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PROTECTION BY VITAMIN B_2 AGAINST OXIDANT-MEDIATED ACUTE LUNG INJURY

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Abstract—The effect of vitamin B_2 (riboflavin) on oxidant-mediated acute lung injury has been examined in three different rat models. Pulmonary injury was induced by intravenous injection of cobra venom factor (CVF), by the intrapulmonary deposition of IgG immune complexes, or by hind limb ischemia-reperfusion. In each of the three models, injury was characterized by increases in vascular permeability (leakage of ^{125}I-labeled bovine serum albumin), alveolar hemorrhage (extravasation of ^{51}Cr-labeled rat erythrocytes), and neutrophil accumulation (myeloperoxidase activity). Intraperitoneal administration of riboflavin at a dose of 6 μmoles/kg body weight reduced vascular leakage by 56% in the CVF model, by 31% in the immune complex model, and by 53% in the lung injury model following ischemia-reperfusion of the hind limbs. Similar treatment reduced hemorrhage by 76%, 51%, and 70% in the three models of lung injury. In the CVF model, riboflavin was also shown to decrease products of lipid peroxidation (conjugated dienes) in lungs (by 45%) and in plasma (by 74%). Neutrophil accumulation in the lungs was not influenced by riboflavin administration in any of the three models. The studies demonstrate that riboflavin can mount a significant protection against oxidant-mediated inflammatory organ injury.

INTRODUCTION

Oxygen-derived free radicals have been shown to participate in many forms of acute inflammatory reactions (1, 2), and have also been related to the development of complement- and neutrophil-dependent acute lung injury in various experimental rat models (3–6). For example, catalase or superoxide dismutase significantly attenuated lung microvascular injury in rats that had developed acute lung injury as a consequence of systemic complement activation follow-

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ing the injection of cobra venom factor (3). Immune complex-induced alveolitis in the rat has also been shown to be oxygen radical-dependent (4). More recently, ischemia-reperfusion injury in rat hind limbs has been shown to cause remote pulmonary injury as demonstrated by the development of lung microvascular damage and alveolar hemorrhage (5). In this model, administration of various antioxidants resulted in a significant reduction in the microvascular injury observed in lungs and limb muscle, suggesting that oxygen radicals are involved as well.

Several natural antioxidants and detoxifying redox enzymes exist in biological systems, including glutathione, ascorbic acid, vitamin E, catalase and superoxide dismutase (7, 8). A derivative of vitamin B_2 has been shown to protect mitochondria from adriamycin-induced oxidative stress (9) and vitamin B_2, riboflavin, has been proposed to function as an antioxidant in protecting against reperfusion injury (10). Riboflavin easily passes into cells, where an intracellular flavin reductase can catalyze its reduction (*rxn* 1). The product of this catalysis, dihydroriboflavin, rapidly reduces the higher oxidation states of a number of hemeproteins (*rxn* 2) and,

$$\text{NADPH} + \text{Riboflavin} \rightarrow \text{NADP}^+ + \text{Dihydroriboflavin} \quad (1)$$

$$\text{Dihydroriboflavin} + \text{Hemeprotein-}H_2O_2 \rightarrow \text{Riboflavin} + \text{Hemeprotein} + 2H_2O \quad (2)$$

$$\text{Hemeprotein} + H_2O_2 \rightarrow \text{Hemeprotein-}H_2O_2 \quad (3)$$

in the case of the reduction of the myoglobin-H_2O_2 complex, protects the protein against oxidative damage (11–13). Since the higher oxidation states of iron (generated in *rxn* 3 by reaction with peroxides) may contribute appreciably to the oxidative damage of cells under pathological conditions, the findings suggested that *rxn*s 1 and 2 might constitute a means by which cells can be protected from such damage. Preliminary studies have indeed provided evidence that riboflavin does protect cells and organs from oxidative damage (10, 13–15).

