Lung injury caused by cobra venom factor is reduced in rats raised on an essential fatty acid-deficient diet

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MORGANROTH, M. L., S. O. SCHOENEICH, G. O. TILL, W. PICKETT, AND P. A. WARD. *Lung injury caused by cobra venom factor is reduced in rats raised on an essential fatty acid-deficient diet.* Am. J. Physiol. 257 (Heart Circ. Physiol. 26): H1192–H1199, 1989.—Arachidonate metabolites appear to be involved in lung injury caused by cobra venom factor (CVF)-induced complement and polymorphonuclear leukocyte (PMN) activation. These studies were designed to assess the effects of a dietary-induced deficiency of arachidonic acid on CVF-induced lung injury. Rats raised on an essential fatty acid-deficient (EFAD) diet exhibited the expected changes in fatty acid composition including decreased plasma levels of arachidonic acid and increased levels of 5,8,11-eicosatrienoic acid. In intact rats raised on the EFAD diet, CVF-induced lung injury was attenuated. When blood and excised lungs from rats raised on the normal diet were used, CVF caused pulmonary vascular constriction and acute lung injury, as evidenced by increased ^{125}I-labeled bovine serum albumin accumulation in lung parenchyma and alveolar lavage fluid. The CVF-induced pulmonary artery pressor response and lung injury were reduced when blood perfusate or blood perfusate and excised lungs were obtained from rats raised on the EFAD diet. The pulmonary vascular constriction and lung injury were not attenuated when the blood perfusate was obtained from rats raised on the normal diet, irrespective of whether the excised lungs were obtained from rats raised on the normal or EFAD diet. PMNs obtained from rats raised on the EFAD diet demonstrated decreased superoxide production as well as impaired random migration and chemotaxis in vitro. In contrast, β-glucuronidase release was quantitatively similar to PMNs from control rats. These data indicate that the EFAD diet-induced attenuation of CVF-induced pulmonary hypertension and acute lung injury is due to defective effector cells in blood rather than modified pulmonary target tissue.

leukotrienes; platelet-activating factor; toxic oxygen metabolites

INTRAVENOUS INJECTION into rats of the complement activator, cobra venom factor (CVF), results in the development of acute pulmonary hypertension followed by lung injury. The lung injury has been demonstrated by morphological changes as well as accumulation of albumin in lung parenchyma and in the alveolar compartment (16). The morphological changes include plugging of pulmonary capillaries with polymorphonuclear leukocytes (PMNs) and platelets as well as destruction of vascular endothelial cells, fibrin deposition, and focal hemorrhage (16, 26). The lung injury is not dependent on the preceding pulmonary vascular constriction but is dependent on toxic oxygen metabolites and the presence of PMNs (16, 26). Cyclooxygenase and lipoxygenase metabolites of arachidonic acid are also involved in CVF-induced lung injury. Leukotrienes B_4 (LTB_4) and C_4 (LTC_4) as well as thromboxane B_2 (TxB_2) are produced in association with the lung injury (15). Production of these metabolites is important because drugs that inhibited their production attenuated the lung injury (15). However, use of drug inhibitors did not allow a determination of whether the attenuated injury was due to inhibition of arachidonate metabolite formation in blood elements or lung parenchymal cells.

In the current study we utilized a dietary approach to inhibit arachidonic acid metabolism. PMNs and chopped lung parenchyma obtained from rats raised on an essential fatty acid-deficient (EFAD) diet displayed impaired production of all biologically active sulfidopeptide leukotrienes, as well as LTB_4 (13, 20, 22). However, stimulated lung production of cyclooxygenase products was not affected (13). Platelet-activating factor (PAF) is another potent lipid, the synthesis of which is often linked to LTB_4 (23). Although the mechanism is unclear, the EFAD diet also resulted in a reduction of PMN and lung parenchymal production of PAF (13, 20, 22). The purpose of this study was to determine whether the EFAD diet attenuated lung injury caused by CVF in intact rats and isolated perfused rat lungs. The isolated lung model, in which the blood perfusate and isolated lungs can be obtained from rats on different diets (control or EFAD), permitted investigation if alterations in lung injury were mediated by effects of the EFAD diet on the lung parenchyma or blood elements. The effect of the EFAD diet on PMN function in vitro was assessed because CVF-induced lung injury is dependent on PMNs (15, 26). In this study we demonstrate that the EFAD diet reduced CVF-induced pulmonary hypertension, lung injury, and leukotriene production. The decreased lung injury could be attributed to defective effector cells in blood rather than modified lung tissue. The EFAD diet impaired PMN superoxide production, chemotaxis, and random migration in vitro.

