Creatine Supplementation Exacerbates Allergic Lung Inflammation and Airway Remodeling in Mice

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Creatine supplement is the most popular nutritional supplement, and has various metabolic functions and sports medicine applications. Creatine supplementation increases muscle mass and can decrease muscular inflammation. Some studies have also suggested a beneficial role of creatine supplementation on chronic pulmonary diseases such as chronic obstructive pulmonary disease and cystic fibrosis. Among athletes, the prevalence of asthma is high, and many of these individuals may be taking creatine. However, the effects of creatine supplementation on chronic pulmonary diseases of allergic origin have not been investigated. In the present study, we analyzed the effects of creatine supplementation on a model of chronic allergic lung inflammation. Thirty-one Balb/c mice were divided into four groups: control, creatine (Cr), ovalbumin (OVA), and OVA+Cr. OVA and OVA+Cr groups were sensitized with intraperitoneal injections of OVA on Days 0, 14, 28, and 42. OVA challenge (OVA 1%) and Cr treatment (0.5 g/kg/d) were initiated on Day 21 and lasted until Day 53. We determined the index of hyperresponsiveness, the serum levels of OVA-specific immunoglobulin (Ig)E and IgG_1, and the total and differential cell counts in bronchoalveolar lavage fluid. We also quantified airway inflammation, and the airway density of IL-4+, IL-5+, IL-2+, IFN-γ+, and insulin-like growth factor (IGF)-1+ cells, collagen and elastic fibers, and airway smooth muscle thickness. Our results showed that creatine in OVA-sensitized mice increased hyperresponsiveness; eosinophilic inflammation; airway density of IL-4+, IL-5+, and IGF-1 inflammatory cells; airway collagen and elastin content; and smooth muscle thickness. The results show that creatine supplementation exacerbates the lung allergic response to OVA through a T helper cell type 2 pathway and increased IGF-1 expression.

Keywords: creatine supplementation; asthma; lung inflammation; Th2 cytokines; insulin-like growth factor-1

CLINICAL RELEVANCE

We show for the first time that creatine supplementation exacerbates the effects of ovalbumin sensitization (airway inflammation, remodeling, and hyperresponsiveness) in mice, suggesting that creatine might have deleterious effects in individuals with asthma.

Creatine is considered a nutritional supplement and is synthesized from three amino acids: arginine, glycine, and methionine. It can be produced endogenously by the liver and kidneys or ingested from exogenous sources such as meat and fish (1, 2). Creatine is a precursor molecule for adenosine triphosphate, which is the basic substrate for intracellular energy generation (3). Cells take creatine from the bloodstream through a creatine transporter and store it as free creatine and its phosphorylated form, creatine phosphate (4). Previous studies demonstrated that creatine supplementation increases the intracellular content of both free creatine and creatine phosphate (5–7).

Initially, creatine supplementation was used mainly by athletes and recreational sportsmen who wanted to increase strength and muscle mass (8–10). In the 1990s creatine became one of the most popular sport supplements, reaching in 1998 global investments of $200 million by the sport supplements industry (11, 12). It is now known that creatine can decrease muscular inflammation after overtraining and overuse, being used in sports medicine practices to improve muscular recovery after training (10, 13, 14). Creatine supplementation given to long-distance endurance runners reduces markers of cell death, such as creatine kinase and lactate dehydrogenase, and decreases muscle soreness and proteolysis, suggesting a positive effect of creatine in maintaining muscle integrity after intense prolonged exercising (14).

Recent data have demonstrated that creatine has important metabolic functions such as on myocardial stability (15, 16) and on normal cerebral development and function (17, 18), as well as a role in the treatment of neuromuscular diseases (19, 20), suggesting that creatine applications are not necessarily limited to sports practices. Creatine supplementation has also important anti-ischemic effects on the brain tissue, such as a decrease in infarct volume after transient focal cerebral ischemia in mice (21), and inhibition of apoptosis in the striatal ischemic core via a decrease in activation of caspase cell-death pathways (22). Due to its effects on muscular performance, creatine has been administered to patients with chronic diseases, including heart diseases and myopathies (16, 19). Some studies have also suggested a beneficial role of creatine supplementation on chronic obstructive pulmonary diseases, improving health status and general well being (23, 24).

Asthma is a chronic inflammatory disease with a high prevalence in the general population, around 10% (25). Among professional and nonprofessional athletes, the estimated prevalence of asthma is even higher, ranging from 3 to 50%, depending on the sport training environment and the presence of atopy (26). Among adolescents, one of the largest groups of sport supplements consumers, asthma prevalence can be as high as 30% (27–29). Many of these athletes and adolescents are probably taking creatine. However, the effects of creatine supplementation on this population remain unknown.

Taking into account that the uses of creatine in sports medicine are based not only on its effects on muscle mass gain,

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but also on its anti-inflammatory muscular action, and also considering its beneficial role on chronic obstructive pulmonary diseases, we hypothesized that creatine could also have some beneficial effect on chronic pulmonary inflammatory diseases of allergic origin, such as asthma. Furthermore, since professional and nonprofessional athletes with asthma might be using creatine supplementation, we believe it is important to investigate the effects of creatine in this population. Therefore, in the present study, we analyzed the effects of 32 days of oral creatine supplementation on lung inflammatory response in a model of chronic allergic lung inflammation in sensitized mice.