In the present study, we examined the ability of riboflavin to protect rats against oxygen radical-mediated lung injury whether induced by the intravenous injection of cobravenom factor (CVF), the intrapulmonary deposition of IgG immune complexes, or following bilateral hind limb ischemia-reperfusion. We provide evidence that riboflavin, in all three types of rat pulmonary injury, reduces both lung microvascular leakage and alveolar hemorrhage. This, and our observation that riboflavin treatment also attenuates the appearance of lipid peroxidation products in lungs and plasma of injured animals, suggests that riboflavin may play a role as antioxidant in inflammatory reactions.

METHODS

Animal Models. For all experimental models, specific pathogen-free male Long-Evans rats (300–350 g) (Charles River Laboratories, Portage, Michigan) were used. Rats were anesthetized initially by an intraperitoneal injection of ketamine hydrochloride (150 mg/kg body weight (BW); Parke Davis, Morris Plains, New Jersey). Rats subjected to ischemia-reperfusion injury received an additional injection of butorphanol (2 mg/kg BW; Bristol-Myers, Evansville, Indiana). Subsequent doses of ketamine (50 mg/kg) and butorphanol (2 mg/kg per 4 h) were given to sustain anesthesia throughout the experiments.

All experiments were performed according to the standards in "The Guide for the Care and Use of Laboratory Animals," DHEW Publication No. (NIH) 78/23, and were supervised by the Unit for Laboratory Animal Care of the University of Michigan Medical School. Unless otherwise indicated, all reagents were purchased from Sigma Chemical Corp. (St. Louis, Missouri).

CVF-Induced Lung Injury. Anesthetized rats were injected intravenously with 20 units of cobra venom factor (CVF) per kg body weight together with an aliquot of ^{125}I-labeled bovine serum albumin (^{125}I-BSA) and ^{51}Cr-labeled rat erythrocytes (^{51}Cr-RBC) (see below). After 30 min, animals were exsanguinated via the posterior vena cava. The lungs were then removed and the pulmonary vasculature was flushed with 10 ml phosphate-buffered saline (PBS). The amount of radioactivity remaining within the lung tissue was assessed with a gamma scintillation counter. Lung damage was defined by increases in lung vascular leakage (^{125}I-BSA) and lung hemorrhage (^{51}Cr-RBC). Further details are given below.

IgG Immune Complex Alveolitis. Anesthetized rats simultaneously received 2.5 mg anti-BSA (rabbit polyclonal IgG, Organon Teknika, West Chester, Pennsylvania) in 0.3 ml saline by intratracheal administration and 10 mg BSA in 0.5 ml saline by intravenous injection. Animals were killed after 4 h, the lungs were removed and the vasculature was flushed with 10 ml of PBS. Vascular permeability and hemorrhage were assessed as mentioned below.

Ischemia-Reperfusion Injury. Anesthetized rats received a tourniquet which was placed on both hind limbs proximal to the trochanter major. After 4 h of ischemia, the tourniquet was released and reperfusion proceeded for up to 4 h. During ischemia, no blood flow was detectable using a laser Doppler velocimeter (LD 6000 Med Pacific, Seattle, Washington), whereas normal blood flow was detectable within the first 10 minutes of reperfusion. Animals were killed after 4 h of reperfusion by an overdose of ketamine. Again, the lungs were removed for injury determinations, and blood was drawn from the posterior vena cava. Furthermore, muscle specimens (5 g) from each hind limb were taken.

Vascular Permeability and Hemorrhage. For assessment of lung and muscle vascular permeability, 0.5 ml of sterile 0.9% saline containing 0.5 μCi ^{125}Iodine-labeled bovine serum albumin (^{125}I-BSA) was injected intravenously at the time of tourniquet release. The ^{125}I-BSA was prepared by incubating 100 μCi ^{125}I in 10 ml of 2.5% BSA-phosphate-buffered saline solution (PBS) for 30 min at 21°C. Hemorrhage was measured by intravenous injection of 0.5 ml ^{51}Cr-labeled rat erythrocytes (RBC) at the time of tourniquet release. To prepare ^{51}Cr-labeled rat RBC, 9 ml of rat blood were diluted with 40 ml saline containing heparin (10 U/ml). Then 100 μCi ^{51}Cr were added, and the RBC were incubated for 1 h at 37°C with continuous shaking. After centrifugation at 200 × g (at 4°C) for 6 min, the red cells were washed three times in PBS and the radioactivity determined (approx. 80,000 cpm/0.5 ml).

