activity in the kidney and brain; regulation of protein phosphorylation in brain, retina, and heart; a regulatory role in calcium dynamics in retina, brain, and heart; and antioxidant activities in lung tissue (reviewed in Refs. 2 and 3). Among cardiovascular actions, taurine plays a modulatory role in myocardial contraction; protects cardiac tissue against myocardial calcium overload; and has antihypertensive effects (reviewed in Refs. 2 and 3).

Atherosclerosis is one of the leading causes of cardiovascular disease. Numerous researchers have studied the development, distribution, and composition of atherosclerotic lesions in both humans and animal models in an effort to understand better how the process might be prevented or slowed [4–7]. Thickening of the vessel wall intima has been observed, and constituents of human atherosclerotic lesions composed of lipid deposits, macrophages, and VSMCs§ have been described. Since the first demonstration that VSMCs represent the principal mesenchymal cell in human atherosclerotic plaques [8], the accelerated proliferation of VSMCs has been suggested to be a central and characteristic feature of atherogenesis [8–10]. Several investigators have suggested that studying growth rates and other metabolic processes of cultured VSMCs represents a reasonable method for the investigation of vascular dysfunction resulting from the exposure to atherogenic stimuli *in vivo* [10–13], and the ability of VSMCs to proliferate *in vitro* has been interpreted to be a reflection of the pathologic stimulation that the cells received *in vivo* [14].

Because of the varied influences of taurine on the cardiovascular system, it was of interest to determine if taurine might also influence the rate of proliferation of

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§ *Abbreviations:* VSMCs, vascular smooth muscle cells; GES, guanidinoethanesulfonic acid; ISA, isethionic acid; HBSS, Hanks' balanced salt solution; ALA, alanine; GLY, glycine; and SER, serine.

VSMCs. In this study, VSMCs isolated from rat aorta were cultured in the presence of various pharmacological concentrations of taurine and single concentrations of GES and other amino acids. The object of the present study was to determine if taurine alters proliferation of VSMCs over a 14-day period as determined by the increase in cell count, incorporation of [^{3}H]thymidine into DNA, and incorporation of [^{3}H]leucine into protein. Other taurine analogues and amino acids also were tested for their effects on the growth of VSMCs in order to control for osmotic effects and to compare with the taurine results.

MATERIALS AND METHODS

Materials

Taurine, β-alanine, L-alanine, glycine, L-serine, L-glutamate, L-phenylalanine, collagenase, trypsin, and α-smooth muscle actin specific antibody (1A4) were purchased from the Sigma Chemical Co. Trypsin was also purchased from Difco Laboratories. ISA was purchased from the Aldrich Chemical Co. [^{3}H]Thymidine and [^{3}H]leucine were purchased from New England Nuclear. Fetal bovine serum was purchased from Atlantic Biologicals. Medium 199 and penicillin/streptomycin mixture were purchased from GIBCO. GES was synthesized according to the procedure of Morrison *et al.* [15].

Cell Culture

VSMCs were harvested for primary cell culture according to previously described methods [16]. Briefly, aortae isolated from five female Sprague–Dawley rats (200 g) were placed in HBSS with Ca^{2+} and Mg^{2+}. Connective tissue and fat were removed from the aortae, which were then digested with 1 mg/1 mL of collagenase for 20 min at 37°. The aortic segments were removed from the collagenase solution, placed in a flask containing 1.25 mg/mL of trypsin (Sigma) in 15 mL HBSS, and incubated for 15 min at 37° with gentle agitation. Then the aortic segments were removed from the flask (supernatant discarded), placed in a glass Petri dish, minced into small segments, and incubated with fresh collagenase solution (15 min, 37°). The supernatant was discarded. Fifteen milliliters of trypsin solution (1.25 mg/mL, Difco Laboratories) was added to the aortic segments and incubated for 10 min at 37° with gentle agitation. This digestive procedure was repeated three additional times, and the supernatant from each was collected. All supernatants were combined and filtered through a Gelman filter to collect the VSMCs. Then the VSMC suspension was diluted to 2×10^5 cells/mL with growth medium (medium 199 containing 10% fetal bovine serum including antibiotics and supplemented with 2 mM glutamine). VSMCs were cultured in 75-cm flasks at a density of 10,500 cells/cm^2. The cultures were kept at 37° in an incubator with an atmosphere of 5% CO_2:95% air. Cells have been identified as VSMCs, using immunohistochemistry with α-smooth muscle actin specific antibody (1A4) and muscle specific myosin antibody. Greater than 95% of the cells took up both antibodies (Fig. 1).

