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Short Communication

Reduction of Hexa (sulfobutyl) Fullerence on Oxidative Injury by Xanthine and Xanthine Oxidase in Isolated Rat Lungs

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Abstract

The present study was undertaken to evaluate whether some fullerenols could effectively reduce direct damages of free radicals produced by xanthine/xanthine oxidase (X/XO) in isolated rat lungs. Methods: Female Wistar rats (205 ± 4 g) were used in studies in pulmonary vascular response to the challenge of xanthine/xanthine oxidase by an isolated-perfused lung method. Free radicals were determined by chemiluminescence (CL) to confirm the release of free radicals after X/XO treatment. The CL count in the lung perfusate was 737 ± 213 (CL/10 sec); 5 min and 45 min after X/XO administration, the CL counts were 3,778 ± 425 (CL/10 sec) and 1,183 ± 193 (CL/10 sec), respectively. Challenge with X/XO caused a mild but significant increase in pulmonary arterial pressure (P_{pa}) and a marked increase of filtration coefficient (K_{fc}). The pretreatment of Hexa (sulfobutyl) fullerence antioxidant, K_{fc} became insignificantly increased in pretreated lungs. In conclusion, We found that hexa(sulfobutyl) fullerene, but not C_{60}(glucosamine)$_6$, nor superoxide dismutase could attenuate the oxidative stress, judged from the attenuated increase in pulmonary filtration coefficient after challenge.

Key Words: fullerenols, isolated lung, superoxide dismutase, xanthine oxidase

Introduction

Free radicals, continually generated in all aerobic biological systems, are a potential cause of tissue injury. They are also important causative factors of many lung diseases, for instance, pulmonary emphysema, adult respiratory distress syndrome, lung fibrosis or rejection of transplanted lung (15) and pulmonary hypertension (8). Oxidative stress can arise through increased production of reactive oxygen species (ROS) and/or because of deficiencies in antioxidant defenses. Antioxidant deficiencies can develop as a result of decreased intake of antioxidants such as vitamins C and E, defective synthesis of enzymes (such as superoxide dismutase and glutathione peroxidase or increased antioxidant utilization. Consequently, there is a constant search for new and more effective means of counteracting the toxic effects of oxidants. Fullerene (C_{60}) represents a group of nanoparticles (11). They are spherical molecules consisting entirely of carbon atoms (Cx) to which side chains can be added, furnishing compounds with widely different properties. Chemical modification of the hydrophobic C_{60} yielded many water-soluble adducts that exhibit a variety of biological activities. For example, administration of fullerenol-1 significantly ameliorated the exsanguination-induced bronchoconstriction (12), and a water-soluble fullerene vesicle alleviated angiotensin II-induced oxidative stress in human umbilical venous

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endothelial cells (17). The polyhydroxylated C_{60} (fullerenol) elicited an antiproliferation effect on vascular of atherosclerosis (16) and prevented degeneration of articular cartilage in osteoarthritis (21). Fullerenol has neuroprotective potency (9) and is also able to ameliorate ischemia reperfusion injury in the kidney (4, 5). Lai *et al*. reported that fullerene derivative attenuates ischemia-reperfusion-induced lung injury (13, 14). The protective effect of these water-soluble fullerene derivatives on pulmonary circulation remains unknown.

The aim of the present study was to evaluate whether [hexa(sulfobutyl)fullerene, (H C_{60})] or [C_{60}(glucosamine)$_6$, (G C_{60})] could effectively reduce direct damages of free radicals produced by xanthine/xanthine oxidase in isolated rat lungs.