METHODS

Rats were fed ad libitum either a normal diet of rat chow (control diet) or a vitamin-supplemented EFAD

diet (ICN Biochemical). The rat chow diet was 4.5% fat with the following distribution: 12:0, 0.1%; 14:0, 0.9%; 16:0, 14.9%; 16:1, 1.4%; 18:0, 3.6%; 18:1, 20.8%; 18:2, 47.2%; 18:3, 5.7%; 20:0, 0.3%; 20:2, 0.4%; 20:4, 0.3%; 22:6, 0.8%; unknowns and fatty acids <0.1%, 3.6%. The EFAD diet was 0.21% fat with the following distribution: 12:0, 2.5%; 14:0, 11.1%; 16:0, 29.0%; 16:1, 4.6%; 18:0, 9.2%; 18:1, 36.7%; 18:2, 33.6%; 18:3, 2.0%; 20:0, <0.1%; 20:2, 0.5%; 20:4, 0.3%; 22:6, 0.2%; unknowns and fatty acids <0.1%, 0.3%. Rats were started on the EFAD diet 3 wk after birth and continued for 4–8 mo. This duration for the diet was chosen because plasma fatty acid composition rapidly changed during the first 2 mo and then remained stable over the subsequent 7 mo.

The effect of the EFAD diet on CVF-induced lung injury in the intact rat was determined. The effect of the EFAD diet on pulmonary vascular constriction, lung injury, and arachidonic acid metabolite formation after CVF infusion was also assessed in an ex vivo isolated blood-perfused rat lung model. The isolated lung model allowed the blood perfusate and isolated lungs to be obtained from different rats. This model thus allowed an investigation of whether the effects of the EFAD diet on lung injury were due to alterations in lung parenchyma or blood elements by varying the source (control or EFAD rat) of the blood perfusate and lungs. In vitro studies were also used to assess the effect of the EFAD diet on PMN function.

Effect of EFAD Diet on Plasma Polyenoic Fatty Acid Composition

Plasma levels of 20:3 and 20:4 were monitored in a subgroup of rats maintained on the EFAD diet to determine that there was a >70% decrease in plasma arachidonic acid before use of the rat in the experimental protocols. At the time the rats were used in the experimental protocol, 500 μl of rat plasma were obtained. Ten to twenty microliters of the rat plasma were transesterified to the methyl esters by heating in methanol (8% in HCl) at 60°C for 40 min. Heptadecanoic acid (10 μg) was used as an internal standard. Analysis of the fatty acid methyl esters was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a 7673A autosampler-injector and a 3393A integrator. A DB 225 30 meter capillary column with a film thickness of 0.25 μm (J & W Scientific), utilizing helium as the carrier gas, was used to separate the methyl esters. Total fatty acids were expressed as nanograms per microliter of plasma. We have previously demonstrated that rats raised on the EFAD diet in a comparable fashion had equivalent changes in their PMN fatty acid composition (22).

Effect of EFAD Diet on CVF-Induced Lung Injury in Intact Rats

The effect of the EFAD diet on CVF-induced acute lung injury was assessed in intact Sprague-Dawley rats. CVF (20 U/kg body wt), purified and separated from cobra species *Naja naja* venom, was injected intravenously into control rats (rats raised on the normal diet) and treated rats (rats raised on the EFAD diet) to induce lung injury, as previously described (26). Thirty minutes after the concomitant injection of the CVF and ^{125}I-labeled bovine serum albumin (BSA), the anesthetized (ketamine) rats were exsanguinated. Lung injury was measured by the accumulation of ^{125}I-labeled BSA into lung parenchyma during that 30-min period as previously described (26). Lung injury was expressed as the albumin leak index, which is the ratio of radioactive counts remaining in the lung after vascular perfusion with 10 ml of saline to the radioactive counts present in 1.0 ml of blood obtained from the animal at the time of exsanguination.

Effect of EFAD Diet on CVF-Induced Pulmonary Vascular Constriction, Lung Injury, and Arachidonic Acid Metabolite Formation in Isolated Perfused Rat Lungs

Ex vivo lung preparation. An ex vivo model was used to assess lung injury and pulmonary vascular constriction after infusion of CVF (15, 26). Lungs were isolated from male Sprague-Dawley rats that were anesthetized with pentobarbital sodium (40 mg/kg ip). Lungs were isolated and perfused with heparinized homologous blood at a constant flow rate of 0.03 ml·g rat body wt^{-1}·min^{-1} as previously described (14, 16). Mean pulmonary artery inflow pressure (PA) was measured with a Statham transducer and recorded on a Grass recorder. Perfusion pressure was proportional to pulmonary vascular resistance, because the flow for a given lung was held constant. Left atrial pressure (pulmonary outflow) was set by adjusting the height of the outflow reservoir and was kept at 0 cmH_2O unless otherwise stated. The lungs were ventilated via a tracheal cannula with an air-gas mixture containing 21% O_2 and 5% CO_2 at a rate of 60 strokes/min, using a Harvard animal respirator. The lungs and perfusate reservoir were kept at a temperature of 37–40°C. Lungs were perfused for 30 min to reach a stable perfusion pressure and temperature before the initiation of all experimental protocols.

Lung perfusion. The isolated lungs and the whole blood perfusate were obtained from rats raised on the same diet (EFAD or control). Whole blood was obtained from three rats by cardiac puncture. Body weights of the control rats and treated rats from which the lungs were excised were 272 ± 13 and 437 ± 28 g, respectively.