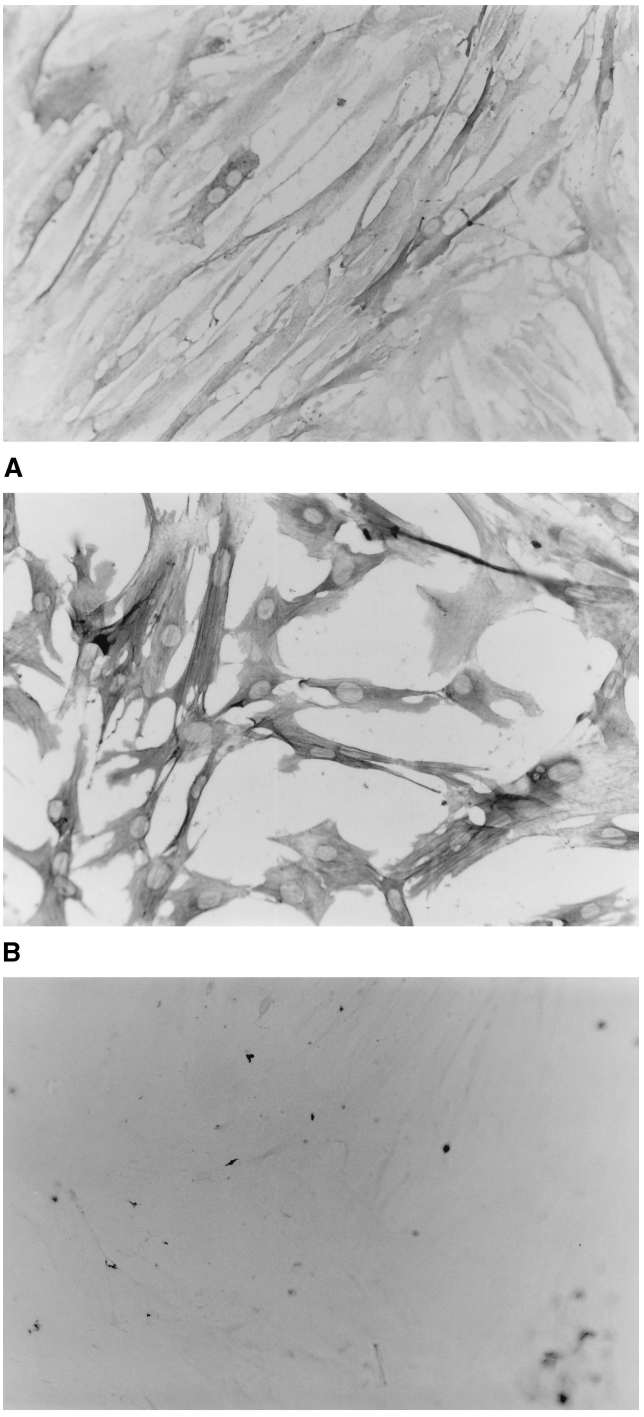

FIG. 1. Immunostaining of rat aortic VSMCs. Upper panel: muscle specific myosin antibody; middle panel: α-actin antibody; and lower panel: control.

VSMCs were grown in the presence of taurine, analogues of taurine, or other amino acids as follows: VSMCs were seeded with serum-free medium 199 into 24-well multidishes at 20,000 cells/well. After 2 days, medium in the control group was changed to growth medium. Medium in

TABLE 1. Concentration-response of taurine on the growth (cell count) of rat aortic VSMCs

Day	Cell count			
	Control	Tau (0.3 mM)	Tau (3.0 mM)	Tau (30 mM)
1	9,660 ± 926	9,640 ± 773	9,320 ± 782	9,990 ± 856
3	50,200 ± 10,500	40,100 ± 8,130	41,600 ± 8,210	37,100 ± 7,700
5	201,200 ± 29,500	150,800 ± 24,400	135,900 ± 21,400	105,600 ± 18,200*
7	337,500 ± 36,800	246,100 ± 34,100*	200,100 ± 32,000*	144,100 ± 23,700*
9	511,200 ± 41,900	393,900 ± 40,200*	301,000 ± 26,800*	201,000 ± 29,500*
14	839,100 ± 41,400	701,800 ± 42,500*	617,100 ± 42,800*	481,300 ± 50,300*

Data are presented as means ± SEM of 7 experiments.
*Significant difference ($P < 0.05$) for each concentration of taurine on a specific day compared with the corresponding control value.

the experimental groups was changed to growth medium plus taurine, taurine analogue, or other amino acid. Medium was changed on days 1, 3, 5, 7, 9, 11, and 13. Cells were counted on days 1, 3, 5, 7, 9, and 14. Culture doubling time was determined using the logarithmic portion of the growth curve as described by Alipui *et al.* [16].