MATERIALS AND METHODS

This study was approved by the review board for human and animal studies of the School of Medicine of the University of Sao Paulo, process number 503/05. All animals in the study received humane care in compliance with the "Guide for care and use of laboratory animals" (NIH publication 85–23, revised 1985).

Experimental Groups, Antigen Sensitization, and Creatine Treatment

Thirty-one male Balb/c mice (20–25 g) were divided into four groups: control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$). OVA and OVA+Cr groups were sensitized using an intraperitoneal injection of OVA (10 mg) diluted in 3 ml of sterile saline and 2 ml of alum hydroxy solution (200 μl/mouse, corresponding to 400 μg of OVA/mouse) on Days 0, 14, 28, and 42. From Day 21 on, OVA and OVA+Cr groups received aerosolized OVA (1 g OVA in 100 ml of sterile saline) three times per week until Day 53. The oral creatine supplement (0.5 g/kg/mouse/d, five times per week) was diluted in sterile water (total volume 50 μl per animal, via gavage), started at Day 21 and lasting until Day 53. Control and Cr groups were submitted to the same protocol using sterile saline.

Airway Hyperresponsiveness

Seventy-two hours after the last OVA or saline inhalation, responsiveness to methacholine (MCh) was assessed in conscious mice using a flow whole body plethysmograph (FWBP) (Buxco Europe, Winchester, UK), as previously described (30–32). Briefly, the unrestrained, spontaneously breathing mice were placed in the main chamber of the FWBP. The box pressure in the FWBP was recorded and it was proportional to the flow into and out of the chamber through a pneumotachograph due to animal's respiration. To estimate the flow and volume units from the box pressure signal, the plethysmograph was calibrated by injecting 1 ml of air before the measurements. Expiratory (TE) and relaxation (TR, defined as the time until 36% of the total box pressure decay during expiration) times, and peak inspiratory and expiratory pressures were extracted from the box pressure recordings. Peak expiratory (PEF) and inspiratory (PIF) flows were calculated from peak inspiratory and expiratory pressures, respectively (30). Enhanced pause (Penh) values were calculated according to the formula:

$$Penh = \frac{PEF}{PIF} \times \left(\frac{TE}{TR} - 1\right)$$

Penh has a theoretical relationship with airway obstruction (30–32). Therefore, airway hyperresponsiveness (AHR) was assessed using calculated values of Penh as an index. Baseline measurements were taken and averaged for 3 minutes after acclimation of the animals to the FWBP. Mice were exposed to nebulized physiologic saline for 3 minutes and then to increasing concentrations of nebulized methacholine (6.25, 12.5, 25, 50 mg/ml), by use of an ultrasonic nebulizer. Measurements were obtained for 5 minutes (3 min during nebulization and 2 min after). Penh values were calculated and averaged approximately every 25 breaths, and the cumulative values were averaged and expressed for each concentration (30–32).

Total and Differential Bronchoalveolar Lavage Fluid Cell Counts

Approximately 3 hours after hyperresponsiveness data was collected, the animals were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (40 mg/kg), tracheostomized, and cannulated for bronchoalveolar lavage fluid (BALF) collection. BALF samples (1 ml) were collected after washing the lungs with 1.5 ml of sterile saline. BALF samples were centrifuged at 800 rpm for 10 minutes at −10°C, supernatant was stored at −70°C, and the cell pellet was resuspended in sterile saline. Total cell count was performed in Laser Blood Cell Counter (Model JXJ-402 Automatic; Shanghai Sichuang Instrument Co., Shanghai, China). Differential cell count was performed by microscopic examination of BALF samples prepared in cytocentrifuge slides, stained with May-Grünwald-Giemsa, and 300 cells were counted per slide (33).

Passive Cutaneous Anaphylaxis to OVA-Specific IgE and IgG_1

Blood samples were collected from the cava vein and serum was obtained for the measurement of titers of anaphylactic OVA-specific immunoglobulin (Ig)E and IgG_1 antibodies by the passive cutaneous anaphylaxis (PCA) technique, as previously described (34, 35). Briefly, an aliquot of 0.1 ml of serum was submitted to different dilutions and injected intradermally into a naive Wistar rat. After passive sensitization (46 h for OVA-specific IgE), these rats were challenged with an intravenous injection of 1 ml 0.9% NaCl solution containing 1 mg of OVA and 10 mg of Evans blue dye. One hour later, rats were killed and the diameters of the blue spots on the inner surface of the flayed skin were measured. For OVA-specific IgG_1 analysis, 0.1 ml of serum was incubated for 3 hours at 56°C to neutralize IgE antibodies. These samples were submitted to different dilutions and injected intradermally into naive Balb/c mice. After passive sensitization (24 h for OVA-specific IgG_1) these Balb/c mice were challenged and analyzed as described above for Wistar rats. IgE and IgG_1 PCA titers were defined as the highest dilution that presented a blue spot of at least 10 mm in diameter.