CVF administration. CVF (15 U) was administered as a bolus injection into the pulmonary artery. Mean pulmonary artery perfusion pressure was measured before and for 60 min after the injection of CVF or normal saline. Pulmonary venous effluent was sampled before and 10 min after the injection of CVF to determine the number of circulating PMNs present, as previously described (16, 26). Because increased left atrial pressure will markedly increase lung water and accumulation of albumin in lungs with increased vascular permeability but will cause only small changes in lungs exhibiting normal vascular permeability (16), we subjected each lung to a hydrostatic challenge. Thirty minutes after the intra-arterial injection of CVF or normal saline, the reservoir draining the pulmonary venous effluent was raised 10 cm above the level of the left atrium and

remained there for the remainder of the experiment (30 min). Lung injury was measured by the amount of ^{125}I-labeled BSA that accumulated in lung parenchyma and alveolar lavage fluid, as described below.

Measurement of lung injury. ^{125}I-labeled BSA was prepared by the standard chloramine-T method. Unbound iodine was removed by gel filtration using a G-75 column. ^{125}I-labeled BSA was added to the blood reservoir 30 min after the injection of CVF. This was done in part so that the ^{125}I-labeled BSA could be added to the system after the transient pressor response to CVF, thus minimizing the effects of change in perfusion pressure on the accumulation of ^{125}I-labeled BSA in lung tissue. Thirty minutes after the addition of the ^{125}I-labeled BSA, 1 ml of pulmonary venous effluent was obtained, weighed, and counted for 2 min in a gamma counter. This allowed the determination of the number of ^{125}I-labeled BSA counts in 1.0 g of circulating perfusate.

The reservoir supplying perfusate to the lung was then changed to contain physiological salt solution osmotically stabilized with Ficoll (4 g/100 ml). After the lungs were perfused with 15 ml of this perfusate, 1.0 ml of pulmonary venous effluent was obtained and assessed for radioactivity in a gamma counter. This was done to confirm that minimal ^{125}I-labeled BSA counts (<1% of the counts present in 1.0 g of the circulating perfusate) remained within the intravascular space of the lung. Bronchoalveolar lavage was then done by repeatedly instilling and withdrawing 3.0 ml of normal saline into the tracheal cannula. One milliliter of the lavage fluid recovered was then counted for 2 min in a gamma counter to assess the amount of ^{125}I-labeled BSA that had accumulated in alveolar lavage fluid. The lung was dissected free of the attached structures. The lung tissue was then counted for 2 min in a gamma counter to assess the amount of ^{125}I-labeled BSA that had accumulated in lung parenchyma. ^{125}I-labeled BSA counts present in alveolar lavage fluid, lung tissue, and pulmonary venous effluent were normalized by dividing the number of counts measured by the number of ^{125}I-labeled BSA counts present in 1.0 g of circulating perfusate.

Measurement of arachidonate metabolites. In a separate series of experiments LTB_4, LTC_4, and TxB_2 were measured in bronchoalveolar lavage fluid obtained 45 min after the injection of CVF. This time and these metabolites were chosen because we have previously demonstrated that CVF induced their production at that time point in isolated blood-perfused rat lungs (15). Bronchoalveolar lung lavage was performed by instilling and withdrawing 10 ml of normal saline into and out of the lung via the tracheal cannula. A total of 30 ml was instilled, and 26–28 ml was recovered. After adding 2 vol of 95% ethanol to the lavage fluid sample and centrifuging it at 600 *g* for 20 min, the supernatant was evaporated to dryness in a rotoevaporator. After resuspension, the samples were extracted by passage through a C_{18} Sep-Pack (Waters). An aliquot of the extracted sample from each lung lavage sample was resuspended in 1 ml of 0.1% phosphate-buffered saline. TxB_2 was measured in that aliquot by radioimmunoassay (RIA), in duplicate, as previously described (15). Another aliquot from each extracted lavage sample was separated using reverse-phase high-performance liquid chromatography (RP-HPLC) with a gradient mobile phase of acetonitrile and water with 0.1% trifluoroacetic acid (15). An ultraviolet monitor continuously examined the eluate from the RP-HPLC column for absorbance at 230 nm for the first 30 min then 280 nm from 30 to 55 min postinjection. The retention times for synthetic LTC_4, LTD_4, and LTB_4 were established before analysis of the lavage fluid. Eluate fractions were collected each minute (1 ml). Five-hundred microliters of the eluate fractions collected were evaporated to dryness and resuspended in 1 ml of 0.1% phosphate-buffered saline and assayed in duplicate for LTB_4 and LTC_4 by RIA as previously described (15). The RIAs utilized antibodies with sensitivities of 37 and 14 pg/ml, respectively, for LTC_4 and LTB_4. Eluate fractions collected at the retention times determined for LTC_4 and LTB_4 were used to quantitate the amount of LTC_4 and LTB_4 present.