DNA and Protein Synthesis

DNA and protein synthesis were measured as described by Palmberg *et al.* [17]. Briefly, 5 μCi of [^{3}H]thymidine or [^{3}H]leucine was added to each well and incubated for 1 hr with the VSMCs at 37°. Then the VSMCs in each well were washed with phosphate-buffered saline (1 mL/well) two times. The wash was discarded. Trichloroacetic acid (TCA, 5%) was added to each well for 5 min to precipitate the DNA and protein, the process was repeated once, and then the TCA was discarded. Sodium hydroxide (0.2 mL of 0.5 M) was then added to each well and allowed to dissolve the precipitate overnight. Next the NaOH solution was collected into scintillation vials. An additional 0.2 mL of the NaOH solution was used to wash the wells, added to the appropriate scintillation vial, and counted.

Protein Assay

Each well was washed twice with phosphate-buffered saline (2 mL). NaOH (0.2 mL of 0.5 M) was added to dissolve the VSMCs present in each well. Then an aliquot was assayed by the method of Lowry *et al.* [18] for the quantitative measurement of the protein content.

TABLE 2. Effects of various levels of taurine on the doubling time of VSMCs during a 7-day period

Taurine (mM)	Doubling time (hr)
0	28.4 ± 0.9
0.3	31.4 ± 1.0
3.0	33.6 ± 1.6
30	39.3 ± 2.9*

*Analysis of variance and Duncan's multiple-range test were used to determine significant differences between the control value and the values obtained in the presence of different concentrations of taurine ($P < 0.05$).

Statistical Analysis

Analysis of variance and Duncan's multiple-range test were used to determine significant differences ($P < 0.05$) between the control value and the various experimental conditions.

RESULTS

Effects of Various Concentrations of Taurine on the Growth of VSMCs

The effects of various concentrations of taurine from 0 to 30 mM on the growth of VSMCs over a 14-day period are shown in Table 1. Concentrations of 0.3, 3, and 30 mM taurine significantly inhibited the growth of the VSMCs at days 7, 9, and 14. Thirty millimolar taurine also inhibited growth on day 5. Culture doubling time for VSMCs was increased in a concentration-dependent fashion as the concentration of taurine was increased (Table 2). Cell growth, monitored by quantitatively measuring the amount of protein per well, as a function of the concentration of taurine in the cell culture medium, is shown in Fig. 2. Only 30 mM taurine at days 7, 9, and 14 significantly inhibited the increase in protein content.

Effects of Taurine on the Incorporation of [^{3}H]Thymidine and [^{3}H]Leucine into DNA and Protein of VSMCs

Figure 3 shows that [^{3}H]thymidine incorporation (cpm/μg protein) into VSMCs was consistent with DNA synthesis being maximal at day 3. The rate of DNA synthesis was also inhibited by all concentrations of taurine (0.3, 3, and 30 mM) at day 3 and by 30 mM taurine on day 5. However, [^{3}H]leucine incorporation (cpm/μg protein) into newly synthesized protein was not affected by any of the concentrations of taurine tested (Fig. 4).

Effects of Analogues of Taurine and Amino Acids on the Growth of VSMCs

The effects of taurine, GES, and ISA (2-hydroxyethanesulfonic acid) and of alanine (ALA), glycine (GLY), and serine (SER) on the growth of the VSMCs (cell count) are

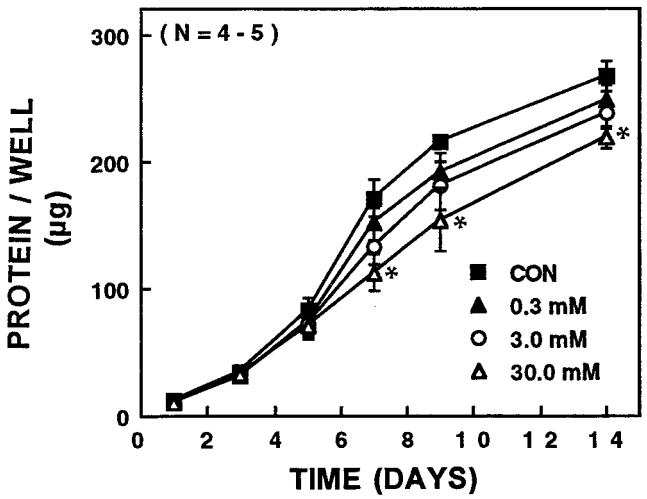

FIG. 2. Concentration-response of three levels of taurine (0.3, 3.0, and 30 mM) on the accumulation of protein in cultures of VSMCs. The data points are presented as means ± SEM of four to five separate experiments. Asterisks denote a significant difference ($P < 0.05$) of the data compared with the corresponding control value.

reported in Table 3. The effects of 20 mM GES and ISA are shown to be inhibitory at days 3, 5, 7, 9, and 14. Taurine was inhibitory at days 3, 5, 7, and 9. GLY (20 mM) had no effect at any time point. However, ALA inhibited cell growth at day 7, while SER had an effect at days 5 and 7.

