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Antioxidant Diet Protects Against Emphysema, but Increases Mortality in Cigarette Smoke-Exposed Mice

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Abstract

Oxidative stress plays an important role in cigarette smoke-induced lung inflammation and emphysema. We produced an enriched diet by adding freeze-dried fruits and vegetables and additional supplements to the 8604 Teklad Rodent Diet, a standard rodent diet. In this study, we examined the effects of the antioxidant-enriched diet on cigarette smoke-induced lung inflammation and emphysema. CH3/HeN mice were fed either a regular diet or an antioxidant diet. These mice were exposed to filtered air, a low concentration of cigarette smoke (total particulate matter: 100 mg/m³) or a high concentration of cigarette smoke (total particulate matter: 250mg/m³) for 6 hours/day, 5 days/week for total 16 weeks. Surprisingly, increased mortality (53%) was observed in the high concentration of cigarette smoke-exposed mice fed the antioxidant diet compared to the high concentration of cigarette smoke-exposed mice that were fed a regular diet (13%). The necropsy analysis revealed nasal passage obstruction due to mucous plugging in cigarette smoke-exposed mice on the antioxidant diet. However, the antioxidant diet significantly reduced neutrophilic inflammation and emphysema in the high concentration of cigarette smoke-exposed mice as compared to the regular diet /high concentration of cigarette smoke controls. The antioxidant capacity in the bronchoalveolar fluid or oxidative damage to the lung tissue was not affected by the antioxidant diet. Pro-MMP-2, MMP-2, and MMP-9 activity did not correlate with the protective effects of AOD on cigarette smoke-induced emphysema. These data suggest that the antioxidant diet reduced cigarette smoke -induced inflammation and emphysema, but increased mortality in the obligate nose-breathing mice.

Keywords

cigarette smoke; emphysema; oxidative stress; lung inflammation; adverse effects; lipid peroxidation; metalloproteinase

Introduction

Chronic obstructive pulmonary disease is the 4th leading cause of death in the US [1]. Chronic obstructive pulmonary disease is characterized by an irreversible airflow limitation and includes heterogenous clinical phenotypes, such as chronic bronchitis (persistent mucus

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production) and emphysema (destruction of alveolar sacs) that is primarily caused by long-term cigarette smoking [2]. Oxidative stress is believed to play an important role in cigarette smoke (CS)-induced emphysema [3]. Nuclear factor erythroid-derived 2, like 2 (NRF-2), is a redox-sensitive basic leucine zipper protein transcriptional factor that is responsible for upregulating many antioxidant enzymes. Mice deficient in this nuclear factor are more susceptible to CS-induced emphysema than wild-type mice [3-4]. In contrast, transgenic mice that overexpress copper, zinc superoxide dismutase exhibit resistance to CS-induced emphysema [4]. These studies suggest that redox regulation can modulate susceptibility to CS-induced emphysema.

Oxidative stress occurs in the setting of oxidant/antioxidant imbalance and may be involved in lung inflammation in CS-exposed mice [3]. Oxidative stress may activate redox-sensitive transcriptional factors, such as nuclear factor- κ B and Activator Protein-1 to upregulate expression of pro-inflammatory mediators [5]. To counteract the redox imbalance, antioxidant therapies have been successfully developed to attenuate CS-induced emphysema in mice [6-7]. The antioxidant therapies include pharmacological mimetics to augment activity of extracellular superoxide dismutase (SOD) [7] or NRF-2 [6] and dietary antioxidants, such as curcumin [8], and tomato juice [9].

In humans, there is substantial evidence that a “healthy diet,” particularly one rich in fruits [10] and possibly vegetables [11-12] is protective against CS-induced emphysema and chronic obstructive pulmonary disease. However, the specific components responsible for these effects have not been defined. It is possible that the protection results from synergistic interactions among antioxidants and other bio-active compounds in the supplemented diet.