Effects of Obtaining only Lung Parenchyma or Blood from Treated Rats on CVF-Induced Vascular Constriction and Lung Injury

In these isolated perfused lung experiments the same protocols for lung preparation, CVF administration, and lung injury measurements were followed. However, for each of these experiments, the lungs and whole blood were obtained from rats raised on different diets (EFAD or control). Lungs obtained from control rats were perfused with whole blood from treated rats. Alternatively, lungs obtained from treated rats were perfused with whole blood from control rats. Because the whole blood perfusate and isolated lungs were obtained from rats raised on different diets, the first 5 ml of pulmonary venous effluent was discarded to remove blood present in the intravascular space of the excised lung. Body weights of the control rats and treated rats from which the lungs were excised were 307 ± 27 and 500 ± 12 g, respectively.

Effects of the EFAD Diet on PMN Function In Vitro

Chemotaxis assays were carried out with Boyden chambers. Activated rat serum diluted to 10% in Hanks balanced salt solution (HBSS) was added to the lower compartment of a chemotaxis chamber. Rat PMNs, obtained from glycogen (1.0%)-induced peritoneal exudates (4 h postinjection of glycogen) were washed, suspended in HBSS at a concentration of 1.5×10^6 ml^{-1}, and added to the upper compartment of the chamber. Both compartments were separated by a cellulose nitrate filter of 3-μm average pore size. After incubation in humidified air at 37°C for 90 min, the filter was removed, stained with hematoxylin, dried in isopropanol, and then cleared with xylene. The distance of migration of the PMNs within the filter was measured under light microscopy by the so-called "leading front" method of Zigmond and Hirsch (30). Tests were performed in triplicate and the mean value ± SE of three to five measurements calculated.

Superoxide anion generation was measured by the

superoxide dismutase-inhibitable reduction of ferricytochrome c according to the method of Babior, Kipnes, and Curnutte (1). The reaction mixture consists of 5×10^5 unstimulated or phorbol 12-myristate 13-acetate (PMA)-stimulated PMNs and 80 μmol ferricytochrome c (Sigma Chemical) in 0.9 ml HBSS. To one set of duplicate tubes is added 0.1 ml HBSS, and 85 U of superoxide dismutase (SOD) was added to the other set. The tubes were incubated for 60 min at 37°C after which the total volume was brought to 1.8 ml. The tubes were centrifuged and the absorbance of the supernatant fluids at 550 nm determined. The difference in absorbance between the presence and absence of SOD was determined, and the amount of ferricytochrome c reduced was calculated based on an extinction coefficient of 18.5 mM^{-1} for ferricytochrome c.

β-Glucuronidase activity was used as an indicator of PMN lysosomal enzyme release. PMN-derived β-glucuronidase activity after PMA stimulation was determined in vitro by the method of Fishman et al. (5) using phenolphthalein glucuronidate as a substrate.

Statistical Analysis

Values are expressed as means ± SE. One-way analysis of variance and the Student-Newman-Keuls test were used when multiple groups were compared. A paired t test was used when responses in the same lung were compared. $P \leq 0.05$ was accepted as significant.

RESULTS

Effect of EFAD Diet on Plasma Polyenoic Fatty Acid Composition

Plasma levels of 20:3 and 20:4 were monitored in a subgroup of the rats maintained on the EFAD diet. Plasma fatty acid composition (in ng/μl) in weaned rats before initiation ($n = 3$) of the EFAD diet was 16:0, 0.8 ± 0.1; 16:1, 0.04 ± 0.05; 18:0, 0.45 ± 0.02; 18:1, 0.79 ± 0.12; 18:2ω6, 0.81 ± 0.12; 18:2ω9, <0.01; 18:3, 0.04 ± 0.02; 20:3ω9, <0.01; 20:4, 0.72 ± 0.3; 20:5, 0.04 ± 0.02; and 22:6, 0.1 ± 0.05. The ratio of 20:3 to 20:4 and the percent depletion of arachidonic acid in plasma were 0.25 ± 0.2 and 11 ± 3% 2 wk ($n = 4$) after the initiation of the EFAD diet. The ratio of 20:3 to 20:4 and the percent depletion of arachidonic acid increased to 1.1 ± 0.4 and 60 ± 15%, respectively, 1 mo ($n = 5$) after the start of the EFAD diet and to 4.1 ± 0.3 and 80 ± 6%, respectively, 2 mo ($n = 5$) after the initiation of the EFAD diet. The ratio of 20:3 to 20:4 and the percent depletion of arachidonic acid was 5.2 ± 1.2 and 90 ± 5%, respectively 9 mo ($n = 3$) after the initiation of the EFAD diet.

Plasma fatty acid composition was also determined in each rat at the time the rat was used in the experimental protocol (Table 1). Plasma from rats raised on the EFAD diet ($n = 10$) had 23 ± 2% (0.16 ± 0.01 ng/μl) of the mean amount of arachidonic acid found in the plasma of rats raised on the control diet (0.68 ± 0.11 ng/μl, $n = 10$). In contrast, 5,8,11-eicosatrienoate was present in the plasma (0.79 ± 0.10 ng/μl) of the rats raised on the EFAD diet but was below (<0.01 ng/μl) the level of detection in plasma from rats raised on the control diet.