When the protein content of the VSMCs contained in the wells was determined during the 14-day incubation period, it was demonstrated that 20 mM GES and 20 mM ISA decreased the quantity of protein at days 7, 9, and 14 (Fig. 5; asterisks are omitted in upper panel). ALA had an effect on day 14, and SER had an effect on days 9 and 14. (Fig. 5B; asterisks are omitted in upper panel).

β-Alanine, a close structural analogue of taurine, also was tested in the VSMC system (data not shown). Growth of the VSMCs was inhibited at days 3, 5, 7, 9, and 14 by 20 mM β-alanine. The doubling time of the VSMCs was increased by 13.6% from 28.6 ± 0.3 to 32.5 ± 0.6 hr ($P < 0.05$). The protein content of the VSMCs was reduced by β-alanine on days 5, 7, 9, and 14.

Phenylalanine (20 mM) completely prevented the growth of the VSMCs but appeared not to kill the cells (data not shown). Thus, the cell count on day 14 (20,200 ± 100 cells/well) was the same as at the start of the experiment on day 0 (20,600 ± 350 cells/well). On the contrary, glutamate (20 mM) killed the cells, which was expected (data not shown) since glutamate is a known toxin [19].

Effects of Analogues of Taurine and Amino Acids on the Incorporation of [^{3}H]Thymidine and [^{3}H]Leucine into DNA and Protein of VSMCs

GES, ISA, and taurine at a concentration of 20 mM inhibited the incorporation of [^{3}H]thymidine into DNA at day 3 (Fig. 6A). GES also inhibited [^{3}H]thymidine incorporation on days 1 and 14, while ISA also inhibited

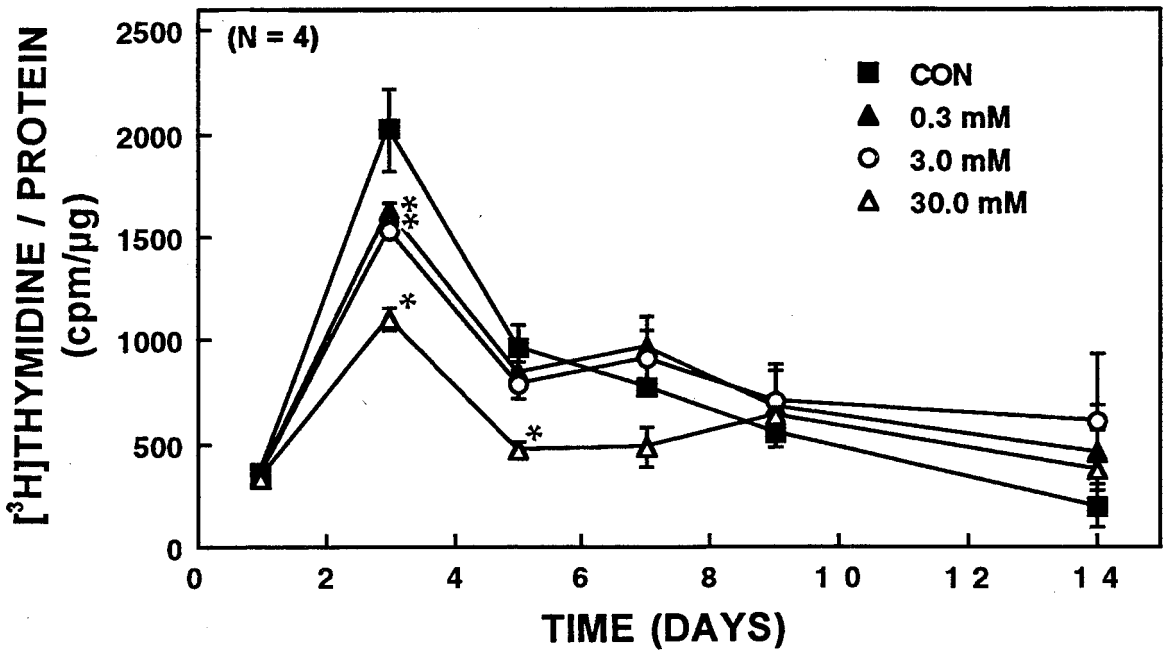

FIG. 3. Concentration-response of three levels of taurine (0.3, 3.0, and 30 mM) on the incorporation of [^{3}H]thymidine into VSMCs during a 14-day growth period. Thymidine incorporation (cpm) was normalized to the quantity of protein (µg) in each well. The data points are presented as means ± SEM of four separate experiments. Asterisks denote a significant difference ($P < 0.05$) of the data compared with the corresponding control value.

