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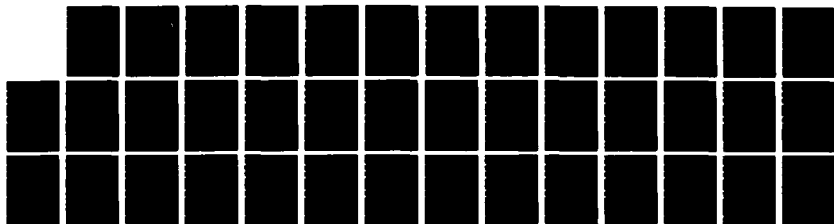
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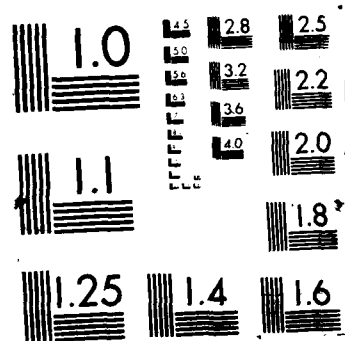
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THROMBOXANE-MEDIATED INJURY FOLLOWING RADIATION

Annual Final Report

Peter A. Kot, M.D.

June 18, 1986

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Previous studies from this laboratory demonstrated an increased <u>in vivo</u> and <u>in vitro</u> release of immunoreactive thromboxane B2 (TXB2) four hours after 20.0 Gy whole body irradiation. It was also shown that this radiation-induced increase in TXB2 release was of extrarenal origin. The present report confirms last year's observations that whole body ionizing radiation exposure results in an increased pulmonary TXB2 release. Further, since radiation exposure is associated with an increased release of TXB2, the present studies sought to determine if tissue responsiveness was altered to this cyclooxygenase product. Rats were anesthetized (30 mg/kg sodium pentobarbital, i.p.) and exposed to 20.0 Gy whole body gamma irradiation. The aortic vascular reactivity to the TXA2 mimic, U46619, was assessed four, 24, and 48 hours post irradiation. Vascular reactivity to this agonist was unchanged at four and 24 hours after irradiation, but was significantly depressed 48 hours after exposure. This reduced vascular responsiveness to U46619 was not due to the (OVER)						
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19. Abstract, continued

destruction of vascular smooth muscle as the vascular reactivity to the non-receptor mediated agonist, KCl, was unchanged 48 hours post irradiation.

The effect of the radioprotectant, WR2721, on the radiation-induced depression in vascular reactivity was assessed next. Rats were pretreated with WR2721 (200 mg/kg, i.p.) 20 minutes prior to exposure to 20.0 Gy whole body irradiation. Forty-eight hours later, the aortic vascular reactivity to U46619 was determined and compared to untreated irradiated vascular tissue. Pretreatment with WR2721 prevented the radiation-induced depression in vascular reactivity.

Finally, the release of TXB2 from the pulmonary bed of irradiated animals was determined. Rats were exposed to 20.0 Gy whole body gamma irradiation and four hours later the lungs were isolated and perfused with either Krebs-Ringer bicarbonate (KRB), KRB plus 3% bovine serum albumin (KRB-BSA), or KRB plus 3% Dextran 70 (KRB-Dextran 70). An observable radiation-induced increase in pulmonary TXB2 release could only be demonstrated when the lungs were perfused with KRB-BSA.

The data presented in this report confirm previous findings in that whole body gamma irradiation increases pulmonary TXB2 release. These results indicate the importance of a plasma-like perfusate in studying cyclooxygenase product release from isolated organs. The data presented in this report also show that radiation exposure will depress vascular reactivity to the TXA2 mimic, U46619. This radiation-induced alteration in vascular reactivity was preventable by treatment of the animals with the radioprotectant, WR2721, prior to irradiation. These results suggest that the altered release of TXB2 seen following lethal doses of gamma radiation may have both physiological ramifications and pharmacological applications.