In this study, we developed a mixed antioxidant diet (AOD) containing four different freeze-dried fruits, four different freeze-dried vegetables, and other various antioxidants. We hypothesized that the mixed AOD would attenuate emphysema in CS-exposed mice. To test this hypothesis, we chose female C3H/HeN mice for two reasons. First, female C3H mice are more susceptible to CS-induced emphysema than male C3H/HeN mice or any gender of C57/Bl6 mice (personal observation). Second, a relatively short period of CS exposure (16 weeks) is sufficient to induce significant emphysema in female C3H mice [13]. To examine a possible dose-response relation to a concentration of CS, we used a low (LCS; total particulate matter [TPM] 100mg/m³) and a high (HCS; TPM 250mg/m³) concentration of CS. We found that AOD significantly protects against emphysema, but increases mortality in HCS-exposed mice. Although the autopsy analysis suggests that mucus plugging in the nasal passage might have contributed to more aerodigestion, resulting in circulatory failure, the main cause of death is unclear. This study indicates that AOD may alter the impact of CS on the alveolar destruction by reducing lung inflammation and possibly metalloproteinase activity. However, in the obligate nose-breathing mice, the AOD increased mortality.

Materials and Methods

Mice

Female CH3/HeN mice, 8–10 weeks of age, were purchased from Charles River. All experiments were approved by the Institutional Animal Care and Use Committee and were performed at Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International.

Antioxidant diet

The following fruits and vegetables were purchased at a local supermarket: oranges, apples, grapes, carrots, spinach, tomato paste, and broccoli. Each of these was freeze dried using a

LabConCo lyophilizer. Freeze-dried blueberries were purchased from Honeyville Farms. Additional supplements (curcumin, mixed tocopherols, vitamin C [as Stay-C, an esterified stable form of the vitamin], sodium selenite, taurine, rosemary extract, acetyl carnitine, α -lipoic acid, and epigallocatechin gallate [EGCG]) were purchased from Sigma Chemical Co, except for Stay-C (provided by Harlan Teklad), and rosemary extract (Guardian Extract #11) and mixed tocopherols (Grindox Toco 70), which were purchased from Anisco.

To produce the supplemented diet, for each kg of diet, 10 g each of the freeze dried oranges, apples, grapes, carrots, spinach, tomato paste, broccoli, and blueberries, 380 mg of curcumin, 320 mg of tocopherols, 242 mg of Stay-C, 1.7 mg of sodium selenite, 1 g of taurine, 2.5 g of rosemary extract, 300 mg of acetyl carnitine, 125 mg of α -lipoic acid, and 60 mg of EGCG were combined with 915 g of powdered Harlan Teklad Rodent diet 8604. The detailed ingredient of the 8604 Teklad Rodent Diet are available at the website: <http://www.harlan.com>. The powdered mixture was thoroughly combined and reformed into pellets (Harlan Teklad). The diet was stored at -20°C until use, and fresh food was provided daily. It is important to note that although designated “antioxidant diet” for simplicity, this diet also includes numerous additional phytochemicals with properties beyond simple antioxidant capacity.

Cigarette smoke exposure (whole-body inhalation)

Type 2R4F research cigarettes (Kentucky Tobacco Research and Development Center) were used in this study. The mice were exposed for 6 h/d, 5 d/week in Hazelton 1000 chambers. Exposure concentrations of TPM were kept either at 100 mg/m^3 for the first week and 250 mg/m^3 for subsequent weeks (HCS) or the chamber was kept at 100 mg TPM/m^3 for all of the 16 weeks of exposure (LCS). Air control mice were housed in similar exposure chambers and exposed to filtered air (FA). From a total of 90 mice, 30 mice were exposed to each concentration of CS and to FA. Of them, 15 were fed the base rodent diet (Harlan Teklad 8604) and the other 15 were fed the AOD. From the group of 15, seven mice were designated for bronchoalveolar lavage (BAL) and the other eight were designated for fixation of the lungs and histopathology. The whole body weight of the mice was monitored every 2 weeks.

Bronchoalveolar lavage and histology

BAL was performed as previously described [14-15]. Briefly, the lungs of surviving mice from each exposure group were lavaged with phosphate buffered saline, and total cells were evaluated with a hemocytometer; differential cell types were evaluated on a Diff-Quick-stained cytocentrifuge preparation. The lungs of the remaining mice in each group were fixed under a constant pressure ($25\text{ cm H}_2\text{O}$) in neutral buffered formalin, embedded in paraffin, and sectioned at $3\text{ }\mu\text{m}$ thickness as described (12).