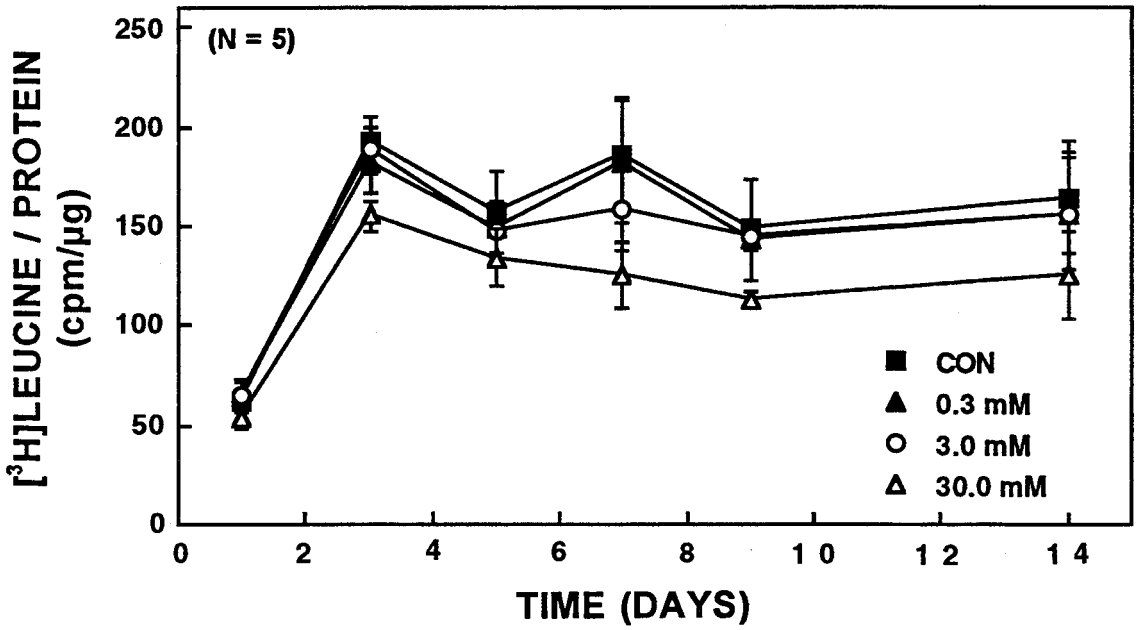

FIG. 4. Concentration-response of three levels of taurine (0.3, 3.0, and 30 mM) on the incorporation of [^{3}H]leucine into VSMCs during a 14-day growth period. Leucine incorporation (cpm) was normalized to the quantity of protein (μg) in each well. The data points are presented as means ± SEM of five separate experiments.

[^{3}H]thymidine incorporation on day 1. [^{3}H]Thymidine incorporation was not affected by ALA, GLY, or SER on day 3 when DNA was maximally synthesized (Fig. 6B).

The incorporation of [^{3}H]leucine into protein was not affected by taurine or any of the taurine analogues (GES, ISA) or the amino acids (GLY, ALA, SER) (Fig. 7, A and B).

β-Alanine (20 mM) inhibited the incorporation of [^{3}H]thymidine into DNA on all days that were measured (1, 3, 5, 7, 9, and 14; data not shown). However, when protein synthesis was measured it was observed that β-alanine had a significantly different effect than taurine or the other amino acids in that [^{3}H]leucine incorporation was diminished at days 5, 7, 9, and 14 (data not shown).

DISCUSSION

Taurine and its analogues inhibited VSMC proliferation as determined by cell count, thymidine incorporation into DNA, and protein content per well. However, they did not

TABLE 3. Effects of 20 mM taurine, taurine analogues, and amino acids on the growth (cell count) of VSMCs during a 14-day period

	Cell count			
Day	Control	GES	ISA	Taurine
1	13,700 ± 507	13,400 ± 1,020	12,800 ± 1,440	11,800 ± 661
3	83,100 ± 8,310	47,700 ± 7,630*	56,400 ± 8,000*	57,700 ± 4,900*
5	272,000 ± 30,300	136,500 ± 18,100*	184,100 ± 9,790*	193,400 ± 17,300*
7	454,800 ± 18,300	222,800 ± 44,400*	298,000 ± 42,900*	284,700 ± 28,900*
9	684,200 ± 26,300	341,300 ± 68,700*	484,500 ± 39,800*	531,200 ± 68,000*
14	1,114,000 ± 71,800	543,800 ± 70,600*	793,500 ± 104,100*	831,100 ± 155,800

	Cell count			
Day	Control	Alanine	Glycine	Serine
1	11,800 ± 520	10,900 ± 712	11,800 ± 947	10,800 ± 500
3	83,600 ± 6,880	80,200 ± 15,100	72,000 ± 15,200	54,100 ± 9,030
5	242,900 ± 13,900	256,400 ± 24,000	276,100 ± 22,000	171,200 ± 15,700*
7	522,300 ± 24,000	406,700 ± 42,600*	473,600 ± 22,900	354,400 ± 15,000*
9	691,900 ± 44,600	574,000 ± 31,700	522,800 ± 105,600	451,300 ± 84,000
14	1,126,000 ± 66,300	984,400 ± 9,710	1,214,000 ± 52,400	1,155,000 ± 51,200

Data are presented as means ± SEM of 3 experiments.
*Significant difference ($P < 0.05$) for each compound on a specific day compared with the corresponding control value.