SUMMARY

Previous studies from this laboratory demonstrated an increased in vivo and in vitro release of immunoreactive thromboxane B₂ (TXB₂) four hours after 20.0 Gy whole body irradiation. ~~It was also shown that this radiation-induced increase in TXB₂ release was of extrarenal origin. The present report confirms last year's observations~~ that whole body ionizing radiation exposure results in an increased pulmonary TXB₂ release. ~~Further, since radiation exposure is associated with an increased release of TXB₂, the present studies sought to determine if tissue responsiveness was altered to this cyclooxygenase product.~~

Rats were anesthetized (30 mg/kg sodium pentobarbital, i.p.) and exposed to 20.0 Gy whole body gamma irradiation. The aortic vascular reactivity to the TXA₂ mimic, U46619, was assessed four, 24, and 48 hours post irradiation. Vascular reactivity to this agonist was unchanged at four and 24 hours after irradiation, but was significantly depressed 48 hours after exposure. This reduced vascular responsiveness to U46619 was not due to the destruction of vascular smooth muscle as the vascular reactivity to the non-receptor mediated agonist EC_{50} , was unchanged 48 hours post irradiation.

The effect of the radioprotectant, WR2721, on the radiation-induced depression in vascular reactivity was assessed next. Rats were pretreated with WR2721 (200 mg/kg, i.p.) 20 minutes prior to exposure to 20.0 Gy whole body irradiation. Forty-eight hours later, the aortic vascular reactivity to U46619 was determined and compared to untreated irradiated vascular tissue. Pretreatment with WR2721 prevented the radiation-induced depression in vascular reactivity.

Finally, the release of TXB₂ from the pulmonary bed of irradiated animals was determined. Rats were exposed to 20.0 Gy whole body gamma irradiation and four hours later the lungs were isolated and perfused with either Krebs-Ringer bicarbonate (KRB), KRB plus 3% bovine serum albumin (KRB-BSA), or KRB plus 3% Dextran 70 (KRB-Dextran 70). An observable radiation-induced increase in pulmonary TXB₂ release could only be demonstrated when the lungs were perfused with KRB-BSA.

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Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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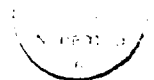


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BACKGROUND:

Observations on the cellular and tissue effects of ionizing radiation are numerous but the molecular manifestations of radiation injury are poorly understood. Radiation injury is associated with the formation of free radicals and lipid peroxides (1). Several groups of investigators have demonstrated a pivotal role for peroxides in the acceleration of cyclooxygenase activity (2-5). Since cyclooxygenase activity may be one of the rate limiting steps in arachidonate metabolism, and since the cyclooxygenase products have powerful vasoactive and platelet aggregatory properties (6-18), it is possible that these arachidonate metabolites play an important role in radiation-induced vascular injury.

In recognition of this possibility, Eisen and Walker (19) showed that exposure of mice to 7.0 Gy x-irradiation resulted in increased pulmonary and splenic PGE-like activity and increased synthesis of PGF_{2a}. Other studies have shown that hepatic and cerebral PGE-like activity and PGF_{2a} synthesis increased after 5.0 to 7.5 Gy (20). Exposure of mice to 9.0 Gy gamma irradiation resulted in a significantly increased in vitro synthesis of PGE₂ and PGF_{2a} by hepatic microsomes and homogenates of brain and testis (21,22). These and other studies demonstrated that in vitro synthesis of the classical products of the cyclooxygenase pathway are increased following exposure to ionizing radiation (19-23).

The effect of ionizing radiation on TXA₂ synthesis is less clear. Maclof et al (24) demonstrated a significant increase in TXB₂ release by splenic microsomes isolated from rats exposed to 9.0 Gy whole body irradiation. Steel and Catravas (25) showed increased TXB₂ release from guinea pig lung parenchymal strips one to three hours post irradiation. On the other hand, Steel et al (26) failed to show an increase in TXB₂ synthesis by guinea pig lung airway tissue following irradiation. Similarly, Allen et al (27) could not demonstrate an alteration in TXB₂ release by platelets irradiated in vitro.