The emphysema quantification was performed by an experienced veterinary pathologist using some modifications of the semi-quantitative method previously described [16-17]. Briefly, severity of emphysema was scored by light microscopy on a subjective scale, where minimal, mild, moderate, and marked corresponded to the ordinal values of one, two, three, and four respectively. The severity was multiplied by a distribution score on a five-point scale where focal, locally extensive, multifocal, multifocal and coalescing, and diffuse corresponded to scores of one, two, three, four, and five, respectively. The maximum possible score would be marked severity and diffuse distribution with a product score of 20.

Trolox equivalent antioxidant content (TEAC)

TEAC in the supernatant of the bronchoalveolar fluid (BALF) was measured by using the method described by Troost et al [18]. This assay compares the antioxidant capacity to

reduce the radical cation of 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) with that of Trolox, a vitamin E analog, which scavenges both superoxide anion and hydrogen peroxide.

Lipid peroxidation assay

The thiobarbituric acid reactive substances (TBARS) assay was performed to measure lipid peroxidation in the lung tissue by using the method previously described [19-20]. After 16 weeks of exposure to FA, LCS, or HCS, the mice were euthanized. Following lavage, the lungs were removed for analysis of TBARS, an alternative measure of oxidative stress as described [21]. The thiobarbituric acid reacts with products from lipid peroxidation. The product was measured spectrophotometrically at 532 nm and compared with a standard curve constructed from 1,1,3,3-tetramethoxypropane.

Metalloproteinase assay by zymography

Zymography was performed as described [22]. Briefly, a 7.5% acrylamide gel containing 1.5 mg/mL molecular-grade gelatin (BioRad, Hercules, CA) was used. Samples (75 μ L) were combined with 25 μ L of 4 $\times$ Laemmli sample buffer without reducing agents, and 20 μ L were loaded onto mini-gels (BioRad Protean IV system). Following resolution by polyacrylamide gel electrophoresis, the gels were washed in 2.5% Triton X-100, and then placed in development buffer consisting of 100 mM Tris buffer, pH 7.4 with 5 mM CaCl_2 , and 1 mM ZnCl_2 . Following continued incubation on a rotating platform for 1 h, the gels were incubated at 37°C for 22–23 h, and then fixed with 40% methanol and 7% acetic acid. The gels were stained in the same solution containing 2 mg/mL Coomassie Brilliant Blue R250 overnight. The gels were de-stained in distilled water. Digital images were taken with a GS-800 Calibrated Densitometer system (PowerLook2100XL, BioRad Laboratories) and analyzed using Quantity One (version 4.5.1; BioRad).

Statistical analysis

Results from six groups of mice (exposed to FA on a regular diet [RD], FA on AOD, LCS on RD, LCS on AOD, HCS on RD, HCS on AOD) were expressed as mean $\pm$ SEM and were analyzed using ANOVA with Bonferroni's post-test for multiple comparisons. For the histopathological scores, the non-parametric Kruskal Wallis test was used, with Dunn's post-test. In addition, pairwise comparisons were performed using Mann Whitney. Survival curves were analyzed by Kaplan Meier tests, using Bonferroni corrections on the Gehan-Breslow-Wilcoxon *P* values.

Results

Antioxidant diet protects against emphysema, but increases HCS-associated mortality

To investigate whether mixed AOD would attenuate CS-induced emphysema, C3H mice fed with RD or AOD were exposed to CS (TPM 100 or 250 mg/m^3) for 16 weeks. Surprisingly, as shown in Figure 1A, eight of the 15 mice (53%) in the group of AOD/HCS died over the exposure period (*P* value; 0.001). The necropsy analysis revealed no clear cause of death although the dead mice on AOD had mucus plugs in the nasal passage and air-filled digestive tracts. Two mice (13%) in the group of RD/HCS and one mouse (7%) in the group of RD/LCS also died over the 16 weeks (Figure 1A). The cause of death in these mice was unknown. These data suggest that AOD may contribute to mortality of HCS-exposed C3H mice due to an unclear etiology.