TABLE 1. *Absolute amounts of fatty acids in plasma obtained from EFAD and control rats when used in experimental protocols*

Fatty Acid*	EFAD, ng/μl plasma	Control, ng/μl plasma
16:0	0.75±0.09	0.75±0.06
16:1	0.41±0.05†	0.09±0.03
18:0	0.46±0.07	0.51±0.02
18:1	1.25±0.16	0.90±0.08
18:2ω9	0.70±0.16	ND
18:2ω6	ND	0.95±0.07
18:3	0.13±0.01	0.08±0.01
20:3ω9	0.79±0.10	ND
20:4	0.16±0.01†	0.68±0.11
20:5	ND	0.04±0.02
22:6	0.16±0.01	0.21±0.06

Values are means ± SE. * Carbon chain length is indicated before colon, and number of unsaturations is indicated after colon. ω indicates number of carbons from end to last double bond. EFAD refers to rats raised on essential fatty acid-deficient diet ($n = 10$); control refers to rats raised on normal diet ($n = 10$). ND, none detected (<0.01 ng/μl plasma). † $P < 0.05$.

TABLE 2. *CVF-induced acute lung injury in EFAD rats*

Animals	n	Treatment	Lung Injury*
Control	4	CVF (15 U/kg)	1.26±0.13
EFAD	4	CVF (15 U/kg)	0.76±0.11†
EFAD	2	Saline	0.16±0.03
Control	2	Saline	0.18±0.04

Values are means ± SE; n, no. of rats per group. * Lung injury equals ^{125}I-bovine serum albumin (BSA) counts in lung parenchyma divided by ^{125}I-BSA counts in 1 ml of blood. EFAD, rats fed an essential fatty acid-deficient diet; CVF, cobra venom factor. † $P < 0.05$ compared with CVF injection in control rats.

Elevated levels of 16:1 and 18:2ω9, as well as decreased levels of 18:2ω6 and 20:5, were also observed in the EFAD plasma.

Effect of EFAD Diet on CVF-Induced Lung Injury in Intact Rat

CVF caused a large increase in ^{125}I-labeled BSA accumulation in the lung parenchyma of rats raised on the control diet (Table 2). CVF caused less ^{125}I-labeled BSA accumulation in the lung parenchyma of treated rats. ^{125}I-labeled BSA accumulation in lungs after the injection of normal saline did not appear different in treated or control rats (Table 2).

Effect of EFAD Diet on CVF-Induced Pulmonary Vascular Constriction, Lung Injury, and Arachidonic Acid Metabolite Formation in Isolated Perfused Lungs

Ten minutes after perfusion of control lungs with blood from control rats containing 15 U CVF, the PMN count in the pulmonary venous effluent decreased (percent of initial pulmonary effluent PMN count, 46 ± 6%; $n = 5$, $P < 0.05$). Addition of CVF to the lung perfusate also resulted in an increase in PA pressure (Fig. 1). The peak increase in PA pressure occurred within 15 min after the administration of CVF (Table 3), with the PA pressure then slowly returning toward base line. Lung injury also occurred as evidenced by increased accumu-