In the experiments, ^{51}Cr-labeled RBC were injected intravenously together with the ^{125}I-BSA. At the end of each experiment, 1 ml of venous blood was drawn from the posterior vena cava after laparotomy. Lungs were then removed and the vasculature flushed with 10 ml of 0.9% saline via the pulmonary artery. From each hind limb the crural muscle group (5.0 g) was harvested after dissection of the skin. Tissue and blood samples were counted using a gamma scintillation counter

(Gamma Trac 1191, TM Analytic, ELS Grove Village, Illinois). Lung or muscle vascular leakage and hemorrhage values were expressed as the tissue to blood ratio of ^{125}I-BSA and ^{51}Cr, respectively.

Riboflavin (Vitamin B_2). Riboflavin was prepared as a saturated solution (approx. 300 μM) in 0.9% saline (at 37°C) containing 50 mM glucose. The glucose served as a metabolite to allow intracellular reduction of riboflavin. The riboflavin solution is characterized by a pH of 7.40, conductivity of 135 μS/cm and an osmolarity of 308 mosmol/l. For dose-response experiments, 150 μM and 75 μM concentrations of riboflavin were also used.

Experimental Groups. In the CVF model, one group of animals (n = 9, 300–350 g body weight) received 2 μmoles riboflavin/kg (between 2 and 2.3 ml of the 300 μM riboflavin solution) intravenously 10 minutes prior to CVF. The positive control group (n = 9) received intravenously glucose-saline solution with no riboflavin prior to CVF. Other groups of rats were injected intraperitoneally 10 minutes prior to CVF with 6 μmoles riboflavin/kg (6–7 ml of a 300 mM riboflavin solution; n = 9), 3 μmoles riboflavin/kg (6–7 ml of a 150 μM riboflavin solution; n = 6), or 1.5 μmoles riboflavin/kg (6–7 ml of a 75 μM riboflavin solution; n = 6). Positive control animals (n = 9) received the glucose-saline solution with no riboflavin intraperitoneally prior to CVF. Negative controls (n = 6) were anesthetized and received glucose-saline but no riboflavin or CVF.

Animals subjected to immune complex-mediated alveolitis received intraperitoneally 1.5 μmoles riboflavin/kg body weight (between 1.5 and 1.75 ml of the 300 μM riboflavin solution) 10 minutes prior to injury, followed by subsequent injections of 1.5 μmoles riboflavin/kg at 30, 60, and 120 minutes (n = 9). Positive control animals were treated identically but without riboflavin (n = 9). A negative control group was anesthetized and received glucose-saline solution only (n = 6).

In the ischemia-reperfusion model, animals (n = 8) were injected intraperitoneally with 1.5 μmoles riboflavin/kg (between 1.5 and 1.75 ml of the 300 μM riboflavin solution) 10 minutes prior to reperfusion, followed by subsequent injections of 1.5 μmoles riboflavin/kg at 30, 60, and 120 min of reperfusion. Positive control animals (n = 6) received only the glucose-saline solution in the same protocol. To assess the effect of ischemia itself, one group (n = 6) of animals was sacrificed at the end of the ischemia phase. A negative control group was anesthetized and received glucose-saline solution only (n = 6).

Tissue Myeloperoxidase Content. Neutrophil sequestration in lungs and muscle was assessed by measuring the myeloperoxidase (MPO) content. As has been demonstrated, MPO activity directly correlates with the number of neutrophils in the tissues (16). The total lung and the dissected muscle were first homogenized (Tissumizer, Tekmar Comp., Cincinnati, Ohio) in 50 mM potassium phosphate buffer (pH 6.0) with 5 mM EDTA and 0.5% hexadecylmethyl ammonium bromide (HTAB). The suspension was then sonicated (W 380, Sonicator, Heat Systems-Ultrasonics Inc., Farmingdale, New York) and centrifuged at 3000 × g for 30 min at 4°C. The supernatant fraction (50 μl aliquot) was added to 1.45 ml of 100 mM potassium phosphate buffer containing 3% hydrogen peroxide and 1% o-dianisidine hydrochloride (ODH). Myeloperoxidase activity was assayed by recording the change in absorbance at 460 nm over 3 min.

