

Original Article

The Role of L-carnitine in Treatment of a Murine Model of Asthma

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Leukotrienes, one of the mediators of inflammation in asthma, have a strong bronchoconstrictive effect. L-carnitine has been reported to influence respiratory functions. It has also been reported that L-carnitine inhibits leukotriene synthesis. To evaluate the effects of L-carnitine on oxygen saturation, urine leukotriene E4 levels and lung histopathology in a murine model of asthma, high IgE responder BALB/c mice (n = 24) were systemically sensitized to ovalbumin and chronically challenged with low particle mass concentrations of aerosolized ovalbumin, and then they were divided into 3 groups (study groups A, B, and C) each including eight mice. After methacholine-induced bronchoconstriction, the mice in groups A and B were given intraperitoneal L-carnitine (250 and 125 mg/kg, respectively), while the mice in group C were given placebo. Oxygen saturation of the mice was measured by pulse oxymeter before and after methacholine and after L-carnitine/placebo application. In addition, urine leukotriene E4 levels were measured before asthma development, and 24-h after L-carnitine injection in asthmatic mice. Inflammation in the lung tissues of the sacrificed animals was scored histopathologically to determine the effect of L-carnitine on tissue level. A control group of non-sensitized mice (n = 8) treated with placebo only was used for comparison of urine leukotriene E4 levels and of histopathological parameters. Oxygen saturation of the mice in the study groups tended to decrease after methacholine and to improve after L-carnitine injection, although these changes were not significant at all time points. Urine leukotriene E4 levels of all 3 study groups increased significantly after asthma development. The rate of increment was smallest in the group given the highest L-carnitine dose (group A). Inflammation at the tissue level was also mildest in group A, and severest in the group that was not given carnitine (group C). All of the study groups and the control group differed significantly with respect to inflammation scores. In conclusion, L-carnitine improved oxygen saturation, and decreased urine leukotriene E4 levels and inflammation in lung tissues in the present murine model of asthma.

Key words: asthma, L-carnitine, leukotriene E4, oxygen saturation

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Asthma is a chronic inflammatory disease of the airways [1]. Various endogenous mediators play a role in inflammation. Leukotrienes are among the mediators which are synthesized in the bronchial mucosa by eosinophils, basophils and mast cells. Leukotrienes

play a very important role in asthma pathogenesis, and are involved in eosinophilic inflammation, bronchoconstriction and edema formation [2].

L-carnitine has been reported to improve the obstructive findings in pulmonary function tests in children undergoing chronic hemodialysis [3]. It has also been reported that L-carnitine decreases leukotriene synthesis by inactivation of lipoxygenase in hemodialysis patients [4]. We previously evaluated the bronchodilator effect of L-carnitine in tracheal and bronchial smooth muscle of Guinea pigs and in human bronchial smooth muscle *in vitro*, but observed no significant effects. However, since these tissues were taken from healthy subjects without asthma, this lack of significance does not exclude a possible relation between asthma and L-carnitine [5].

The aim of this study was to evaluate the effects of L-carnitine on arterial oxygen saturation (SaO₂), urine leukotriene E₄ (LTE₄) levels, and lung histopathology in a murine model of asthma.

Materials and Methods

Experimental animals. BALB/c mice with 87% homogeneity were used for the experiment. The mice were 8- to 10-weeks-old and weighed 28-30 g. They were kept in hygienic macrolane cages and in air-conditioned rooms under a 12-h light/12-h dark cycle. Thirty-two mice were divided into 4 groups, the study groups A, B, C and the control group, each including eight mice. The study was approved by the local ethical committee (00/09-02).

Sensitization and Inhalational Exposure. BALB/c mice are high IgE responders to ovalbumin [6]. The mice in the study groups A, B, and C were sensitized by intraperitoneal injection of 10 μ g of alum precipitated chicken egg ovalbumin (grade V, \geq 98% pure, Sigma, St. Louis, MO, USA) 21 and 7 days before inhalational exposure. The mice in the control group were given saline solution by the same route and dosage.