Materials and Methods

Setup for the Isolated Lungs

Young female Wistar rats weighing 205 ± 4 g were used. All animal experiments and animal care were performed in accordance with the "Guides for the Care and Use of Laboratory Animals" (published by National Academy Press, Washington DC, 1996). All protocols used in this study were approved by the Laboratory Animal Care Committee of the National Taiwan University College of Medicine. The animal was anesthetized with sodium pentobarbital (40 mg/kg). Then the isolated-perfused lungs were prepared as we have described previously (13). Briefly, after insertion of a tracheal cannula, the chest was opened and the lungs were ventilated with a humidified 95% air and 5% CO_2 gas mixture under an end expiratory pressure of 2.5 cm H_2O. After the right ventricle was injected with heparin (150 IU), the pulmonary artery was cannulated and perfused with a perfusate. The perfusate was a mixture of bovine serum albumin (4 mg/100 ml) with Krebs-Henseleit buffer solution containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl{\cdot}2H_2O$, 1.2 mM $MgSO_4{\cdot}7\ H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose. A wide-bore cannula was placed in the left atrium through the left ventricle to collect the effluent perfusate for recirculation. About 50 ml of initial perfusate was discarded to clear the blood before initiating recirculation. A perfusion rate of 3 ml/min/100 g body weight was maintained by a roller pump through an air bubble trap. The heart and the lung were removed en bloc and were placed on a weighing pan, which was mounted on a Grass force transducer for detecting the changes in lung weight, and were suspended in a constant-temperature (37°C) humidified chamber. The weighing system was calibrated by placing a 2-g weight on the pan and adjusting the output to 5 cm of chart deflection. The pulmonary arterial (P_{pa}) and venous pressures (P_v) were continuously monitored with Statham pressure transducers, which were placed at the same height as the heart. The distance between the pressure transducers and the pulmonary artery and vein were 29 and 50 cm, respectively. Resistances of the connecting catheters were measured. The above measured P_{pa} and P_v were then corrected for these resistances of connecting tubings. Changes in lung weight, P_{pa}, and P_v were continuously recorded with a Grass recorder. In addition, the isolated-perfused lungs were continuously ventilated with the 95% air-5% CO_2 gas mixture with an respiratory rate of 60 times/min, a tidal volume of 2 ml and a final expiratory pressure of 2.5 cm water.

Capillary Pressure (P_c)

With a constant-flow perfusion, venous outflow was momentarily stopped for 3-4 sec at end expiration. There was a rapid rise in P_v followed by a slower but steady rise. P_c was obtained by extrapolating the slow rising component back to zero time.

Filtration Coefficient (K_{fc})

K_{fc} was determined by the gravimetric method of Drake *et al*. (6). Upon achieving an isogravimetric state, we raised the P_v rapidly by 10 cm H_2O for 10 min. This hydrostatic pressure caused the lung to gain weight promptly. This was followed by a slow but steady rise in lung weight. The rapid component represents the expansion of pulmonary blood vessels, whereas the slow component was due to fluid filtration into the interstitial space. The initial rate of fluid filtration was estimated by extrapolating the slow component to zero time in a semi-log plot. The value of the y-intercept was divided by the hydrostatic pressure challenge (ΔGPc) and normalized to 100 g of lung weight.

Experimental Protocols

We studied pulmonary vascular response to the challenge of xanthine/xanthine oxidase (X/XO). At the end of 20-min equilibration (baseline) period, baseline values for vascular parameters (P_{pa}, P_v, P_c, K_{fc}) were measured. Xanthine (500 μm) was then added to the perfusate, and 5 min later, xanthine oxidase (5 mU/ml) was also added. The vascular response were determined both before and 45 min after the challenge. Subsequently, arterial resistance [R_a, $R_a = (P_a - P_c)$/perfusion rate], venous resistance [R_v, $R_v = (P_c - P_v)$/perfusion rate], and K_{fc}, were separately calculated.