Airway Inflammation and Remodeling

Four-micrometer thick slides of lung tissue were stained with hematoxylin and eosin for routine histologic analysis, with Luna staining for eosinophils (36, 37), with Weigert's resorcin fuchsin with oxidation for elastic fibers and with picrosirius for collagen fibers (38, 39). Positive inflammatory cells for IL-2, -4, and -5, IFN-γ, and insulin-like growth factor 1 (IGF-1) were assessed with immunohistochemistry. Sections were deparaffinized and a 0.5% peroxidase in methanol solution was applied for 10 minutes to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 30 minutes. Sections were incubated with anti–IL-4 (1:500), anti–IL-5 (1:800), anti–IL-2 (1:400), anti–IFN-γ (1:800) and anti–IGF-1 (1:800) (all from Santa Cruz, Santa Cruz, CA) and left overnight at 4°C. An ABC Vectastain Kit (Vector Elite PK-6105; Vector Laboratories, Burlingame, CA) was used as secondary antibody and 3,3 Diaminobenzidine (Sigma Chemical Co., St. Louis, MO) was used as chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany).

Conventional morphometry was used to determine the airway density of eosinophils, mononuclear cells, and IL-4–, IL-5–, IL-2–, IFN-γ–, and IGF-1–positive inflammatory cells. We also determined the volume proportion of collagen and elastic fibers in airway walls, airway smooth muscle thickness, and a bronchoconstriction index. Using a 100-point grid with a known area (10,000 μm^2 at ×1,000 magnification) attached to the microscope ocular, the number of points hitting the outer area of the airway wall (located between the external limit of smooth muscle layer and the adventitia) was computed. The airway area in each field was calculated according to the number of points hitting the airway, as a proportion of the total grid area. The number of eosinophils, mononuclear cells, and IL-4–, IL-5–, IL-2–, IFN-γ–, and IGF-1–positive cells within that airway wall area was counted. For each cell type, cell density was determined as the number of positive cells in each field, divided by tissue area. Measurements were expressed as cells/μm^2. The results were then transformed to cells/mm^2 by adjusting the units (38, 39). Counting was performed in five airways per animal and five fields per airway, at a ×1,000

magnification. The volume proportion of collagen and elastic fibers in airway walls was calculated as a proportion of number of points hitting collagen or elastic fibers and the number of points hitting the airway wall. Measurements were performed in five airways per animal and five fields per airway, at a ×1,000 magnification, and the results were expressed as percentage (%). The airway smooth muscle thickness index was assessed as the number of points hitting smooth muscle, divided by the number of intercepts between the lines of the grid and the basal membrane. The airway bronchoconstriction index was assessed as the number of points hitting on airway lumen divided by the root square of the number of intercepts between the lines of the grid and the airway basal membrane. Measurements were performed in five airways from each animal at ×400 magnification (40).

Statistical Analysis

One-way ANOVA followed by Student-Newman-Keuls *post hoc* test (parametric data) and one-way ANOVA on Ranks followed by Dunn's *post hoc* test (nonparametric data) were used for comparison of the different parameters among groups. Values were expressed as means ± SD for parametric data and as median (variance) for nonparametric data. The level of significance was set at $P < 0.05$.

RESULTS

AHR

Figure 1 shows airway responsiveness to increasing doses of MCh in all groups. We observed that even at basal values and after saline inhalation, the level of Penh was increased in the Cr, OVA, and OVA+Cr groups when compared with the control group ($P < 0.05$). We also observed that AHR was increased in all groups when compared with the control group at all doses of MCh ($P < 0.05$). We further observed that creatine supplementation exacerbated the effects of OVA, with the OVA+Cr group showing a significant increase in Penh when compared with the Cr and OVA groups ($P < 0.05$).

Total and Differential Cells in BALF

Figure 2 shows total and differential cell counts in BALF in the four groups. The OVA and OVA+Cr groups presented a significant increase in the number of total cells in BALF when compared with the control and Cr groups ($P < 0.05$) (Figure 2A). Creatine supplementation increased lung eosinophil inflammation in sensitized animals. The number of eosinophils was significantly increased in OVA when compared with the control group and in OVA+Cr when compared with all other groups ($P < 0.05$) (Figure 2B). We observed an increased number of neutrophils in the OVA and OVA+Cr groups when compared with the control and Cr groups ($P < 0.05$) (Figure 2C). Mononuclear cell number in BALF was significantly increased in OVA when compared with all other groups ($P < 0.05$) (Figure 2D).

PCA to OVA-Specific IgE and IgG_1

Values of IgE and IgG1 titers in the different groups are presented in Table 1. OVA-specific IgE and IgG_1 titers were significantly increased in the OVA and OVA+Cr groups when compared with the control and Cr groups ($P < 0.001$). However, the creatine treatment did not increase the OVA-specific IgE or IgG_1 titers in either sensitized or nonsensitized mice.