The mice in the study groups were then exposed to aerosolized ovalbumin for 30 min per day on 3 days of the week up to 8 weeks [6]. Exposures were carried out in a whole body inhalation exposure system. Temperature and relative humidity were maintained at 20-25°C and 40-60%, respectively. A solution of 2.5% ovalbumin in normal saline was aerosolized by delivery of compressed air to a sidestream jet nebulizer and injected into a

chamber. The aerosol generated by this nebulizer comprised \geq 80% particles with a diameter of \leq 4 μ m. Particle concentration was maintained in the range of 10-20 mg/m³. The mice in the control group were exposed to saline inhalation by the same system.

Methacholine and L-carnitine Administration. Mice with experimentally induced chronic asthma in the study groups A, B and C were given 3 doses of methacholine (at 6.25-12.5 and 25.0 mg/ml concentrations) for 3 min by the same system used for administration of aerosolized ovalbumin [7]. The time interval between the methacholine doses was 1h. SaO₂ was measured by pulse oxymeter just before (0 min) and 5 min after each dose of methacholine. After that, while intraperitoneal L-carnitine was given at 250 mg/kg and 125 mg/kg to the mice in groups A and B, respectively, the mice in group C were not given L-carnitine. Instead, isotonic saline as placebo was injected by the same route to the animals in group C [8]. A third measurement of SaO₂ was also obtained 15 min after L-carnitine/placebo administration in these 3 study groups.

The control group of mice without asthma were not treated with either methacholine or carnitine. Thus, the responses to methacholine and carnitine, as determined by SaO₂, were compared among the 3 study groups of asthmatic mice.

Measurement of Urinary Leukotriene E₄. Twenty four-hour urine samples of the asthmatic mice in the study groups were collected twice, once before the induction of asthma and once after L-carnitine administration, by using metabolic cages. Similarly, 24-h urine samples of the control mice were collected at the beginning of the study and just before sacrifice. The samples were separated into 2 aliquots. The first aliquot was used to measure urinary creatinine levels as the concentration of picric acid complex in alkaline media using a routine spectrophotometric method (Hitachi 911 autoanalyzer; Hitachi, Tokyo, Japan). The second aliquot was supplemented with 6N hydrochloric acid in order to acidify the urine to pH 3 for measurement of LTE₄, then stored at -70°C till analysis. Urine samples were purified using C₁₈ reverse phase cartridges (500 mg/8.0 ml) (Altech Associates, Inc. 2051 Waukegan Road, Deerfield, IL, USA). During purification the samples were traced with tritium-labeled LTE₄ ([³H]-LTE₄) (Dupont-NE, Boston, MA, USA) with the aim of calculating the recovery factor for LTE₄ [9, 10]. In purified samples LTE₄ levels were determined using an LTE₄ EIA (Enzyme

Immunoassay) kit (Cayman Chemical Company, Ann Arbor, MI, USA). The results were expressed as pg LTE4/mg creatinine in urine.

Histopathologic Examinations. The mice in the study groups were sacrificed 24-h after the last dose of carnitine/placebo. Control mice were sacrificed at the same time as the experimental mice. The trachea and lungs of the mice were inflated with 10% buffered formalin, and after fixation overnight, a horizontal slice was obtained from the left lung. The slices were embedded in paraffin, sectioned into 5- μ m thick sections, and stained with hematoxylin-eosin. The degree of bronchial inflammation was evaluated semi-quantitatively using scores of 0-3 to indicate no, mild, moderate, and severe inflammation [6, 11]. In addition, the distribution and intensity of the following findings were recorded: 1) bronchoconstriction (epithelial shedding or undulation of the nuclei of bronchial epithelial cells); 2) increase in the number of goblet cells; 3) infiltration of inflammatory cells and fibrin from vessels into the mucosal and submucosal area of the bronchus and peribronchial interstitium; and 4) hypertrophy and thickening of the smooth muscle cell layer. A score of 0 indicated normal histology and a score of 3 indicated the greatest degree of alteration from normal.