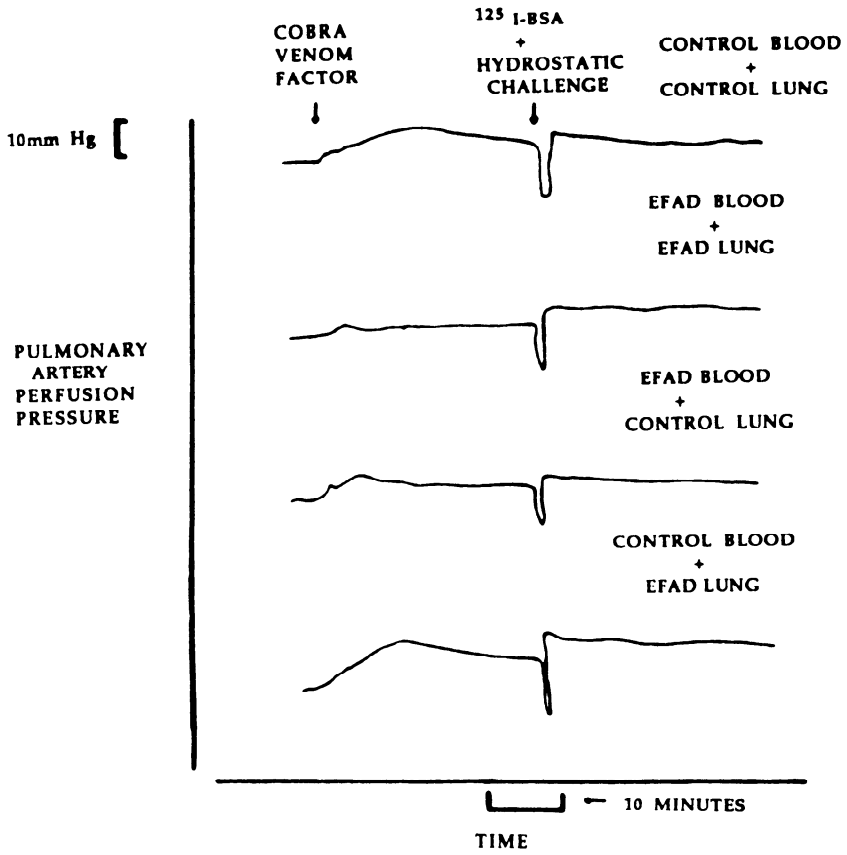

FIG. 1. Typical tracings of effect of cobra venom factor (CVF) on pulmonary artery perfusion pressure. Control blood or essential fatty acid-deficient (EFAD) blood refers to blood perfusate being obtained from rats raised on normal or EFAD diet. Similarly, control lung or EFAD lung refers to excised isolated lung being obtained from a rat raised on normal or EFAD diet. *Top tracing*, control blood and control lung; *second tracing*, EFAD blood and EFAD lung; *third tracing*, EFAD blood and control lung; *bottom tracing*, control blood and EFAD lung. Addition of CVF and ^{125}I-bovine serum albumin to lung perfusate are indicated by downward arrows. Timing and resultant change in pulmonary artery perfusion pressure to hydrostatic challenge are also indicated.

TABLE 3. *Effects of EFAD diet on response to CVF in isolated perfused lungs*

Rat's Diet		Intervention	PA Pressor Response, mmHg		Albumin Leak Index		PA Pressure During Albumin Accumulation, mmHg
Lung	Blood perfusate		Pressure before CVF	Increase in pressure after CVF	Lung parenchyma	Alveolar lavage	
Control	Control	CVF (9)	13±0.4	13±1.9	0.78±0.12	0.093±0.026	26±1.9
Control	Control	Saline (6)	13±0.8†	1±0.2*	0.21±0.02*	0.010±0.002*	26±2.6†
EFAD	EFAD	CVF (4)	14±0.6†	4±0.7*	0.32±0.04*	0.018±0.003	24±0.8†
EFAD	Control	CVF (4)	15±0.5†	14±3.6†	1.02±0.08†	0.050±0.014†	22±1.5†
Control	EFAD	CVF (6)	11±0.6*	6±1.5*	0.47±0.08*	0.025±0.006*	21±2.9†

Values are means ± SE of no. of animals per group given in parentheses. Albumin leak index was calculated by dividing ^{125}I-bovine serum albumin (BSA) counts in lung tissue or alveolar lavage fluid by ^{125}I-BSA counts in 1.0 g of perfusate. EFAD, essential fatty acid-deficient diet. * $P < 0.05$ compared with effect of cobra venom factor (CVF) when blood perfusate and lung were obtained from control rats; † P = NS compared with effect of CVF when blood perfusate and lung were obtained from control rats.

lation of ^{125}I-labeled BSA in lung parenchyma and alveolar lavage fluid (Table 3). The increase in ^{125}I-labeled BSA accumulation did not likely reflect hydrostatic changes, since the PA pressures measured during the period of ^{125}I-labeled BSA accumulation were not different from that measured in the control lungs (Table 3).

When the excised lungs and blood perfusate were obtained from rats raised on the EFAD diet, CVF caused the PMN count in the pulmonary venous effluent to decrease ($P < 0.05$) to 49 ± 9% of the initial value. This decrease in pulmonary venous effluent PMN count was not different compared with the control rats (P = NS). However, CVF caused a smaller peak increase in PA pressure when the blood perfusate and excised lungs were obtained from the treated rats (Fig. 1, Table 3). CVF-induced accumulation of ^{125}I-labeled BSA in lung parenchyma and alveolar lavage fluid was also reduced (Table 3). The decreased ^{125}I-labeled BSA accumulation was not explained by hydrostatic changes because the PA pressures measured during the period of ^{125}I-labeled BSA accumulation were not different in the control or treated rats (Table 3).

Multiple peaks of increased ultraviolet absorbance were present both at and surrounding the retention times determined for LTC_4, LTD_4, and LTB_4 when lavage fluid obtained 45 min after the administration of CVF was separated and analyzed by RP-HPLC. Thus peaks of ultraviolet absorbance caused by LTC_4 or LTB_4 were not identified in lavage fluid from the control perfused rat lungs or the treated perfused rat lungs. However, immunoractive LTC_4 (1,306 ± 307 pg) and LTB_4 (352 ± 32 pg) were identified in the RP-HPLC eluate fractions collected at the respective retention times determined for these lipoxygenase metabolites in the control lungs

($n = 4$). CVF administration in the treated lungs ($n = 4$) resulted in the recovery of less immunoreactive LTC_4 (314 ± 97 pg, $P < 0.05$) and LTB_4 (200 ± 54 pg, $P = 0.05$). Immunoreactive TxB_2 was not different (P = NS) in the control (2,528 ± 335 pg) or treated (2,135 ± 397 pg) rats.