Morphometric Analysis of Airway Inflammatory Changes

Figures 3A and 3B show, respectively, the eosinophil and mononuclear cell densities in airway walls in the four groups. We observed a significant increase in airway eosinophil density in all groups when compared with controls ($P < 0.01$), as well as a significant increase in airway eosinophil density in OVA+Cr when compared with the OVA group ($P < 0.01$). There was a significant increase in airway mononuclear cell density in the Cr and OVA+Cr groups when compared with the control and OVA groups ($P < 0.05$). Figures 4A–4C show, respectively, the airway density of IL-4–, IL-5–, and IGF-1–positive cells in the four groups. In Figure 3A, we observe a significant increase in airway density of IL-4–positive cells in the OVA and OVA+Cr groups when compared with the control and Cr groups ($P < 0.05$) and also in OVA+Cr when compared with the OVA group ($P < 0.001$). Figure 3B shows a significant increase in airway density of IL-5–positive cells in all groups when compared with the control group ($P < 0.05$) and also in OVA+Cr when compared with the Cr and OVA groups ($P < 0.001$). Figure 3C shows a significant increase in airway density of IGF-1–positive cells in all groups when compared with the control group ($P < 0.001$) and also in OVA+Cr when compared with the Cr and OVA groups ($P < 0.001$). Figure 5 shows representative photomicrographs of airway density of IL-4–positive cells in the four groups. There was no significant difference in airway

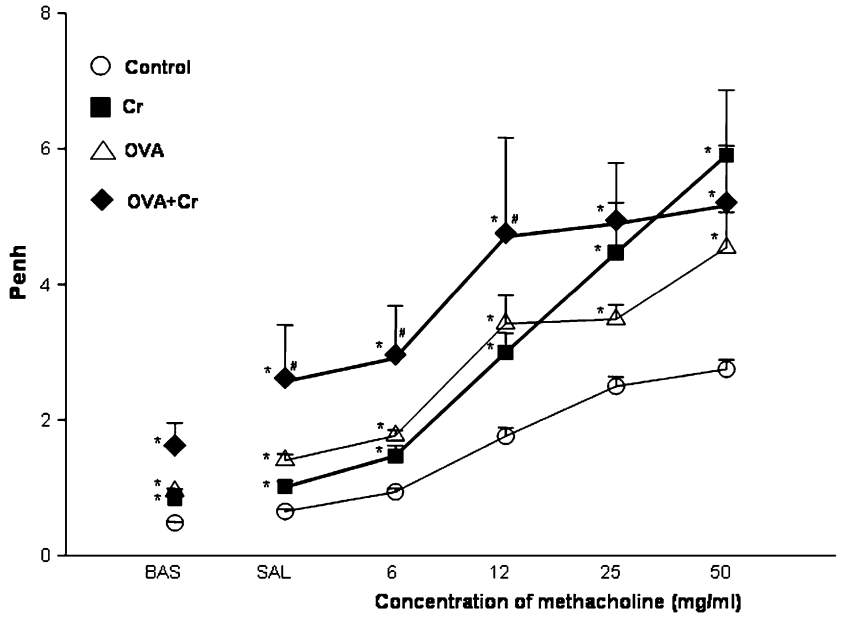

Figure 1. Airway hyperresponsiveness in control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$) groups. OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (0.5 g/kg/d) from Day 21 to Day 53. *$P < 0.05$ when compared with the control group. #$P < 0.05$ when compared with the Cr and OVA groups ($P < 0.05$).