During 1984, studies from this laboratory characterized the effect of whole body gamma irradiation on in vivo synthesis of TXB₂. These studies showed that urine TXB₂ levels were unchanged two to 120 hours following 2.0 Gy ionizing radiation exposure (28,29). Increasing the dose of ionizing radiation to 10.0 Gy significantly ($p < .05$) increased urine TXB₂ concentrations four, 12, 24, 72, and 120 hours after exposure. Rats exposed to 20.0 Gy gamma irradiation showed a 233.1% and 105.3% ($p < .05$) increase in urine TXB₂ four and 12 hours post irradiation respectively (29). These results were subsequently confirmed by Donlon et al (30) who demonstrated a significant increase in the TXB₂ excretion rate 12 and 36 hours after 9.0 Gy whole body irradiation of rats. These studies indicate that in vivo synthesis of TXA₂ is increased acutely after whole body irradiation.

In order to determine if this increase in radioimmunoassayable TXB₂ was due to an alteration in cyclooxygenase pathway activity, a second series of studies was performed in which rats were pre-treated with 5 mg/kg indomethacin or vehicle one hour before exposure to 20.0 Gy. Four hours after irradiation (five hours after indomethacin injection) both irradiated and non-irradiated animals showed greater than a 90% suppression in urine TXB₂ levels compared to their respective vehicle injected groups (29). Thus, the increase in urine TXB₂ levels seen acutely after ionizing radiation exposure was a function of altered cyclooxygenase pathway synthesis and/or metabolism (29).

The role of the kidneys in urine TXB₂ levels was assessed next. In an isogravitometric cross-perfusion system, recipient animals pre-treated with high dose indomethacin had their circulations linked to sham or 20.0 Gy irradiated rats (29). After 60 min of cross-perfusion, urine TXB₂ levels in recipient animals were

significantly higher than pre-cross perfusion levels indicating the source(s) of urine TXB2 was/were the circulation of the untreated donor animals (29). No differences were seen in the urine levels of TXB2 of recipient rats cross-perfused with either control or irradiated animals (29). Thus, urine TXB2 receives a significant contribution from the circulation but the source(s) of the radiation-induced increase could not be determined (29).

During 1985, studies from this laboratory continued to evaluate the source(s) of the radiation-induced increase in urine TXB2 excretion. This was approached, first by regional shielding studies in which rats were exposed to sham irradiation, 15.0 Gy whole body irradiation, or 20.0 Gy irradiation with either the upper abdomen or the thorax shielded (31). These studies showed that the organs of the upper abdomen, including the kidneys, were not the primary source of the radiation-induced increase in urine TXB2 excretion (31). These studies also suggested that the lungs were a major source of the increased urine TXB2 following irradiation (31).

In order to assess the extent of the renal contribution to the radiation-induced increase in TXB2 excretion, an isolated perfused kidney model was developed (31). Animals were exposed to 20.0 Gy whole body irradiation and four hours later the kidneys were isolated and perfused with an acellular perfusate. Isolated kidneys from irradiated animals showed no significant increase in urine TXB2 excretion compared to isolated kidneys from sham irradiated controls (31). The excretion of both PGE2 and 6KPGFla was elevated from kidneys isolated from irradiated animals compared with controls (31).

Other studies were initiated to determine the role of the lungs in the observed increase in TXB2 excretion following irradiation. A study was performed to determine the effects of radiation dose and the time course of release of TXB2 from the pulmonary circulation (32). These experiments showed a significant increase in the pulmonary release of TXB2 12 hours after 5.0 or 10.0 Gy whole body irradiation, and 4 hours after 20 Gy radiation exposure (32). These studies suggest that the lungs contribute to the increased urinary excretion of TXB2 following whole body radiation exposure (32).

In 1986, studies from this laboratory extended the earlier findings that whole body radiation exposure alters the pulmonary release of cyclooxygenase products. Lungs from irradiated or sham irradiated rats were isolated and perfused with three different media. This was done to confirm the earlier work performed by this laboratory in 1985 as well as to determine the effect of the perfusion media on the radiation-induced increase in pulmonary cyclooxygenase product release. In addition, one of the media used had free radical scavenger characteristics. Perfusion of irradiated lungs with this media allowed an evaluation of the effect on free radical scavengers on the radiation-induced alteration in pulmonary cyclooxygenase product release.