The lung pathology seen in Figure 1B was examined, and the severity of emphysema was measured for eight surviving mice in all the groups except for seven mice in the AOD/LCS group, six mice in the RD/HCS group and three mice in the AOD/HCS group. As shown in Figure 1C, exposure to HCS but not to LCS significantly induced emphysema. Although the

effect of the AOD was not significant by Dunn's post-test of the ANOVA, a pairwise comparison of the RD/HCS group with the AOD/HCS group using the Mann Whitney test yielded a *P* value of 0.036. However a non-significant increase in the emphysema score was observed for the AOD/LCS group relative to the RD/LCS group. We also observed that cigarette smoke exposure reduced weight gain of the whole body in the mice in a dose-dependent manner over the 16 weeks. Interestingly, AOD attenuated the effects of cigarette smoke on weight gain in HCS-exposed mice. These data suggest that AOD may modulate susceptibility to CS-induced emphysema and body weight alteration, depending on the concentration of CS.

Antioxidant diet has little effect on oxidative damage of the lungs in cigarette smoke-exposed mice

To examine the effects of AOD on the antioxidant capacity in the lung of FA- or CS-exposed mice, we measured the antioxidant capacity in BALF obtained from seven surviving mice in all the groups except for four mice in the AOD/HCS group by using the TEAC assay. Oxidative damage in the lungs was assessed as TBARS. As shown in Figure 2A, AOD failed to increase the antioxidant capacity in BALF of mice regardless of the smoking status. The TBARS assay in the whole lung tissue obtained from CS-exposed mice also showed no significant increase in oxidative damage (Figure 2B). These data suggest that AOD has little effect on the antioxidant capacity in the BALF and lipid peroxidation in the lung tissues regardless of the smoking status.

Antioxidant diet decreases cigarette smoke-induced lung inflammation

To evaluate the effects of CS and the AOD on inflammation in the lungs, BAL was performed for surviving mice in all six groups of mice. HCS exposure significantly increased neutrophils in the BALF of mice, and AOD blocked the effects of CS (Figure 3B). The number of macrophages and lymphocytes were not affected by CS exposure and by AOD (Figure 3A&C). These data show that AOD reduced CS-induced lung inflammation.

Antioxidant diet minimally modulates metalloproteinase activity in cigarette smoke-exposed mice

We next tested if AOD would alter the activity of a proenzyme form of MMP-2 (pro-MMP-2), MMP-2, and MMP-9 in CS-exposed mice. Exposure to CS appeared to increase the activity of pro-MMP-2, MMP-2, and MMP-9 in the BALF from HCS-exposed mice (Figure 4). Although AOD did not decrease the MMP-9 levels in LCS-exposed mice, pro-MMP-2 and MMP-2 activities in HCS-exposed mice fed the AOD were undetectable, similar to the control animals. These data suggest that AOD may suppress the CS-induced increases in levels of pro-MMP-2 and MMP-2.

Discussion

Long-term exposure to CS induces chronic inflammation and emphysema in the lungs of some individuals [23]. CS-induced inflammation appears to be mediated by oxidative stress [3]. We previously showed that treatment with antioxidants epigallocatechin gallate, a compound that also possesses MMP-inhibitory capacity [24], but not N-acetylcysteine, decreases inflammatory cells in the BALF from CS-exposed A/J mice [14]. In addition, human epidemiological studies suggest that diets rich in antioxidant-containing foods protect against various CS-associated pathologies [10-12]. In this study, we examined whether AOD alters lung inflammation and emphysema in CS-exposed C3H mice because an animal model replicating the human evidence would be useful in identifying the specific mechanisms involved in the protective effects.

The adverse effects of an antioxidant diet: To our knowledge, there have been no reports that antioxidant therapy increases mortality in CS-exposed mice. In general, human studies have emphasized pharmacological doses of a single antioxidant. A major antioxidant trial on human mortality revealed that high doses of beta carotene resulted in the significantly increased mortality of smokers due to various causes, such as lung cancer and cardiovascular disease [25-26]. Moreover, a recent meta-analysis of the human randomized trials of antioxidant supplements on mortality suggested that treatment with beta carotene, vitamin A, or vitamin E could significantly increase mortality [27]. The effects of beta carotene and vitamin A appeared to be dose-dependent. Although the meta-analysis could not identify the detailed cause of mortality, it is likely to be malignancy and cardiovascular diseases [28-29]. However, there were some significant concerns regarding the studies excluded from this meta-analysis. There is increasing evidence that excessive levels of a single antioxidant can actually be harmful, while a balance resulting from an optimal diet is beneficial, possibly in part due to contributions of micronutrients beyond the classical vitamins [27,30-34]. In our study, the main cause of mortality was unclear, but was neither malignancy nor heart failure. It was also unclear how AOD increases the nasal mucous plugging in CS-exposed mice.