Lipid Peroxidation. Lung and plasma samples from rats subjected to CVF-induced lung injury were evaluated for lipid peroxidation by measuring conjugated dienes as previously described (17). Briefly, plasma samples were dissolved in a chloroform/methanol mixture (2 : 1, v/v) and then centrifuged at 1500 × g. The bottom layer was dried under nitrogen gas and its residue then dissolved in heptane for reading the absorbance of conjugated dienes at 233 nm. Tissue samples were first homogenized and then processed as described for plasma.

Statistical Analysis. All data were first subjected to one- and two-way analysis of normal variance (ANOVA). Individual group mean values were then compared using Student's t test. A value of $P < 0.05$ was considered to be significant. All data in the graphs are presented as mean ± standard error of the mean (*SEM*). For calculation of % protection, mean negative control (saline, sham) values or the "ischemia only" values were first subtracted from all positive and treatment

group values. The difference between the positive control and treatment values was then expressed as percentage of the positive control.

RESULTS

CVF Lung Injury Model. Intravenous injection of CVF (20 units/kg BW) resulted in a six-fold increase in lung vascular permeability within 30 minutes. Intravenous injection of riboflavin (2 μmoles/kg body weight) in this model resulted in a 39% reduction (Figure 1) of lung vascular permeability ($P < 0.02$). In order to examine the effect of increased concentrations of riboflavin, larger volumes of the saturated solution were injected. Here, the riboflavin was administered intraperitoneally in order to avoid hemodynamic changes due to increased blood volume. Administration of 6 μmoles riboflavin/kg produced a protection of 56% in vascular permeability ($P < 0.01$, Figure 1) and reduced ^{51}Cr-RBC extravasation by 76% ($P < 0.01$, Table 1). When 3 μmoles riboflavin/kg were

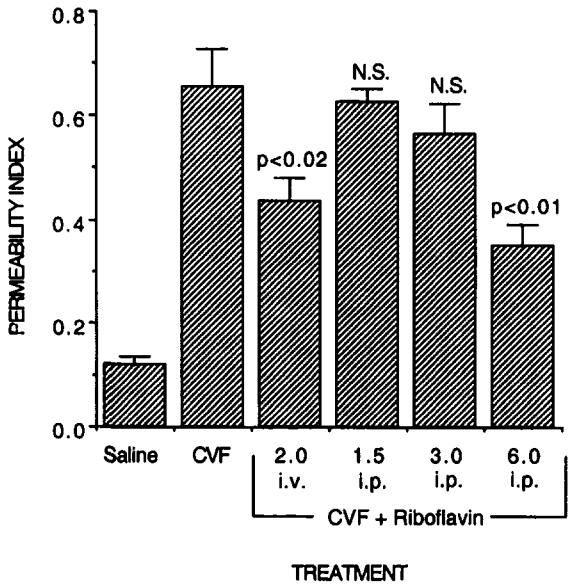

Fig. 1. Dose-dependent protective effect of riboflavin on cobra venom fctor (CVF)-induced acute lung injury in the rat. Intravenous injection of 2 μmoles riboflavin/kg body weight as well as intraperitoneal injection of 6 μmoles riboflavin/kg (but not 3 or 1.5 μmoles/kg of riboflavin) provided significant protection against lung microvascular leakage of ^{125}I-labeled bovine serum albumin as measured at 30 minutes after CVF injection.

Table 1. Effect of Riboflavin on Alveolar Hemorrhage in Oxidant-Mediated Acute Lung Injury in the Rat

Experimental groups	CVF-induced lung injury[a]	Immune complex-induced lung injury[a]	Ischemia/reperfusion-induced lung injury[a]
Negative control group	0.059 ± 0.006	0.062 ± 0.005	0.063 ± 0.005
Positive control group	0.211 ± 0.019	0.236 ± 0.021	0.167 ± 0.018
Riboflavin-treated group[b]	0.095 ± 0.011[c] (76% protection)	0.148 ± 0.018[c] (51% protection)	0.094 ± 0.014[c] (70% protection)

[a]Lung injury (alveolar hemorrhage) was assessed by extravasation of ^{51}Cr-labeled rat erythrocytes.
[b]Riboflavin (6 μmoles/kg BW), given intraperitoneally.
[c]$P < 0.05$.

injected, the protective effect in vascular permeability dropped to 17% ($P = 0.05$) (Figure 1). Administration of 1.5 μmoles/kg (Figure 1) gave only 6% protection (not significant).