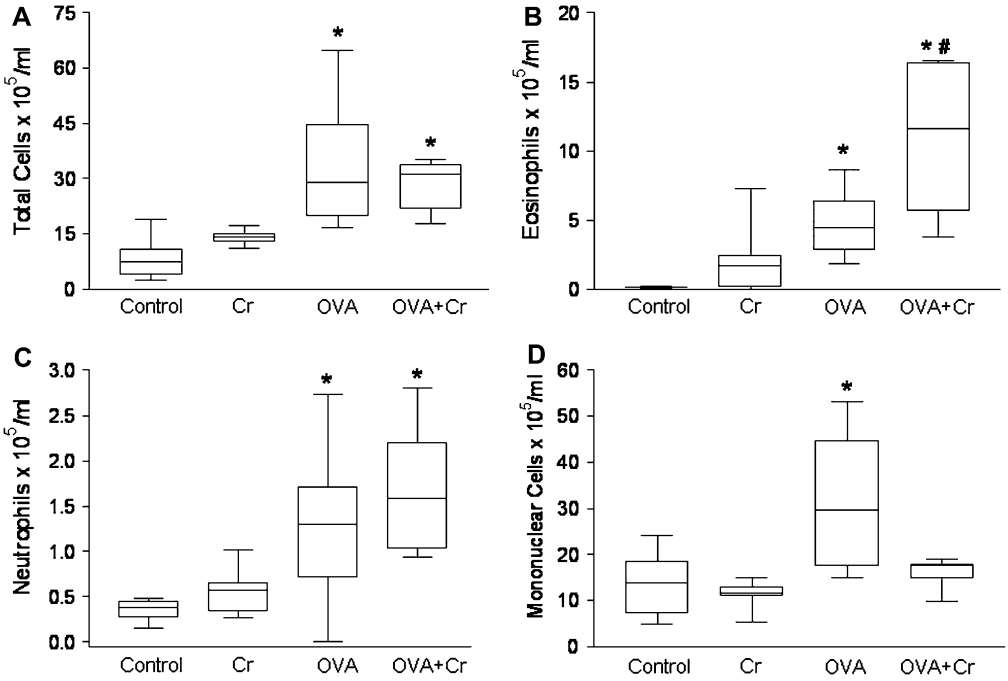

Figure 2. Cell counts in BALF in control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$) groups. OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (0.5 g/kg/d) from Day 21 to Day 53. (*A*) Total cells, $^*P < 0.05$ when compared with control and Cr groups. (*B*) Eosinophils, $^*P < 0.05$ when compared with control and Cr groups; and $^{\#}P < 0.05$ when compared with OVA group. (*C*) Neutrophils, $^*P < 0.05$ when compared with control and Cr groups. (*D*) Mononuclear cells, $^*P < 0.05$ when compared with other groups.

density of IL-2– and IFN-γ–positive cells among groups (data not shown).

Morphometric Analysis of Airway Remodeling and Bronchoconstriction

Figure 6 summarizes the effects of creatine supplementation on airway remodeling and bronchoconstriction. Figure 6A shows a significant increase in the volume proportion of collagen fibers in airway wall in all groups when compared with the control group ($P < 0.05$) and also in the OVA+Cr group when compared with the Cr group ($P < 0.05$). Figure 6B shows a significant increase in the volume proportion of elastic fibers in airway wall in all groups when compared with the control group ($P < 0.001$). Figure 6C shows a significant increase in the smooth muscle thickness index in all groups when compared with the control group ($P < 0.01$) and also in the OVA+Cr group when compared with the Cr group ($P < 0.01$). Figure 6D shows a significant increase in the bronchoconstriction index in the OVA and OVA+Cr groups when compared with the control group ($P < 0.05$).

DISCUSSION

In the present study we evaluated, for the first time, the effects of creatine supplementation on an experimental model of chronic allergic lung inflammation in mice. Our results showed that creatine supplementation exacerbated allergic inflammation, airway responsiveness, and remodeling in sensitized mice, characterized respectively by a significant increase in the density of eosinophils in BALF and airway walls, a significant increase in hyperresponsiveness, and also by a significant increase in the volume proportion of elastic and collagen fibers and smooth muscle thickness. We also showed that creatine-induced changes were mediated by an increase in the release of IL-4, IL-5 and IGF-1 by inflammatory cells. It is interesting to observe that creatine supplementation increased all studied parameters independently of OVA treatment. In other words, creatine administration induced airway inflammation, remodeling, and hyperresponsiveness in nonsensitized mice, and also exacerbated the effects of OVA sensitization.

TABLE 1. PCA TITERS OF OVA-SPECIFIC IgE AND IgG_1 IN CONTROL ($n = 7$), CREATINE (Cr, $n = 8$), OVALBUMIN (OVA, $n = 8$), AND OVA+Cr ($n = 8$) GROUPS

	Control	Cr	OVA	OVA+Cr
IgE	0.0 ± 0.0	0.0 ± 0.0	7.1 ± 0.5*	7.32 ± 0.7*
IgG_1	0.0 ± 0.0	0.0 ± 0.0	11.32 ± 0.0*	12.32 ± 0.0*

Definition of abbreviations: Cr, creatine; Ig, immunoglobulin; OVA, ovalbumin.
OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (05 g/kg/d) from Day 21 to Day 53. Values expressed in logarithm scale (mean ± SD).
* $P < 0001$, comparing OVA and OVA+Cr groups with control and Cr groups.

Creatine supplementation has been used by athletes due to its effects on body weight and muscle mass gain, resulting in a better muscular performance (2, 8, 9). However, Santos and colleagues postulated that creatine supplementation could have other beneficial effects on skeletal muscle. These authors showed an anti-inflammatory effect of creatine supplementation on long distance endurance runners, characterized by a significant decrease in the plasmatic levels of prostaglandin E_2 and tumor necrosis factor-α (14). Nomura and coworkers also suggested an anti-inflammatory effect of creatine supplementation. These authors induced inflammation in human lung endothelial cell culture with serotonin or H_2O_2, and showed a significant decrease in endothelial permeability, neutrophil adhesion, and expression of intercellular adhesion molecule-1 (ICAM-1) and E-selectin at different concentrations of creatine supplementation (41).