Statistical Evaluation. Paired *t*-test (for $n \geq 6$) and Wilcoxon's signed rank test (for $n \leq 5$) were used for the comparison of SaO₂ before and after various methacholine dosages and after L-carnitine administration. Levels of significance was accepted as < 0.05 . Urinary LTE4 levels were compared within each group at the beginning and at the end of the study by paired *t*-test ($n \geq 6$) and Wilcoxon's signed rank tests ($n \leq 5$). Mean increase in LTE4 level was analyzed by one way ANOVA and post-hoc Duncan tests. Histopathological scores were compared among the four groups by Kruskal-

Wallis test. Advanced analyses of the groups were performed by Mann-Whitney *U* test after Bonferroni correction.

Results

A total of 4 mice, 1 in group A and 3 in group B, died due to hypoxia during the study period.

Response to Methacholine and L-Carnitine. SaO₂ measured before and after methacholine administration in all 3 study groups and after L-carnitine administration in groups A and B or placebo in group C are shown in Tables 1 to 3. SaO₂ was found to decrease after all methacholine doses in all 3 groups. However, the decrease in SaO₂ was not always statistically significant. On the other hand, SaO₂ had a tendency to increase after L-carnitine administration, and this increase was significant at the 250 mg/kg dose (in group A).

SaO₂ decreased significantly after administration of a 6.25 mg/ml dose of methacholine ($P = 0.014$), while it increased significantly after administration of 250 mg/kg L-carnitine in group A ($P = 0.027$) (Table 1). Similarly, administration of 25 mg/ml methacholine or 250 mg/kg L-carnitine led to a significant decrease ($P = 0.014$) and a significant increase ($P = 0.012$) in SaO₂, respectively (Table 3).

In the case of group B, 6.25 mg/ml of methacholine decreased SaO₂ significantly ($P = 0.007$) and 125 mg/kg of L-carnitine increased SaO₂, but not significantly (Table 1). Similar results were obtained in this group when the mice were given 12.5 and 25 mg/ml of methacholine (Tables 2 and 3).

In group C, SaO₂ decreased at all doses of methacholine, but the decrease was only significant after a dose of 6.25 mg/ml (Table 1, $P = 0.034$). However, SaO₂ did not tend to increase after placebo administration

Table 1 Oxygen saturation of the mice in groups A, B and C before and after methacholine (6.25 mg/ml) and after L-carnitine/placebo administration

SaO ₂	0 min (before methacholine)	5 min after methacholine	15 min after L-carnitine/placebo*
Group A (n=7)	86.87 \pm 10.43	64.25 \pm 19.08 ^a	85.37 \pm 14.84 ^b
Group B (n=5)	89.57 \pm 5.38	65.71 \pm 11.26 ^c	71.16 \pm 9.06
Group C (n=8)	86.85 \pm 6.36	81.28 \pm 4.57 ^d	80.71 \pm 5.36

*, Group A and B were given 250 and 125 mg/kg L-carnitine, respectively; group C was given placebo. ^a, SaO₂ significantly decreased after methacholine ($P = 0.014$); ^b, SaO₂ significantly increased after L-carnitine ($P = 0.027$); ^c, SaO₂ significantly decreased after methacholine ($P = 0.007$); ^d, SaO₂ significantly decreased after methacholine ($P = 0.034$).