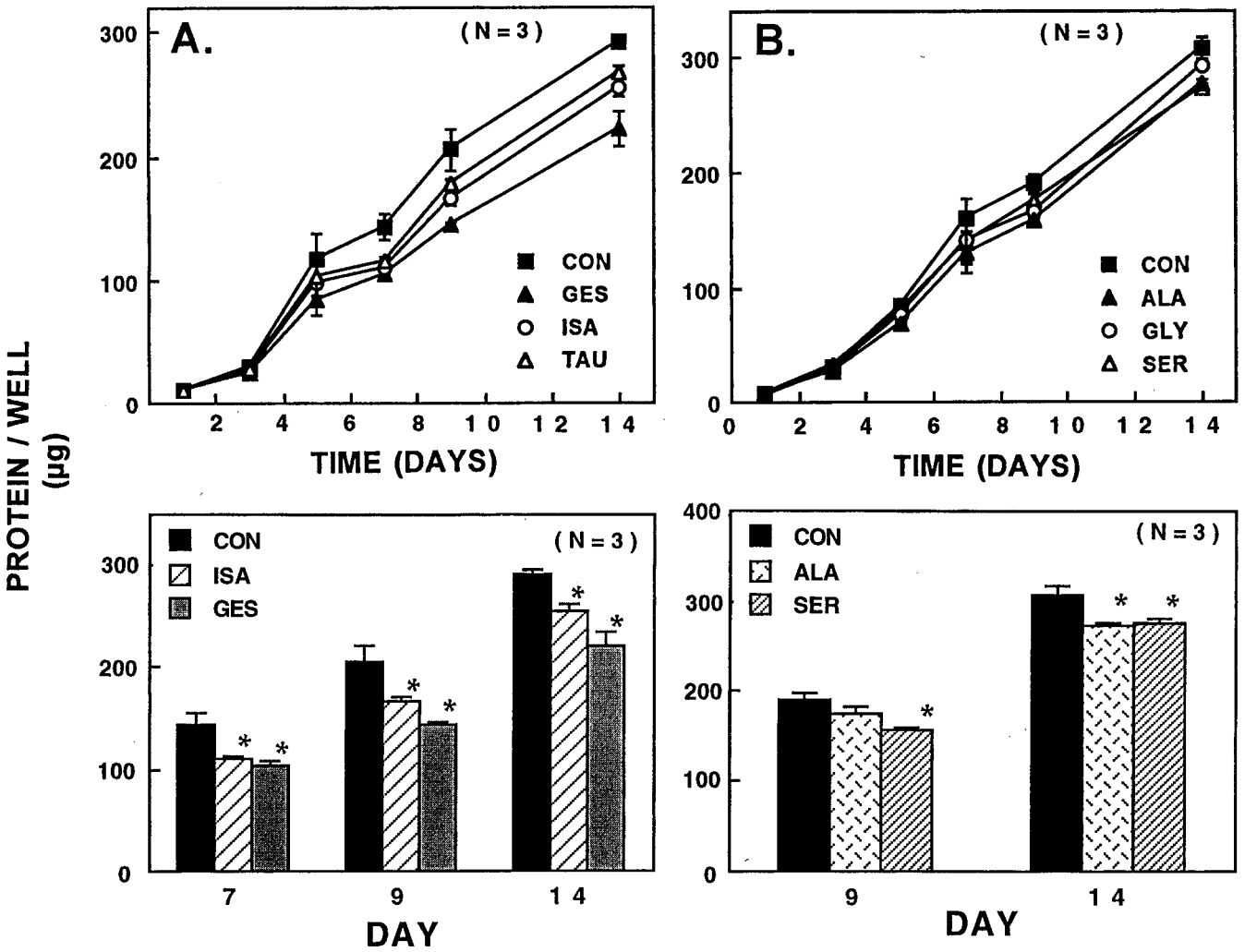

FIG. 5. Effect of 20 mM taurine (TAU), taurine analogues (GES, ISA), and amino acids (ALA, GLY, SER) on the accumulation of protein in cultures of VSMCs. The data points are presented as means ± SEM of three separate experiments. GES and ISA significantly inhibited ($P < 0.05$) the amount of protein per well at days 7, 9, and 14 compared with the corresponding control value. SER was inhibitory at day 9, while SER and ALA were inhibitory at day 14. (Asterisks used for denoting significance are omitted in the upper panels of the figure for clarity.)

appear to inhibit normal cellular protein synthesis. In contrast, β-alanine, a close structural analogue of taurine, was shown to be toxic to VSMCs, inhibiting not only cellular proliferation but also protein synthesis, thereby reducing survivability of VSMCs.