In a second series of studies, the vascular reactivity of irradiated and sham irradiated abdominal aortic rings to a thromboxane A2 mimic (U46619) and to KCl was assessed. These studies were performed to determine if the elevated levels of TXA2 were altering vascular function. In addition, these studies developed a quick, inexpensive means of evaluating the efficacy of radioprotectants in vivo.

Prostaglandins probably play a role in the pathogenesis of radiation injury. Recently, Donlon et al. (33) showed that the radioprotectant WR2721 reduced the radiation-induced increase in TXB2, PGE2, and PGF2a excretion rate. Other studies linking cyclooxygenase product release to tissue injury include the work of Northway et al. (34-37) who demonstrated an attenuation of radiation-induced esophagitis in the opossum by pre-treating animals with indomethacin or aspirin prior to exposure.

Animals pre-treated with 16,16 dimethyl prostaglandin E2 before irradiation had augmented esophageal injury compared to untreated controls (34-37). The precise role prostaglandins play in radiation-induced tissue injury remains to be determined.

APPROACH TO THE PROBLEM

Irradiation: Previous studies have shown that whole body gamma irradiation at a dose of 20.0 Gy results in a consistent increase in urine TXB2 four hours after exposure. As a result, this dose and time frame were used in most of the studies presented in this report. Male Sprague-Dawley rats (200-250 g) were anesthetized with sodium pentobarbital (30 mg/kg i.p.) before irradiation in order to maintain a consistent pattern of exposure in the radiation chamber. The rats were exposed to either sham irradiation or 20.0 Gy gamma irradiation in a ventro-dorsal orientation to a 7.4×10^{13} becquerel ^{137}Cs radiation source (Best Industries Small Animal Irradiator, Arlington, VA). The rate of delivery was previously calibrated at 0.87 Gy per minute.

WR2721 pretreatment: One series of studies evaluated the effect of the radioprotectant, WR2721, on radiation-induced alterations in vascular reactivity. WR2721 was prepared by dissolving 100 mg of the radioprotectant in 1 ml distilled water immediately before injection. Rats were then anesthetized with 30 mg/kg sodium pentobarbital i.p. and pre-treated with either WR2721 (200 mg/kg, i.p.) or the distilled water vehicle. Twenty minutes later, the animals were subjected to 20.0 Gy whole body irradiation or sham irradiation as previously described.

Vascular Reactivity: Four, 24 or 48 hours following irradiation or sham irradiation, the animals were re-anesthetized and a segment of abdominal aorta caudal to the diaphragm and cephalad to the renal arteries was removed. The blood was rinsed from the aortic segment with ice cold Krebs-Ringer bicarbonate solution (KRB). Adherent connective tissue was carefully dissected from the vascular tissue and the section of abdominal aorta was divided into vascular rings segments each two to four millimeters in width.

The isolated ring segments were mounted to a tissue bath at one end and to an isometric tension transducer on the other (Fig. 1). The water-jacketed tissue bath was filled with KRB warmed to 37°C. The pre-load tension was adjusted to 1.0 or 1.5 g. The vascular ring segment was allowed to equilibrate for one hour in this tissue bath. Every 15 minutes during the equilibration period, the pre-load tension was re-adjusted to 1.0 g or 1.5 g and the bath fluid exchanged with fresh KRB.

Following the equilibration period, irradiated and sham irradiated vascular tissue rings were exposed to cumulative doses (10^{-9} - 10^{-6} M) of either 9, 11-dideoxy-11a, 9a-epoxymethano-prostaglandin F2a (U46619) or KCl. The vascular reactivity was evaluated by linear regression analysis of a computer generated semilogarithmic plot of the developed isometric tension versus the log of the drug concentration. The correlation coefficient of all curves was greater than 0.90. The slope of the curve, the maximum developed tension, and the ED50 for irradiated and sham irradiated vascular rings exposed to U46619 or KCl were determined from this same curve. The ED50 was calculated based on the semilogarithmic plot. The dose of vascular agonist that induced a maximal recorded response was entered into the computer and the maximal developed tension determined from the semilogarithmic plot. The maximal developed tension was then divided by two to give the 50% maximum developed tension and this number was entered into the computer. The computer, using the 50% maximum developed tension and the semilogarithmic plot of the particular dose-response curve, determined the concentration of agonist necessary to induce a 50% maximum developed tension or ED50.