Effects of Obtaining Only Lung Parenchyma or Blood from Treated Rats on CVF-Induced Vascular Constriction and Lung Injury

The fall in pulmonary venous effluent PMN counts 10 min after the administration of CVF was not different (P = NS) when the blood perfusate was obtained from control rats, irrespective of whether the excised lung was obtained from a control rat or a rat raised on the EFAD diet. In this latter group, the pulmonary effluent PMN count was 42 ± 10% ($n = 3$) of the initial value at this time point. Similarly, the CVF-induced pulmonary vascular constriction or accumulation of ^{125}I-labeled BSA in lung parenchyma or alveolar lavage fluid were not reduced when lungs from treated rats were perfused with control blood (Table 3). In contrast, when control lungs were perfused with blood from treated rats, the CVF-induced pulmonary vascular constriction and lung injury, as assessed by ^{125}I-labeled BSA accumulation in lung parenchyma and alveolar lavage fluid, were reduced (Table 3). However, there was no effect (P = NS) on pulmonary venous effluent PMN counts measured 10 min after the administration of CVF (percent of initial PMN count, 65 ± 6%; $n = 6$). The decrease in ^{125}I-labeled BSA accumulation was not explained by hydrostatic changes because the PA pressures measured during the period of ^{125}I-labeled BSA accumulation were not different in this group or when treaed lungs were perfused with control blood (Table 3).

Effect of EFAD Diet on PMN Function in Vitro

Addition of PMA to PMNs obtained from control rats resulted in degranulation as evidenced by the release of β-glucuronidase and the generation of superoxide as evidenced by ferricytochrome c reduction (Table 4). PMA-induced β-glucuronidase release was not significantly reduced in PMNs obtained from treated rats. However, PMNs from the treated rats displayed impaired superoxide production, random migration, and chemotaxis compared with PMNs obtained from control rats (Table 4).

TABLE 4. *Neutrophil functions in EFAD rats*

Rat's Diet	β-Glucuronidase, %release	O_2^- Production, nmol·60 min^{-1}·4 × 10^6 PMN^{-1}	Random Migration, μm	Chemotaxis, μm
Control	9.7±0.8 (4)	38.6±6.1 (7)	58.0±1.7 (3)	137.7±1.5 (3)
EFAD	7.6±1.9 (4)*	22.6±4.2 (7)†	36.7±5.5 (3)†	82.8±5.0 (3)†

Values are means ± SE of no. of animals given in parentheses. Values for β-glucuronidase and O_2^- production were determined after stimulation with phorbol 12-myristate 13-acetate. Random migration refers to distance of migration against buffer (Hanks' balanced salt solution). Chemotaxis was induced by 10% zymosan-activated normal rat serum. EFAD, essential fatty acid-deficient diet; PMN, neutrophils. * P = NS; † $P < 0.05$.

DISCUSSION

Although eicosanoids appear to be involved in the full expression of complement and PMN-mediated acute lung injury (3, 7, 15, 17, 24, 27), their source of production as well as their pathophysiolgocial roles are not known. This study demonstrated that CVF-induced lung injury was reduced in isolated perfused rat lungs when the isolated lung and blood perfusate were obtained from rats raised on the EFAD diet. Lung injury was measured by the accumulation of ^{125}I-labeled BSA in lung parenchyma or alveolar lavage fluid. The EFAD diet did not affect pulmonary vascular pressures measured during the period of ^{125}I-labeled BSA accumulation. Therefore, decreased ^{125}I-labeled BSA accumulation caused by the EFAD diet was not explained by hydrostatic pressure changes. The effect of the EFAD diet on CVF-induced lung injury was not peculiar to the ex vivo lung preparation, since CVF also caused less lung injury in intact rats raised on the EFAD diet.

The EFAD diet resulted in the characteristic decrease in plasma arachidonic acid and increased 20:3 as expected (25). We previously demonstrated the effect of the EFAD diet on the phospholipid fatty acid composition and phospholipid subclass distribution in PMNs obtained from rats raised on this diet (22). These PMNs demonstrated similar changes to those we found in rat plasma including decreased arachidonic acid and increased 20:3. Although other fatty acids including 16:1 and 18:2ω9 were elevated, as is characteristic of this deficiency, the increased level of 20:3 is of particular interest. Eicosatrienoic acid (20:3) uniformly replaces arachidonic acid in the major phospholipids and their subclasses (18, 22) but is less efficiently hydrolyzed to the free fatty acid (21). Additionally, eicosatrienoic acid has been shown to inhibit leukotriene A hydrolase, thus diminishing LTB_4 concentration by enzymatic inhibition as well as substrate depletion (25). We have previously demonstrated that these changes are associated with decreased production in stimulated lungs of slow reacting substance and PAF-like material (13). In addition, we have demonstrated that calcium ionophore- and *f*-methionine-leucine-phenylalanine-stimulated PMNs from animals raised on the EFAD diet produced less LTB_4 and PAF (20). Although the EFAD diet is associated with the production of less biologically active lipoxygenase metabolites of arachidonic acid, there was no decrease in stimulated production of cyclooxygenase metabolites of arachidonic acid (13). These effects of the EFAD diet on arachidonate metabolite formation were confirmed in the present study. The EFAD diet inhibited CVF-induced formation of LTB_4 and LTC_4 without affecting the production of TxB_2. The mechanism by which the EFAD diet results in these changes is unclear but indicates a more complex relationship than just substrate depletion (11). Similarly, the mechanisms responsible for the increased plasma levels of 16:1 and 18:2ω9 in EFAD plasma are unclear.