Table 2 Oxygen saturation of the mice in groups A, B and C before and after methacholine (12.5 mg/ml) and after L-carnitine/placebo administration

SaO ₂	0 min (before methacholine)	5 min after methacholine	15 min after L-carnitine/placebo*
Group A (n=7)	85.57 ± 12.36	73.57 ± 15.67	85.10 ± 3.60
Group B (n=5)	87.00 ± 9.29	78.33 ± 9.77 ^a	84.20 ± 14.72
Group C (n=8)	78.71 ± 23.22	68.42 ± 30.81	70.03 ± 15.52

*, Group A and B were given 250 and 125 mg/kg L-carnitine, respectively; group C was given placebo. ^a, SaO₂ significantly decreased after methacholine ($P=0.026$).

Table 3 Oxygen saturation of the mice in groups A, B and C before and after methacholine (25 mg/ml) and after L-carnitine/placebo administration

SaO ₂	0 min (before methacholine)	5 min after methacholine	15 min after L-carnitine/placebo*
Group A (n=7)	85.01 ± 6.09	65.14 ± 19.78 ^a	84.57 ± 11.70 ^b
Group B (n=5)	72.50 ± 9.29	75.75 ± 10.78	79.25 ± 4.11
Group C (n=8)	82.00 ± 12.02	74.47 ± 10.65	72.33 ± 9.48

*, Group A and B were given 250 and 125 mg/kg L-carnitine, respectively; group C was given placebo. ^a, SaO₂ significantly decreased after methacholine ($P=0.014$); ^b, SaO₂ significantly increased after L-carnitine ($P=0.012$).

Table 4 Pre-and post-asthmatic (after a 25 mg/ml dose of methacholine) urinary leukotriene E₄ levels in mice

Groups	Urine Leukotriene E ₄ Levels (Mean ± SD) (pg LTE ₄ /mg creatinine)		
	Pre asthmatic	Post asthmatic (after L-carnitine) ^a	<i>P</i>
A (n=7)	0.64 ± 0.52	12.75 ± 8.80	0.018
B (n=5)	3.53 ± 2.86	20.85 ± 11.89	0.018
C (n=8)	10.71 ± 5.03	55.03 ± 11.15	0.028
Control Group ^b (n=8)	2.76 ± 2.50	11.36 ± 3.73	0.042

^a, The mice in group A were given 250 mg/kg L-carnitine, and those in group B were given 125 mg/kg of L-carnitine. The mice in group C and the control group were not given L-carnitine. ^b, Urine LTE₄ levels were studied at the beginning and end of the study in control group.

(Tables 1-3).

Urinary Leukotriene E₄ Levels. Intragroup evaluation revealed significantly higher levels of urinary LTE₄ after asthma development in all study groups (Table 4). After that, the difference between the post-and pre-asthmatic urinary LTE₄ levels was calculated for each group. The mean differences were 12.11 ± 8.49, 17.32 ± 11.02, 44.32 ± 06.12 and 8.59 ± 2.59 pg LTE₄/mg creatinine in groups A, B, C and the control group, respectively. When all 4 groups were analyzed with respect to these differences, group C had statistically significantly higher levels compared to the others ($P=0.000$).

Histopathological Changes. The mean scores with respect to bronchial inflammation were 1.14 ± 0.37, 1.75 ± 0.88, 1.77 ± 0.75 and 0.33 ± 0.5 in groups A, B, C and the control group, respectively. The intergroup variation was significant ($P=0.005$). In post-hoc analyses, the control group was found to be significantly different from groups B and C, but not from group A ($P=0.008$, $P=0.008$ and $P=0.035$, respectively).