Protective effects of antioxidants on CS-induced emphysema: Despite increased mortality, we found that an AOD significantly blocked the effects of CS on emphysema in the surviving C3H mice. Consistent with our findings, several animal studies have demonstrated protective effects of antioxidants on CS-induced emphysema [6-7]. Sussan, et al demonstrated that a potent NRF-2 activator, 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-lm), significantly attenuates CS-induced emphysema [6]. In addition, Yao, et al reported that extracellular SOD (ECSOD) or pharmacological SOD mimetic protected CS-induced mice [7]. However, neither study showed significant mortality of CS-exposed mice in response to the antioxidant therapy. The discrepancy of the AOD effects on mortality in CS-exposed mice could be due to strain differences because both Sussan and Yao used the C57Bl/6 mice that are relatively resistant to CS-induced emphysema. In addition, we used the AOD consisting of various fruits, vegetables, and antioxidants. It is possible that interactions among the antioxidants may have affected survival due to metabolic effects on CS detoxification. One possibility is that CS produced oxidized forms of phytonutrients that were toxic to the mice and may have possibly increased mucus production.

Effects of antioxidants on CS-induced oxidative stress: Our animal study showed that AOD does not augment the antioxidant capacity in the BALF and fails to protect against lipid peroxidation in the lung of mice regardless of the smoking status. In contrast to our findings, both CDDO-lm [6] and overexpression of extracellular SOD [7] significantly attenuate oxidative stress (less oxidized DNA and protein, respectively) in the lungs of CS-exposed mice. Although it is possible that the contradictory findings could be due to differences in the quantification of the oxidative stress, this discrepancy may also indicate importance of a finely tuned oxidant/antioxidant balance.

The effects of antioxidant on CS-induced lung inflammation: In addition to reducing emphysema, AOD significantly reduced the number of neutrophils in the BALF of surviving CS-exposed mice. Similarly, overexpression of antioxidant enzymes, such as copper, zinc superoxide dismutase [4] and extracellular SOD [7] or dietary antioxidants, such as curcumin [8] significantly attenuate lung inflammation and emphysematous changes. Together, these studies suggest that CS-induced oxidant injury causes chronic inflammation may contribute to the subsequent emphysematous changes in the lung [4,7-8].

The effects of antioxidants on CS-induced MMP regulation: Although differences did not meet the criteria for statistical significance, AOD showed a trend toward reducing CS-induced increases of pro-MMP-2 and MMP-2 in C3H mice. In contrast, we previously showed that treatment with the antioxidant/MMP inhibitor, epigallocatechin gallate did not attenuate the increases pro-MMP-2, MMP-2, and MMP-9 activities in A/J mice [14]. The different response to antioxidant could be due to effects of the specific strain or due to differences in the impact mixed antioxidants have on these enzymes.

Study limitations

The limitations of our study were that the necropsy of the dead mice was not extensive enough to show where in the nasal passages the mucus was obstructing the airways. In addition, the role of distinct components within the AOD have not been elucidated that could explain its inhibitory role on inflammation and emphysema development without having reduced the detectable levels of the antioxidant capacity of the lung. Changes in these endpoints at additional time points and/or in other mouse strains could explain these seemingly contradictory findings.

Conclusions

This study demonstrated for the first time that AOD protects susceptible mice from the development of CS-induced emphysema. However, deaths associated with nasal mucous plugging, occurred in the AOD group exposed to a high level of CS. The protective effects of AOD on emphysema correlated with reduced lung inflammation.