Other amino acids such as ALA, GLY, and SER had significantly lesser effects on the growth of the VSMCs. The testing of these other amino acids in the culture system is an important control for eliminating the effects of ionic strength and changes in osmolarity as a cause of the inhibitory growth effects of taurine, GES, and β-alanine.

Taurine is utilized presently as a nutritional supplement in over-the-counter high energy drinks and in most, if not all, infant formulas manufactured in the United States, Europe, and Japan. The evidence for the requirement of taurine in the human diet comes from many studies involving animal models (rats, cats, and monkeys) (reviewed in Refs. 2, 3, and 20), and a few clinical studies involving children on total parenteral nutrition for long periods (1 year or longer) [21, 22]. It is apparent from these experiments that taurine is an essential amino acid under certain environmental conditions. In addition, taurine is known to have cardioprotective effects and is used currently as a therapeutic agent in patients with congestive heart failure in Japan [23].

The concentration of taurine in animal tissues is reported to be one of the highest of any of the amino acids, attaining millimolar concentrations (25–35 mM in the heart) [1]. Only levels of glutamate and glutamine in mammalian tissues appear to be higher than the levels of taurine [24, 25]. Taurine, when administered physiologically or pharmacologically as a test substance in various animal models, also is used in millimolar concentrations, usually in a range from 10 to 30 mM (reviewed in Refs. 2 and 3). Consequently, because of the high tissue levels of taurine it is assumed that when taurine is added to an organ system at

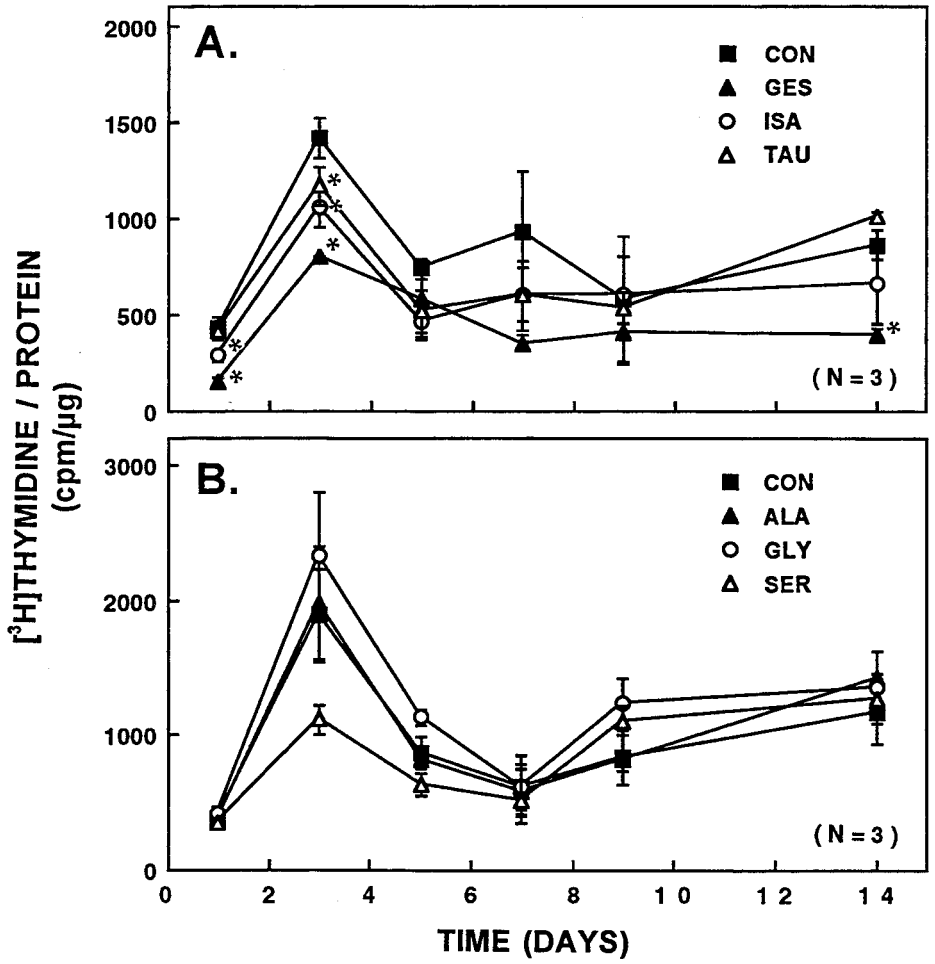

FIG. 6. Effect of 20 mM taurine (TAU), taurine analogues (GES, ISA), and amino acids (ALA, GLY, SER) on the incorporation of [^{3}H]thymidine into VSMCs during a 14-day growth period. Thymidine incorporation (cpm) was normalized to the quantity of protein (μg) in each well. The data points are presented as means ± SEM of three separate experiments. Asterisks denote a significant difference ($P < 0.05$) of the data compared with the corresponding control value.

these high concentrations, it will have no adverse effects. In the current study, while taurine decreased proliferation of VSMCs and [^{3}H]thymidine incorporation at all concentrations studied, it did not inhibit protein synthesis. As such, no direct toxic effect of taurine was observed at the concentrations studied.