Due to its effects on muscular performance, creatine has been administered to patients with chronic diseases, such as chronic heart failure, mitochondrial myopathies, and after rehabilitation from disuse atrophy (16, 19). Few studies have also investigated the effects of creatine supplementation on chronic pulmonary diseases, suggesting a beneficial role. Creatine has been shown to improve health status in patients with chronic obstructive pulmonary disease, mainly by increase of peripheral muscle

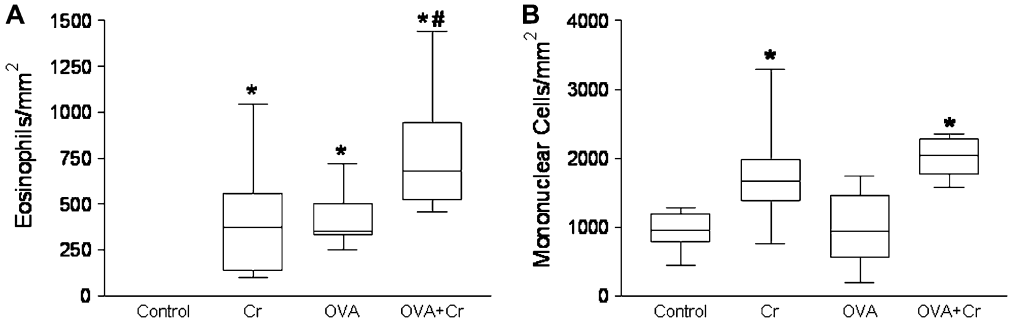

Figure 3. Airway density of eosinophils and mononuclear cells in control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$) groups. OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (0.5 g/kg/d) from Day 21 to Day 53. (*A*) Eosinophils, $*P < 0.01$ when compared with control group, and $^{\#}P < 0.01$ when compared with OVA group. (*B*) Mononuclear cells, $*P < 0.05$ when compared with control and OVA groups.

strength (23). In patients with cystic fibrosis, creatine supplementation has been shown to improve general well being and muscle strength, with no effects on lung function (24).

Based on its reported anti-inflammatory effects and on the improvements in health status observed in patients with chronic pulmonary diseases, we expected to find a beneficial effect of creatine administration in this model of chronic allergic lung inflammation. In contrast, our results showed that creatine supplementation exacerbates inflammation in sensitized mice, with increase in the density of eosinophils in BALF (Figure 2B) and airway walls (Figure 3A), and also in the expression of IL-4 (Figure 4A) and IL-5 (Figure 4B) by inflammatory cells, suggesting a boost of T helper cell type 2 (Th2) response after creatine supplementation.

It is well known that the asthmatic allergic response is, to a great extent, determined by a Th1–Th2 imbalance, characterized by an immune response with a predominance of Th2 lymphocytes and eosinophils, which can secret an array of cytokines, such as IL-4, IL-5, and IL-13, capable of promoting T cell proliferation and differentiation, and Th1/Th2 polarization (42, 43). Experimental models of chronic allergic lung inflammation demonstrated that OVA sensitization induces the release of Th2 cytokines, particularly IL-4 and IL-5 (42, 43). IL-4 perpetuates mast cell activation, which is responsible for IgE and IgG_1 release, and induces eosinophil activation and recruitment as well as mucus production (42, 44). IL-5 stimulates the proliferation, differentiation, survival, and activation of eosinophils in the bone marrow, the release of mature eosinophils from the bone marrow into the blood, and it is also a chemoattractant to eosinophils (42, 45). IL-5 also eliminates the inhibitory effects of IL-2 on eosinophils migration (46, 47).

The immunomodulatory effects of amino acid supplementation are not completely understood and may vary in different clinical conditions. For example, preoperative supplementation with arginine, one of creatine components, improves the immunologic response and decreases the infection rate in patients with colorectal resection (48). On the other hand, it has also been suggested that arginine supplementation has no beneficial effects on infection complications and may even increase mortality in critically ill patients (49). Yeh and colleagues have recently described that pretreatment with an arginine-supplemented diet resulted in more pronounced Th2 cytokine production in an experimental model of sepsis in mice (50). In this study, the authors observed that arginine supplementation resulted in increased circulating ICAM-1 levels, and increased IL-6 and neutrophil myeloperoxidase activities in various organs, and suggested that the use of arginine in a critical condition should be carefully evaluated. In our present study, we showed that creatine supplementation increased the Th2 response, exacerbating the allergic inflammation, with an increase in the release of IL-4 and IL-5. However, the mechanisms responsible for this effect are not clear. One possible explanation could be a direct effect of creatine on inflammatory cells metabolism. It is known that cell survival is highly dependent on the preservation of intracellular bioenergetics and that inflammatory cells have a large intracellular storage of creatine phosphate (4). It is possible that creatine supplementation could lead to an increase in the intracellular creatine phosphate storage of inflammatory cells, resulting in a more prolonged period of activation, or even delaying cell death, which could result in a perpetuation of the inflammatory state (4, 51–53). In fact, it has been demonstrated that some amino acids can present significant effects on inflammatory cells function. For example, glutamine supplementation in rats submitted to exercise-induced neutrophils apoptosis results in increased neutrophils phagocytosis capacity, nitric oxide and reactive oxygen species production, and partially prevents neutrophil apoptosis (54, 55). Although an anti-inflammatory effect of creatine supplementation has been previously reported,