Lung and plasma samples obtained from CVF-treated rats showed a two-fold incresae in conjugated dienes compared to negative controls (Figure 2). The intraperitoneal administration of 6 μmoles riboflavin/kg body weight prior to CVF injection reduced conjugated dienes in lung tissue by 45% ($P < 0.01$) and in plasma by 75% ($P < 0.01$).

Measurements of myeloperoxidase (MPO) content in the lungs of experimental animals revealed that CVF alone caused a two-fold increase in lung neutrophil accumulation (Table 2). When administered at the protective dose shown in Table 1, riboflavin did not cause a significant reduction in lung myeloperoxidase content.

IgG Immune Complex Alveolitis Model. The simultaneous administration of intravenous BSA (10 mg) and intratracheal anti-BSA (2.5 mg) resulted in a seven-fold increase in lung vascular permeability (Figure 3). When rats were treated with riboflavin at the same concentration that was effective in the CVF model (6 μmoles/kg, given intraperitoneally), vascular permeability was reduced by 44% ($P < 0.01$; Figure 3) and hemorrhage was reduced by 51% ($P < 0.01$; Table 1). Neutrophil accumulation increased four-fold in this model. This increase was not influenced by the administration of riboflavin (Table 2).

Ischemia-Reperfusion Model. Four hours of bilateral hind limb ischemia followed by 4 h of reperfusion caused a seven-fold increase in muscle vascular permeability (Figure 4A) and a four-fold increase in lung vascular permeability (Figure 4B), compared to 4 h of ischemia only. Since the major damage

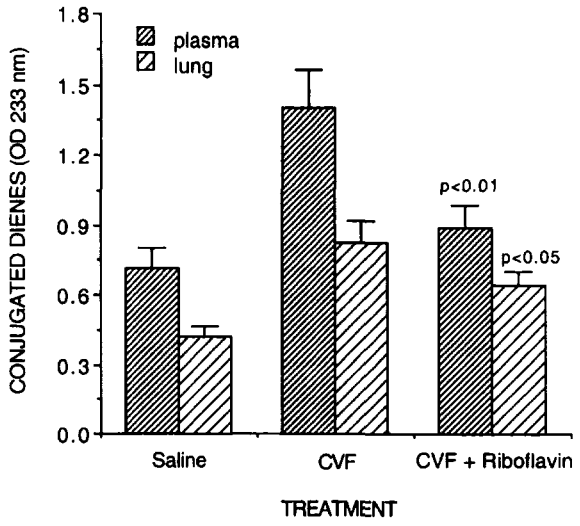

Fig. 2. Effect of riboflavin on lung and plasma levels of conjugated dienes in rats injected with cobra venom factor (CVF). Riboflavin was injected intraperitoneally at a dose of 6 μmoles/kg body weight, causing reductions in lung and plasma levels of conjugated dienes by 45% and 75%, respectively, as measured at 30 min after CVF injection.

occurred during the reperfusion phase, intraperitoneal treatment with riboflavin was started 10 minutes prior to the reperfusion. In this type of injury, treatment with the highest dose of riboflavin tested in the CVF model (6 μmoles/kg body weight) reduced muscle vascular permeability by 22% ($P < 0.05$) (Figure 4A) and lung vascular permeability by 58% ($P < 0.01$) (Figure 4B). Muscle hemor-

Table 2. Effect of Riboflavin on Lung Myeloperoxidase Content in Rats Submitted to Oxidant-Mediated Acute Lung Injury

Experimental groups	CVF-induced lung injury	Immune complex-induced lung injury	Ischemia/reprefusion-induced lung injury
Negative control group	0.10 ± 0.012	0.09 ± 0.015	0.11 ± 0.021
Positive control group	0.22 ± 0.023	0.48 ± 0.025	0.45 ± 0.021
Riboflavin-treated group[a]	0.21 ± 0.019 (N.S.)	0.50 ± 0.031 (N.S.)	0.44 ± 0.029 (N.S.)