Lung sections in group A mice revealed mild inflammation in peribronchial and perivascular areas. In addition, there was mild constriction and thickening of the walls in a small number of bronchi (Fig. 1). Vascular

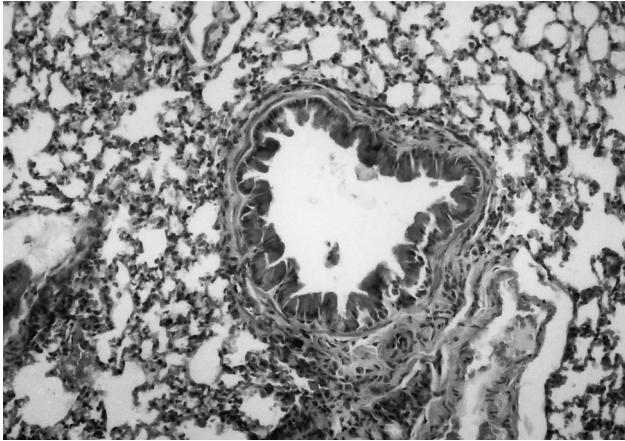


Fig. 1 Peribronchial mild inflammation (H&E \times 100, group A).

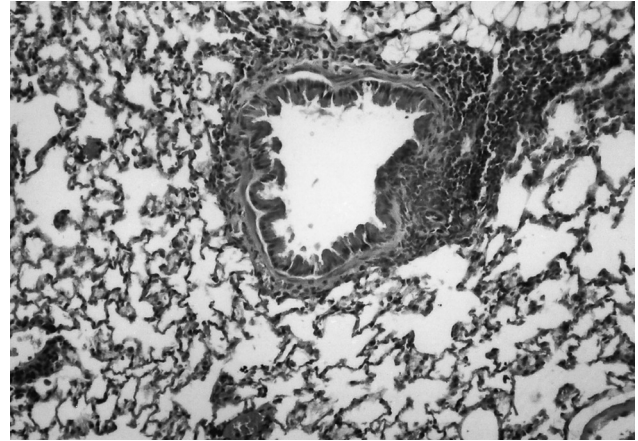


Fig. 2 Peribronchial moderate inflammation (H&E \times 100, group B).

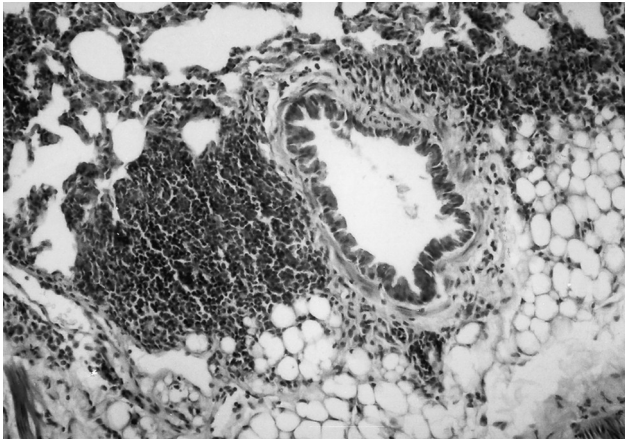


Fig. 3 Peribronchial intense inflammation and thickening of the bronchial walls (H&E \times 40, group C).

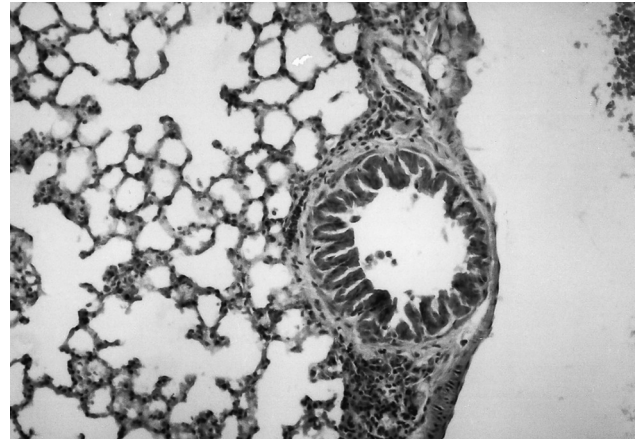


Fig. 4 Bronchoconstriction and mild peribronchial inflammation (H&E \times 40, group C).