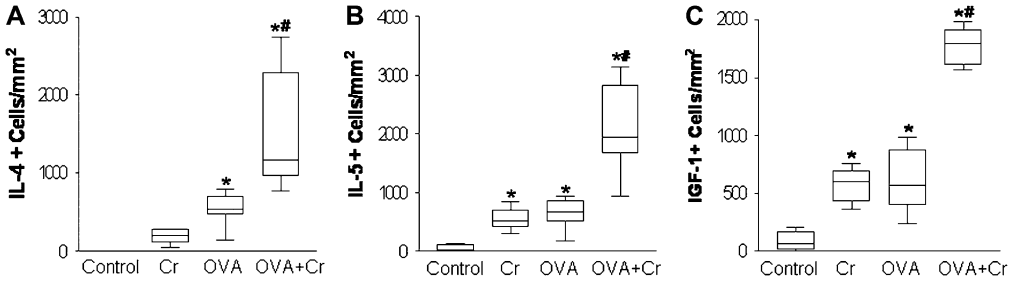

Figure 4. Airway density of IL-4–, IL-5–, and IGF-1–positive cells in control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$) groups. OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (0.5 g/kg/d) from Day 21 to Day 53. (*A*) IL-4–positive cells, $*P < 0.05$ when compared with control and Cr groups, and $^{\#}P < 0.001$ when compared with Cr and OVA groups. (*B*) IL-5–positive cells, $*P < 0.05$ when compared with control group, and $^{\#}P < 0.001$ when compared with Cr and OVA groups. (*C*) IGF-1–positive cells, $*P < 0.001$ when compared with control group, and $^{\#}P < 0.001$ when compared with Cr and OVA groups.

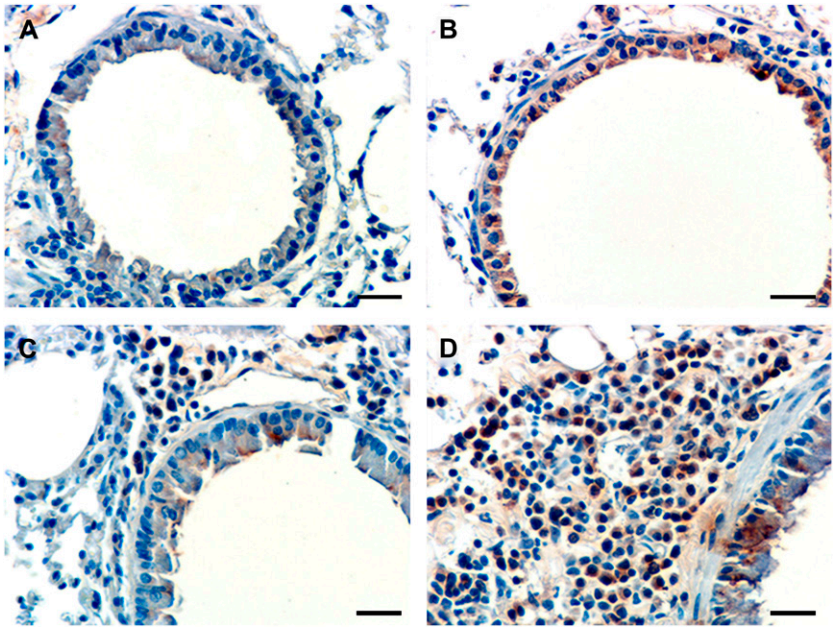

Figure 5. Photomicrographs of airway density of IL-4–positive cells in control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$) groups. OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (0.5 g/kg/d) from Day 21 to Day 53. *A*, Control; *B*, Cr; *C*, OVA; *D*, OVA+Cr. *Scale bar* = 25 μm.

the effect of creatine supplementation on inflammatory cells function under allergic conditions remains to be elucidated.

Interestingly, besides exacerbating lung inflammation, creatine supplementation further increased the collagen and elastic fibers deposition in airway walls, the smooth muscle thickness, and also the AHR. We suggest that these changes might have been mediated by an increase in IGF-1 expression by inflammatory cells, observed in our animals after creatine administration. IGF-1 is known to activate fibroblasts in experimental models of allergic lung inflammation and in fibroblasts cells culture (56, 57). In cell culture, IGF-1 also stimulates airway smooth muscle cells proliferation (58). It was previously suggested that muscle mass gain after creatine administration in rats is mediated by increased IGF-1 expression by skeletal muscle (9). In a murine model of allergic lung inflammation, the expression of IGF-1 by airway epithelial cells and macrophages was increased. In the same model, the blockade of IGF-1 inhibited the elevation of airway resistance, airway inflammation, the increase in airway wall thickening, and the expression of ICAM-1, suggesting that IGF-1 is likely to be an important mediator of inflammation and remodeling in the airways of sensitized mice (56). In humans, IGF-1 leads to an increased bronchial smooth muscle sustained contraction via Rho-kinase–dependent pathway, suggesting an important role in the mechanism of airway hyperresponsiveness (59). Our results demonstrated that also in nonsensitized mice, creatine supplementation increased Penh in basal conditions. We believe that this increase in basal values of Penh induced by creatine might be mediated by the increased expression of IGF-1.