Five min before the administration of X/XO,

Table 1. Effects of some oxidative scavengers on pulmonary hemodynamic changes after xanthin/xanthine oxidase challenge in isolated rat lungs

Parameters / Groups	P_a (mmHg)		P_v (mmHg)		P_c (mmHg)		K_{fc} (g/min·mmHg·100g)		R_a (mmHg/ml·min)		R_v (mmHg/ml·min)	
	baseline	X/XO	baseline	X/XO	baseline	X/XO	baseline	X/XO	baseline	X/XO	basline	X/XO
Control (n = 7)	10.60 ± 0.41	11.14 ± 0.35*	0.46 ± 0.05	0.23 ± 0.04*	3.33 ± 0.27	3.10 ± 0.15	0.34 ± 0.06	0.89 ± 0.10*	0.97 ± 0.03	1.07 ± 0.03*	0.38 ± 0.02	0.38 ± 0.01
Superoxide dismutase (n = 7)	9.91 ± 0.24	11.10 ± 0.27*	0.15 ± 0.06	0.30 ± 0.12	2.75 ± 0.22	2.91 ± 0.15	0.48 ± 0.04	1.31 ± 0.18*	1.23 ± 0.05	1.39 ± 0.08*	0.45 ± 0.02	0.47 ± 0.02
Hexa(sulfobutyl) fullerene (n = 6)	11.98 ± 0.33	13.21 ± 0.52*	0.31 ± 0.07	0.41 ± 0.08	3.30 ± 0.11	3.51 ± 0.14	0.33 ± 0.03	0.45 ± 0.07	1.35 ± 0.04	1.51 ± 0.08	0.46 ± 0.01	0.48 ± 0.02
C_{60}(glucosamine)$_6$ (n = 6)	11.4 ± 0.74	12.16 ± 0.82*	0.65 ± 0.08	0.55 ± 0.07	3.06 ± 0.19	3.03 ± 0.12	0.24 ± 0.02	0.55 ± 0.04*	1.50 ± 0.13	1.64 ± 0.13	0.43 ± 0.03	0.45 ± 0.01

P_{pa}: pulmonary arterial pressure; P_v: pulmonary venous pressure; P_c: pulmonary capillary pressure; R_a: pulmonary arterial resistance; R_v: pulmonary venous resistance; K_{fc}: pulmonary filtration coefficient; SOD (superoxide dismutase, 150 U/ml, n = 7), Hexa(sulfobutyl)fullerene (200 μg/ml, n = 6), C_{60}(glucosamine)$_6$ (200 μg/ml, n = 6), xanthine (500 μM/xanthine oxidase (5 mU/ml)). *P < 0.05, compared with baseline.

superoxide dismutase (150 U/ml, SOD, n = 7); Hexa (sulfobutyl)fullerence, (200 μg/ml, H C_{60}, n = 6); C_{60} (glucosamine)$_6$, (200 μg/ml, G C_{60}, n = 6) or saline (as the control, SAL, n = 7), was separately given.

In order to confirm the release of free radicals after X/XO administration, free radicals were determined by the Chemiluminescence (CL) Analyzing System (CLD-110, Tohoku Electronic Industrial Co., Sendai, Japan). Samples (0.2 ml) obtained from the buffer and the perfusate 5 min and 45 min after addition of X/XO were immediately wrapped with aluminum foils and kept in an ice box until CL measurement which was usually done within 2 h. The CL was measured in an absolutely dark chamber of the Chemiluminescence Analyzing System. After 100 s, 1.0 ml of 0.1 mM lucigenin (bis-N-methylacridinium nitrate, Sigma, St. Louis, MO, USA) in PBS (pH 7.4) was injected into the cell. The CL in the sample was continuously measured for a total of 600 s. Total amount of CL was calculated by integrating the area under the curve and subtracting it from the background level which is equivalent to the dark average. The assay was performed in duplicates for the samples obtained from the buffer and perfusate and was expressed as CL counts/10 s. A means ± SE (standard error) of CL level of each sample was calculated.

Statistical Analysis

Values are expressed as means ± SE. Differences in parameters among groups were analyzed by analysis of variance. If significant differences existed among groups, statistical differences between any two groups were analyzed by the Newman-Keuls test. Differences were considered significant if $P < 0.05$. Differences between values before and after the X/XO challenge were analyzed by Student's paired *t*-test.