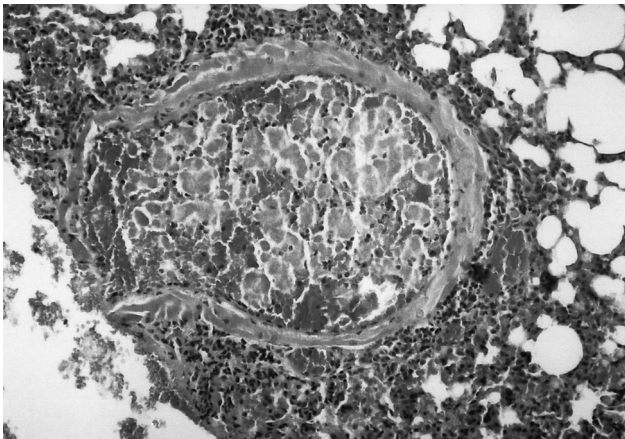


Fig. 5 Thickening of and fibrin deposition in the vessel walls, and perivascular mild inflammation (H&E \times 400, group C).

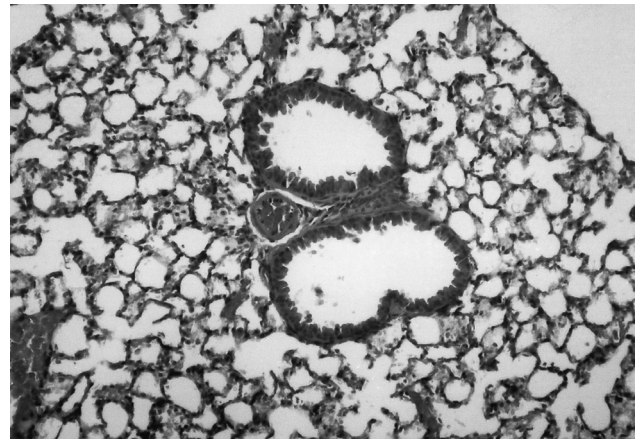


Fig. 6 Mild focal peribronchial and perivascular inflammation (H&E \times 40, control group).

changes like thickening of vessel walls and fibrin deposition were mild and present in a small number of vessels. Bronchial inflammation was moderate in group B mice (Fig. 2). Bronchoconstriction and perivascular inflammation were also more pronounced in group B than in group A mice. Thickening of bronchial and vascular walls were not different between these 2 groups.

Group C mice showed severe peribronchial inflammation, and the other histopathological changes, including bronchoconstriction (Fig. 3) and perivascular inflammation (Fig. 4), were also more pronounced in this group. In addition, the vessel walls of group C mice showed thickening and fibrin deposition (Fig. 5). There were no histopathologic changes other than mild focal inflammation in the lung tissues of the control mice (Fig. 6). None of the groups showed any increase in the number of goblet cells.

Discussion

Leukotrienes, a product of the lipoxygenase pathway, bind to the leukotrien receptors present in bronchial smooth muscle and are found in increased concentrations in the bronchoalveolar fluid of asthmatic patients [12, 13, 14].

In hemodialysis patients, the metabolism of arachidonic acid has been shown to shift from the cyclooxygenase to the lipoxygenase pathway [4]. When L-carnitine was administered to children undergoing hemodialysis, the obstructive pattern in respiratory functions was shown to improve [3]. Carnitine was demonstrated to prevent bronchospasm due to Cys LT by blocking the lipoxygenase pathway in these patients. L-carnitine causes partial restoration of the depleted essential fatty acids (linoleic and linolenic acids) observed in untreated dialysis patients [4]. Although the mechanism by which carnitine corrects these abnormalities is unclear, it has been shown that dietary sources of alpha-linolenic acid may have the capacity to inhibit the generation of leukotrienes by leucocytes in patients with asthma [15]. Thus, carnitine might act on leukotriene metabolism by altering the ratio of essential fatty acids.