[a]Riboflavin (6 μmoles/kg BW), given intraperitoneally. N.S. = not significant.

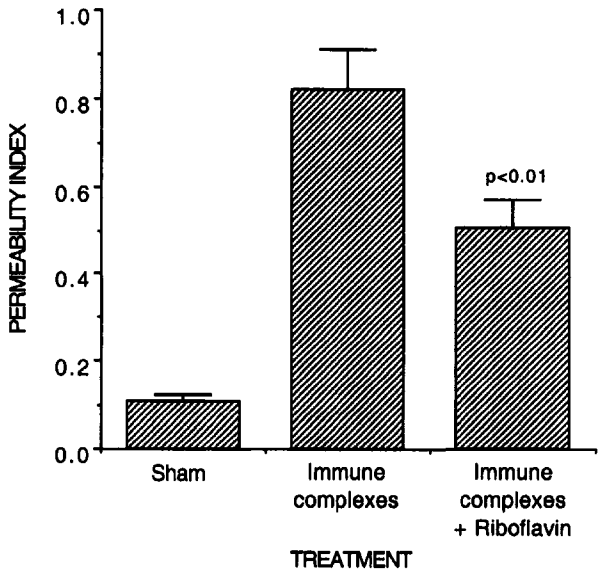

Fig. 3. Protective effect of riboflavin (6 μmoles/kg, intraperitoneally) on lung vascular leakage (^{125}I-BSA) in immune-complex alveolitis in the rat. Vascular permeability was measured at 4 h after antigen and antibody injection.

rhage was reduced by 30% ($P < 0.05$; data not shown) and lung hemorrhage by 70% ($P < 0.01$, Table 1). During reperfusion of the limbs, neutrophil sequestration increased more than four-fold in the lung as well as in the muscle and, again, as seen in the other inflammatory models, lung content of myeloperoxidase was not altered by riboflavin treatment (Table 2).

DISCUSSION

Riboflavin in its reduced form (dihydroriboflavin) has been shown to reduce ferric hemeproteins (18) and the products resulting from the reactions of hemeproteins with peroxides (11). Since dihydroriboflavin is spontaneously oxidized to riboflavin the oxidized form was administered to rats in the present experiments. Our hypothesis is that riboflavin penetrates into cells (e.g., the endothelial cell) and is converted to dihydroriboflavin by the enzyme, flavin reductase. By such a mechanism, hydroxyl radical and Fe(IV)O- and Fe(V)-hemeproteins will be scavenged within the cell, (10, 11) but oxygen radicals that are already released by a cell would not be affected by riboflavin.