Results

The CL count in the buffer was 157 ± 31(CL/10 sec); 5 min and 45 min after application of X/XO, CL counts were 7,737 ± 960 (CL/10 sec) and 2,494 ± 258 (CL/10 sec), respectively. The CL count in the lung perfusate was 737 ± 213 (CL/10 sec); 5 min and 45 min after X/XO administration, the counts were 3,778 ± 425 (CL/10 sec) and 1,183 ± 193 (CL/10 sec), respectively. This confirmed the release of free radicals to challenge the lungs.

Challenge with X/XO caused a mild but significant increase in Ppa which might be due to an enhancement of R_a. A drop of P_v was found in these lungs but without changes in Rv; the capillary pressure did not alter, however, a marked increase of K_{fc} was noticed (Table 1).

Table 1 and Fig. 1 also show that pretreatment of antioxidant, did not significantly influence the hemodynamic changes after X/XO administration in all 3 groups of studies. However, K_{fc} became insignificantly increased in Hexa(sulfobutyl)fullerence-pretreated lungs.

Discussion

Recent evidence indicates that after a variety of systemic insults including thermal injury to the skin (19) and reperfusion of the ischemic liver (20), small quantities of XO my be released into the circulation. In the presence of xanthine, XO reduces oxygen to superoxide. We mimicked this action by perfusion of isolated lungs with solutions containing xanthine and xanthine oxidase resulted in changes on pulmonary circulation. The present study shows that hexa(sulfobutyl)fullerene, but not C_{60}(glucosamine)$_6$, nor SOD could attenuate oxidative stress in isolated rat lungs after challenging by X/XO.