These are several mechanisms by which the EFAD diet could attenuate CVF-induced lung injury. The EFAD diet may be inhibiting the CVF-induced lung injury by inhibiting production of lipoxygenase metabo-

lites. Our data supports this hypothesis because the EFAD diet inhibited the lung injury as well as the production of leukotrienes. This is also consistent with previous data demonstrating that drug inhibitors of the 5-lipoxygenase pathway inhibited CVF-induced lung injury and the associated leukotriene production (15). Lipoxygenase metabolites have also been postulated to be involved in other models of complement and PMN-dependent lung injury. For example, endotoxin-induced lung injury, was associated with production of the lipoxygenase metabolite 5-hydroxyeicosatetraenoic acid (17). The EFAD diet could also have inhibited CVF-induced lung injury by inhibiting PAF production. PAF production has been demonstrated after endotoxin-induced lung injury in isolated rat lungs (2). In addition, PAF receptor antagonists reduced the endotoxin-induced hemodynamic changes and lung injury. Evaluation of whether PAF is involved in CVF-induced lung injury will require determining whether PAF is produced in association with the injury and whether PAF receptor antagonists or synthesis blockers (when available) attenuate the injury. The EFAD diet was not likely reducing the injury by inhibiting the cyclooxygenase pathway of arachidonic acid metabolism because TxB_2 production was not affected. Effects of decreased arachidonic acid on phosphotidylinositol (4) and changes in calcium flux (8) or membrane fluidity could also be involved in the attenuation of CVF-induced lung injury. Finally, the effects of increased 16:1 and 18:2ω9 on arachidonate or PAF metabolism as well as on the lung injury are unclear.

The major finding of this study was that a defect in blood constituents rather than a modification in the lung was responsible for the attentuation of the CVF-induced lung injury caused by the EFAD diet. However, the precise defect is unclear. Impaired release of toxic granule constituents by activated PMNs was an unlikely mechanism because their release by PMNs in vitro was not reduced by the EFAD diet. Impaired PMN production of toxic oxygen metabolites secondary to the EFAD diet may be in part responsible for the attenuated lung injury. However, the degree of impairment in oxygen metabolite production demonstrated in vitro was not proportional to the reduction in injury. Furthermore, toxic oxygen metabolite production must be markedly inhibited to attenuate lung injury or endothelial cell cytotoxicity caused by activated PMNs or chemically generated oxygen metabolites (10, 12, 16). The ability of PMNs to inflict lung injury might also be attenuated by mechanisms independent of reduced toxic oxygen metabolite release. For instance, it is possible that the EFAD diet-induced decrease in chemotaxis and random migration of PMNs in vitro was due to a defect in the cells ability to adhere or undergo shape change. We have previously demonstrated that interference with PMN adhesion can reduce lung injury, despite ongoing PMN production of toxic oxygen metabolites and granule constituents (9). The defects present in vitro, however, did not clearly affect the decrease in pulmonary venous effluent PMN counts in the ex vivo model after activation by CVF. Alternatively, blood constituents other than PMNs or their interactions with PMNs may have been affected by the EFAD diet. Finally, because PAF and leukotrienes can directly cause microvascular injury (19, 28), the EFAD diets' potential inhibition of PAF and leukotriene production may have directly attenuated the lung injury.

The mechanism by which the EFAD diet attenuates the CVF-induced pulmonary hypertension is also unclear. The effect of the EFAD diet was dependent on an effect on blood constituents rather than the lung itself. Thus altered lung vascular reactivity shown previously to result from the EFAD diet (13) is not a likely explanation for the reduced CVF-induced PA pressor response. Because inhibition of the lipoxygenase pathway did not attenuate CVF-induced pulmonary hypertension (15), it is unlikely that the EFAD diet is inhibiting the CVF-induced pressor response by decreasing production of sulfidopeptide leukotrienes. Similarly, because catalase does not inhibit the CVF-induced PA pressor response (16), impaired toxic oxygen metabolite production is not likely responsible for decreased CVF-induced pulmonary hypertension. PAF is a potent pulmonary vasoconstrictor (28). Because the EFAD diet has been shown to decrease production of PAF (13, 20), we cannot exclude that this effect of the EFAD diet resulted in reduced CVF-induced pulmonary vascular constriction. Further investigation of the potential role of PAF in CVF-induced pulmonary hypertension will require studies using specific pharmacological inhibitors of PAF when such agents become available as well as the determination of whether PAF production is temporally related to CVF-induced changes in PA pressure.

When taken together, the EFAD diet resulted in reduced CVF-induced lung injury by impairing the ability of complement to stimulate blood constituents to cause lung injury. Whether these changes are caused by decreased production of lipoxygenase metabolites of arachidonic acid or PAF or other potential effects of the EFAD diet is unknown. In addition, the precise function of these mediator substances as well as their cell of origin are not known.

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