In a previous *in vitro* study, we showed that L-carnitine did not affect bronchoconstriction caused by methacholine in guinea pig tracheal and bronchial smooth muscle, or in human bronchial smooth muscle. However, since there was no asthma pathology in the smooth muscles in that study, it was speculated that L-carnitine

did not affect the bronchial smooth muscles under physiologic conditions [5].

We performed this study to investigate the effects of L-carnitine on acute attacks in a chronic asthma model in mice, and whether leukotriene synthesis inhibition was involved in these effects, if any. In this study, acute attacks were induced by methacholine after the chronic asthma model was developed, and the effect of L-carnitine on SaO₂, as an indirect indicator of bronchoconstriction, during acute attacks was observed. In addition, the role of L-carnitine on leukotriene synthesis was evaluated by measuring urinary LTE4 levels. Moreover, the effect of L-carnitine on acute attacks in chronic asthma was investigated at the tissue level.

Post-methacholine SaO₂ decreased, although not significantly at all times, in the study groups (Tables 1-3). This finding indicates indirectly that there is bronchial hyperreactivity and inflammation in these animals with acute bronchospasm. After L-carnitine administration, SaO₂ increased significantly in group A (given 250 mg/kg L-carnitine), but increased insignificantly in group B (given 125 mg/kg L-carnitine). These findings show that higher doses of L-carnitine had a more pronounced bronchodilator effect in this chronic murine asthma model.

LTE4 is the major metabolite of leukotriene metabolism and is excreted in urine. While other leukotriene metabolites are rapidly metabolized *in vivo*, LTE4 is more stable. Thus, LTE4 levels are often used as a marker of *in vivo* leukotriene production [16], as they were here. We measured urine LTE4 levels before the development of asthma and after L-carnitine administration in asthmatic mice. Ideally, we would also have measured LTE4 levels in asthmatic mice before carnitine treatment. Unfortunately, we were unable to do this due to the limited materials for LTE4 measurement. However, we found significantly increased levels of urine LTE4 even after L-carnitine administration in asthmatic mice in all study groups. We then calculated the mean differences between the post- and pre-asthmatic urine LTE4 levels for each group. The control group showed the lowest difference (8.59 ± 2.59 pg LTE4/mg creatinine), followed in increasing order by group A (12.11 ± 8.49), group B (17.32 ± 11.02) and group C (44.32 ± 06.12). These findings indicated that L-carnitine treatment decreased urinary LTE4 excretion, and suggested that L-carnitine might be effective in reducing the inflammatory process in asthma.

There were statistically significant differences among

the 4 groups with respect to the intensity of bronchial inflammation. The lowest inflammatory scores were in the control group (0.33 ± 0.5) and group A (1.14 ± 0.37). These 2 groups did not differ significantly. On the other hand, the scores in group B (1.75 ± 0.88) and group C (1.77 ± 0.75) were significantly different from those in the control group. Peribronchial and perivascular inflammation was significantly lower in group A than in group B or group C, which had the highest scores. As such, bronchoconstriction and thickening of bronchial walls were prominent in group C, but were present in only a small number of bronchi in group A. Likewise, thickening of vessel walls and fibrin deposition were mild in group A and extensive in group C. These findings show that the histopathological changes of asthma were attenuated in the mice given L-carnitine (groups A and B), and were particularly small in group A, which was given the higher dose of L-carnitine. These findings, in turn, support our hypothesis that L-carnitine has bronchodilator and anti-inflammatory properties.

In conclusion, intraperitoneal administration followed by aerosolized ovalbumin resulted in asthma development in BALB/c mice, and while methacholine administration decreased SaO_2 , L-carnitine administration increased SaO_2 in these asthmatic mice. In addition, urinary LTE4 levels and histopathological injury scores were lower in the asthmatic mice given L-carnitine. These results suggest that L-carnitine might have a role in the treatment of experimentally induced asthma in mice. Additional, larger-scale studies will be needed to confirm the effectiveness of L-carnitine on inhibition of leukotrienes.

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