The effects of creatine supplementation in our animals could also be partially mediated by an increased availability of

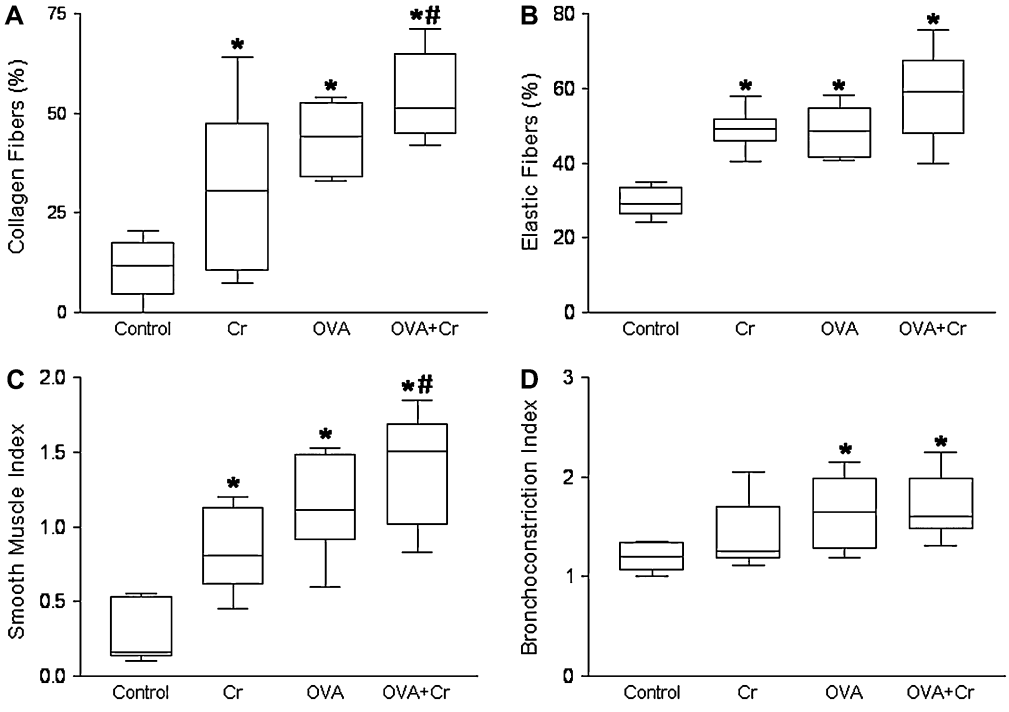

Figure 6. Volume proportion of airway collagen and elastic fibers, smooth muscle thickness, and bronchoconstriction in control ($n = 7$), creatine (Cr, $n = 8$), ovalbumin (OVA, $n = 8$), and OVA+Cr ($n = 8$) groups. OVA groups were sensitized with four intraperitoneal OVA injections and challenged with aerosolized OVA from Day 21 to Day 53. Creatine groups received creatine supplementation (0.5 g/kg/d) from Day 21 to Day 53. (*A*) Collagen fibers, $^{*}P < 0.05$ when compared with control group, and $^{\#}P < 0.05$ when compared with Cr group. (*B*) Elastic fibers, $^{*}P < 0.001$ when compared with control group. (*C*) Smooth muscle thickness, $^{*}P < 0.01$ when compared with control group, and $^{\#}P < 0.01$ when compared with Cr group. (*D*) Bronchoconstriction index, $^{*}P < 0.05$ when compared with control group.

L-arginine and consequently increased synthesis of nitric oxide (NO). The role of NO in asthmatic airways is not completely understood, as it may act either as a protective or a stimulatory factor (60). There are few experimental studies evaluating the effect of exogenous L-arginine in sensitized animals. Maarsingh and colleagues have demonstrated that the administration of exogenous L-arginine to tracheal open-ring preparations reversed the impaired relaxation after allergen challenge, suggesting that increased availability of L-arginine activates the NO synthase pathway and NO synthesis (61). A clinical study that evaluated the effects of L-arginine supplementation in patients with asthma showed that exhaled NO increased after L-arginine supplementation and that NO synthesis inhibition by N-monomethyl-L-arginine (L-NMMA) did not significantly attenuate the exercise-induced bronchoconstriction compared with supplementation of NO synthase substrate (L-arginine). These results suggest that endogenous NO does not inhibit the bronchoconstrictor response to exercise in asthma (60). Experimental models of asthma have demonstrated that the increased availability of L-arginine could also activate the arginase pathway leading to increased airway tonus, mucus production, and airway remodeling with increased collagen production (61–63). The activation of the arginase pathway could be at least partially responsible for our findings of airway remodeling. The involvement of the arginase pathway in the effects of creatine supplementation in our model of chronic allergic lung inflammation should be further investigated.

Taken together, our results suggest that creatine supplementation exacerbates the effects of OVA sensitization (airway inflammation, remodeling, and hyperresponsiveness) via Th2 and IGF-1 pathways. However, the extent to which the results obtained in this murine model of allergic inflammation can be transposed to patients with asthma is unclear.

Creatine has been used indiscriminately and to high dosages by individuals who intend to improve their muscular performance. To date, no deleterious effects of creatine supplementation were confirmed. However, the observed exacerbation of allergic chronic lung inflammation suggests that creatine supplementation might have deleterious effects in individuals with allergic conditions such as asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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