To examine if riboflavin may protect from oxygen radical-mediated mi-

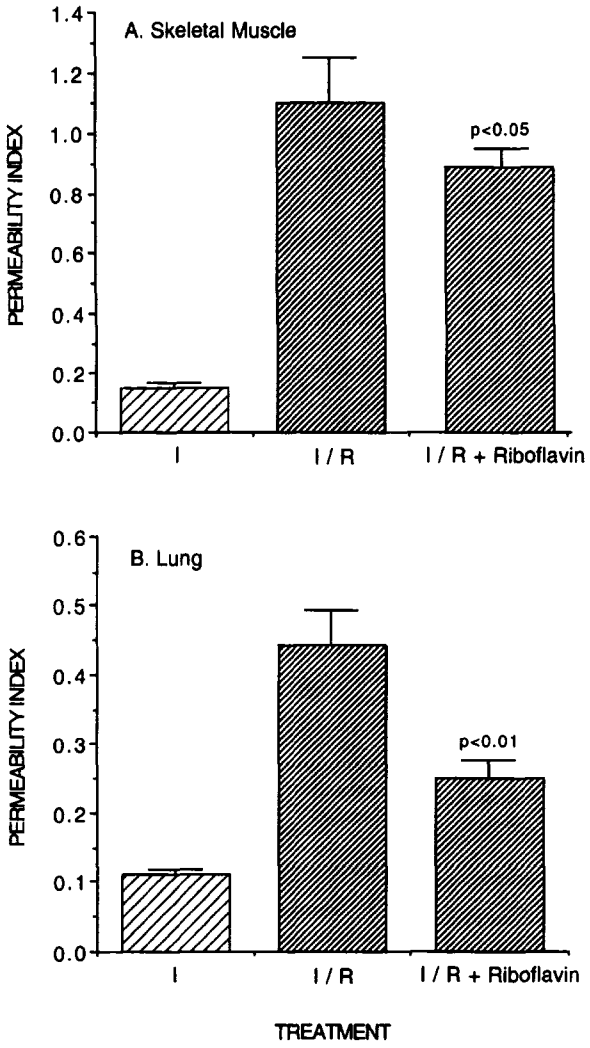

Fig. 4. Protective effects of riboflavin (6 μmoles/kg, intraperitoneally) on muscle (A) and lung (B) vascular leakage (^{125}I-BSA) after bilateral hind limb ischemia-reperfusion (I/R). Permeability measurements were performed after 4 h of ischemia (I) and 4 h of reperfusion.

crovascular injury in vivo, well-characterized rat models of oxidant-mediated lung injury were employed. In the CVF model, systemic complement activation due to cobra venom factor initiates lung injury, which is characterized by neutrophil accumulation along the vascular endothelium leading to subsequent

endothelial cell and basement membrane damage resulting in increased lung vascular leakage and alveolar hemorrhage within 30 min after CVF injection (3). Furthermore, the lung microvascular injury is accompanied by the appearance in plasma and lung tissues of lipid peroxidation products (17). Both lung injury and lipid peroxide generation are significantly attenuated by pretreatment of experimental animals with catalase, hydroxyl radical scavengers or iron chelators, (3, 17) suggesting that complement-mediated activation of blood neutrophils and their generation of an iron-catalyzed conversion product of hydrogen peroxide, most likely the hydroxyl radical, are directly responsible for the observed lung injury. The IgG immune complex-induced lung injury results in extensive neutrophil accumulation, much of it being extravascular. Similar to the CVF model, the accompanying microvascular damage can largely be attenuated by pretreatment of experimental animals with catalase, hydroxyl radical scavengers, or iron chelators (4). Ischemia of hind limbs of rats followed by reperfusion is characterized by increased vascular leakage locally in the muscle and distantly in lungs (5). As in the previous two models, the microvascular injury can be significantly reduced by treatments of rats with antioxidants (5).

In all three of our in vivo models we have shown that vascular leakage and hemorrhage are significantly attenuated by riboflavin. The decrease in lipid peroxidation in the CVF model suggests that the mechanism of riboflavin-mediated protection is releated to oxygen radical scavenging. Previous studies have shown that scavenging of oxygen radicals not ony protects from vascular injury but also can decrease neutrophil accumulation (19). Oxidants released by endothelial cells (20) may be involved in the generation of chemotactic factors (21) and have recently been shown to up-regulate neutrophil adhesion molecules (22, 23). Since riboflavin had no effect on neutrophil sequestration (Table 2), it appears that riboflavin may not scavenge extracellular oxygen radicals released from endothelial cells. Most likely, intracellular reactive oxygen species that directly damage endothelial cells are scavenged by the reduced form of riboflavin, dihydroriboflavin. Furthermore, riboflavin has no effect on the generation of superoxide or hydrogen peroxide by activated rat neutrophils in vitro (A. Seekamp, unpublished observation), suggesting that the protective effect of riboflavin is not due to inhibition of oxidant production by activated neutrophils.

In summary, our results demonstrate that riboflavin, in a dose-dependent manner, protects the lungs from oxygen radical-mediated injury without affectig neutrophil accumulation in vivo. We propose that in order to scavenge oxygen radicals and protect vascular endothelial cells from injury, riboflavin is converted into dihydroriboflavin by flavin reductase, an intracellular enzyme.

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