Previous studies used very large doses of XO

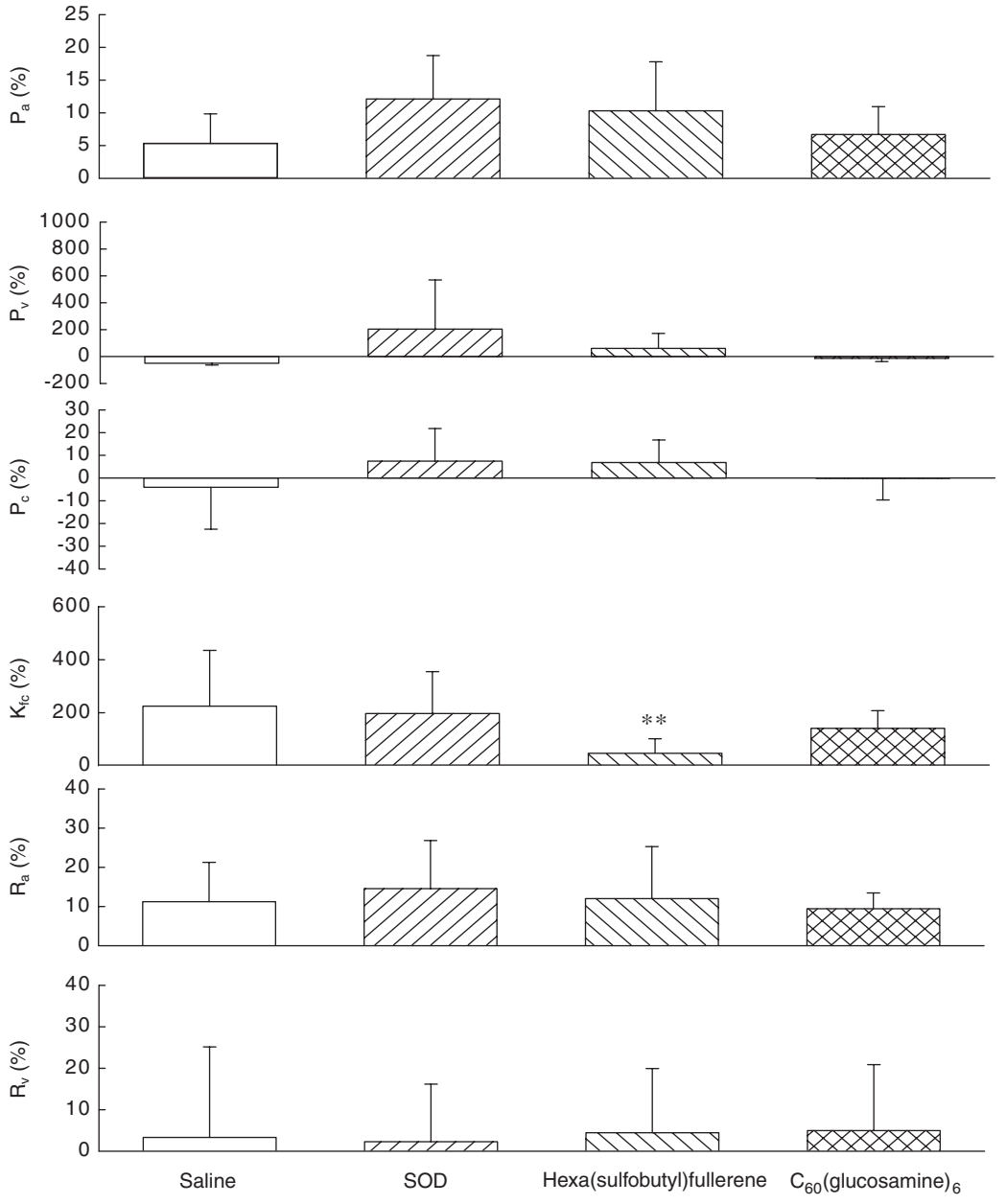

Fig. 1. Comparison of percent-changes of pulmonary hemodynamic by some scavengers in rat isolated lungs after challenges by xanthine/xanthine oxidase (X/XO).
P_{pa}: pulmonary arterial pressure; P_v: pulmonary venous pressure; P_c: pulmonary capillary pressure; R_a: pulmonary arterial resistance; R_v: pulmonary venous resistance; K_{fc}: pulmonary filtration coefficient; SOD (superoxide dismutase, 150 U/ml, n = 7), Hexa(sulfobutyl)fullerene (200 μg/ml, n = 6), C_{60}(glucosamine)$_6$ (200 μg/ml, n =6), xanthine (500 μM)/xanthine oxidase (5 mU/mL). *$P < 0.05$, between groups.

(20-100 mU), which resulted in severe pulmonary vasoconstriction and lung edema (2, 3, 9, 15). The concentration of XO (5 mU/ml) is similar to that released into the circulation after gastrointestinal ischemia and reperfusion (20). We demonstrated that instillation of this small dose of XO into the perfusate increased the pulmonary microvascular endothelial permeability as measured by the capillary filtration coefficient, which is a more sensitive index of endothelial permeability that is independent of changes in vascular pressures. Challenge with X/XO caused a marked increase of K_{fc} which might be due to this effect.

Pretreatment of SOD was ineffective (even worse) in preventing endothelial dysfunction. This result has been reported previously (1, 10). The reaction between xanthine and xanthine oxidase triggers the generation of several reactive species of

oxygen beginning with superoxide and, in subsequent reactions, hydrogen peroxide, hydrogen radical and peroxynitrite anion (7, 18). Superoxide may react directly with cellular targets dismutating to hydrogen peroxide. In the presence of certain transitional metals such as iron, superoxide and hydrogen peroxide combine to form hydroxy radicals. In addition, superoxide combines with nitric oxide to form peroxynitrite. These free radicals are all highly toxic to cells.

The protective effect of the compound may be due to its ROS removing properties, or modification of the oxidative action of X/XO. The reason why it was only effective in hexa (sulfobutyl) fullerene, but not in C_{60}(glucosamine)$_6$ is not known. Further studies are necessary.

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