GES, the taurine transport inhibitor that many investigators have utilized to deplete animals of their body stores of taurine, is also given to animals in high concentrations ([26]; reviewed in Ref. 3). GES is administered to rats in their drinking water usually at concentrations of 1 to 1.5%. These concentrations are calculated to be 60–90 mM.

As a consequence of taurine depletion through the use of GES, we have demonstrated previously that the phosphorylation of a specific ~20-kDa protein in the rat retina and one of ~44 kDa in the rat heart is increased approximately 2-fold [27, 28]. However, GES also has other effects on animal tissues, such as depleting phosphocreatine levels in cardiac tissue [29] and altering myocardial high energy phosphate metabolism by elevating long chain acylcarnitine content and reducing long chain fatty acyl CoA levels [30]. Consequently, high levels of GES could potentially be toxic.

An alternative compound used to deplete animal tissues of their taurine content, but which does not have an effect on cardiac phosphocreatine levels, is β-alanine [29]. β-Alanine was also demonstrated to inhibit VSMC growth, doubling time, and DNA synthesis (data not shown). However, β-alanine also inhibited protein synthesis as measured by the incorporation of [^{3}H]leucine into protein.

Consequentially, the current study suggests that taurine might be a candidate for use as a nutritional supplement to protect against atherosclerosis. Whether taurine exerts its selective inhibition of proliferation by influencing the cell cycle or perhaps through its antioxidant effect remains to be determined. Future studies are planned to determine if, in fact, taurine can prevent or delay atherosclerosis by inhibiting VSMC proliferation.

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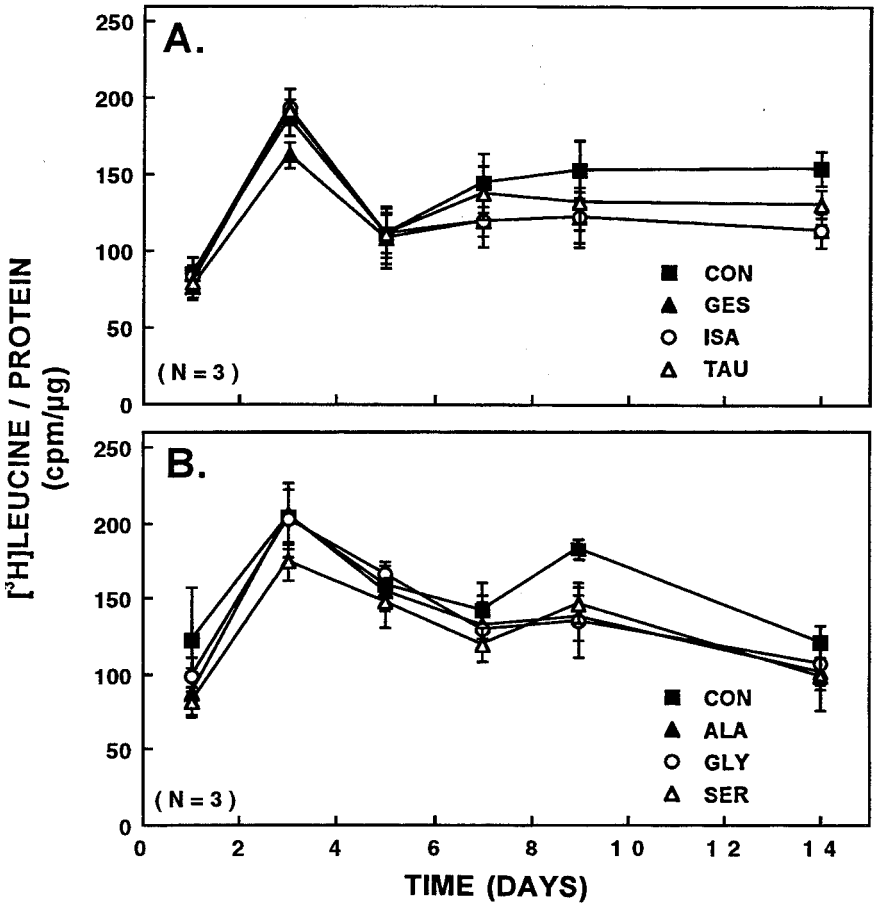

FIG. 7. Effect of 20 mM taurine (TAU), taurine analogues (GES, ISA), and amino acids (ALA, GLY, SER) on the incorporation of [^{3}H]leucine into VSMCs during a 14-day growth period. Leucine incorporation (cpm) was normalized to the quantity of protein (μg) in each well. The data points are presented as means ± SEM of three separate experiments.

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