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Abbreviations

AOD	antioxidant diet
BALF	bronchoalveolar fluid
COPD	chronic obstructive pulmonary disease
CS	cigarette smoke
FA	filtered air
HCS	high concentration of cigarette smoke
LCS	low concentration of cigarette smoke
MMP	metalloproteinase
NRF-2	nuclear factor erythroid-derived 2, like 2
RD	regular diet
SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
TEAC	trolox equivalent antioxidant capacity
TPM	total particulate matter

FA/RD

LCS/RD

HCS/RD

FA/AOD

LCS/AOD

HCS/AOD

Emphysema severity x distribution

Figure 1. Antioxidant diet protects against cigarette smoke-induced emphysema, but potentiates CS-induced mortality

(A) Survival. Each group of 15 age-matched C3H mice fed with regular diet (RD) or AOD were exposed to filtered air (FA), a low concentration of CS (TPM 100 mg/m³; LCS) or a high concentration of CS (TPM 250 mg/m³; HCS) and survival was monitored over 16 weeks. Only the numbers of CS-exposed mice are shown because there was no death in filtered air-exposed mice. Survival curves were analyzed by Kaplan Meier tests, using Bonferroni corrections on the Gehan-Breslow-Wilcoxon *P* values.

(B) Representative photomicrographs of lungs from surviving mice processed for H & E staining (10 × original magnification).

(C) Quantification of emphysema scores. The values in panel C are the mean ± SEM of the emphysema score in the surviving mice in the groups (n = eight except for seven mice in the AOD/LCS group, six mice in the RD/HCS group, and three mice in the AOD/HCS group). The bracketed value is from a pairwise (Mann Whitney) comparison (**P* < 0.05).

(D) The whole body weight of the surviving mice in each group was monitored every 2 weeks. Data are expressed as mean ± SEM.

	FA	LCS	HCS
RD	100 ± 6.08	108.9 ± 8.9	117.1 ± 8.3
AOD	98.1 ± 5.4	121.7 ± 5.1	131.8 ± 6.0

Figure 2. Antioxidant diet has little effect on oxidative damage of the lungs of CS-exposed mice
(A) C3H mice fed with RD or AOD were exposed to FA, LCS or HCS for 16 weeks. The antioxidant capacity in the BALF obtained from all the 6 groups of surviving mice (n = seven except for four mice in the AOD/HCS group) for 16 weeks was measured by using the trolox equivalent antioxidant content (TEAC).
(B) Oxidative damage in the whole lung tissue obtained from surviving mice for 16 weeks was measured as thiobarbituric acid reactive substances (TBARS). Data are expressed as mean ± SEM.

Figure 3. Antioxidant diet decreases CS-induced lung inflammation

C3H mice fed with RD or AOD were exposed to FA, LCS or HCS for 16 weeks.

Bronchoalveolar lavage was performed for all six groups of surviving mice for 16 weeks ($n =$ seven except for four mice in the AOD/HCS group). A total number of macrophages (A), neutrophils (B), and lymphocytes (C) in the BALF were measured. Data are expressed as mean $\pm$ SEM for three independent experiments (* significant difference from FA/RD $P < 0.05$; + significantly different from HCS/RD).

Pro-MMP-2	FA	LCS	HCS
RD	(undetectable)	0.003857 ± 0.00225	0.00414 ± 0.00172
AOD	0.000143 ± 0.000143	0.00429 ± 0.00245	(undetectable)

MMP-2	FA	LCS	HCS
RD	(undetectable)	0.00071 ± 0.00047	0.00200 ± 0.00125
AOD	(undetectable)	0.00186 ± 0.00124	(undetectable)

MMP-9	FA	LCS	HCS
RD	(undetectable)	0.0066 ± 0.0030	0.0126 ± 0.005
AOD	0.0051 ± 0.0034	0.0064 ± 0.0030	0.0122 ± 0.008

Figure 4. Antioxidant diet minimally modulates metalloproteinase activity in CS-exposed mice
C3H mice fed with RD or AOD were exposed to FA, LCS or HCS for 16 weeks. The activity of pro-MMP2 (A), MMP-2 (B), and MMP-9 (C) in the BALF obtained from all the six groups of survived mice was measured by using zymography. Data are expressed as mean ± SEM for three independent experiments.