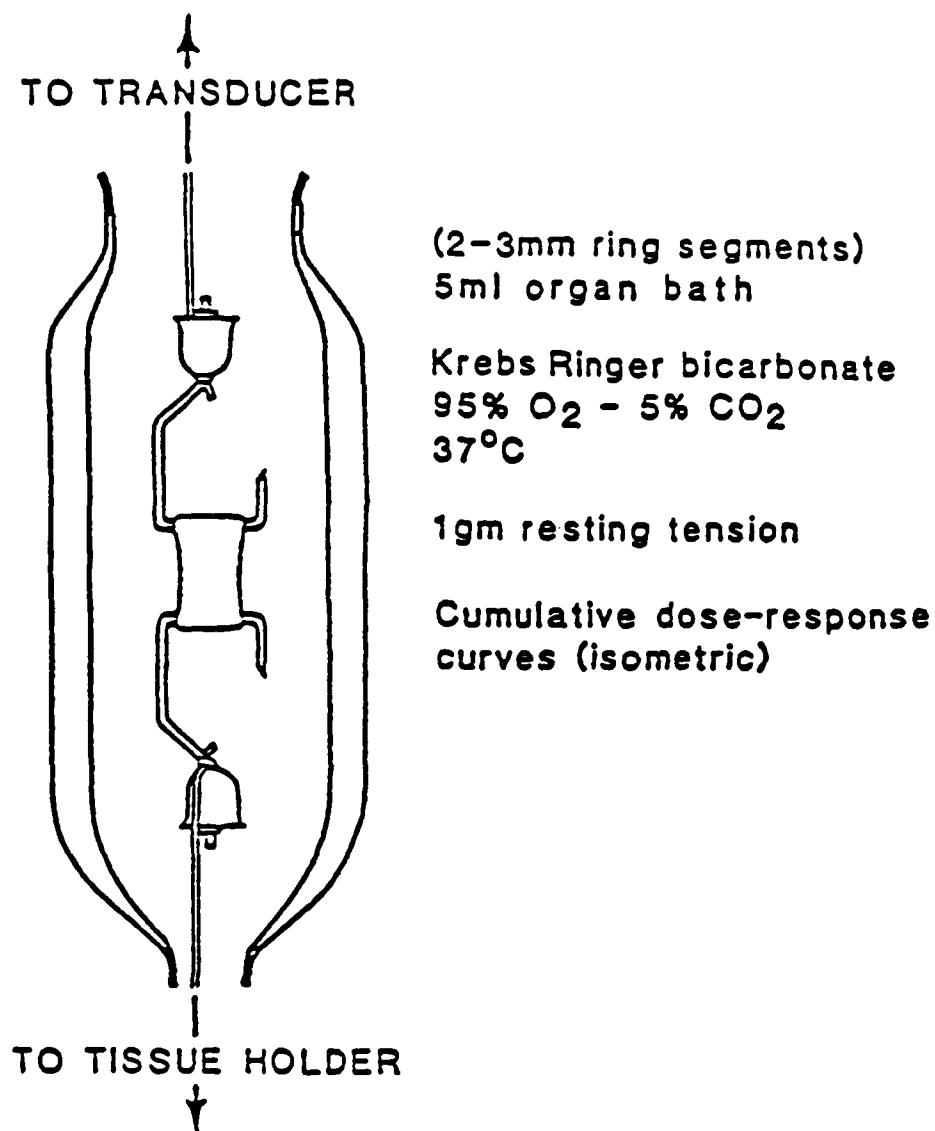


Figure 1: Water-jacketed incubation chamber used in vascular reactivity studies. The chamber was filled with KRB warmed to 37° C. Aortic rings from irradiated or sham irradiated animals were challenged with cumulative concentrations of U46619 or KCl and the developed isometric tension recorded.

Lung Perfusion: Animals were re-anesthetized four hours after exposure to 20.0 Gy whole body gamma irradiation. The trachea was cannulated and the lungs were ventilated with 95% air and 5% CO₂ at a tidal volume of 2.5 ml and a frequency of 55 breaths per minute (Harvard Rodent Respirator, Boston, MA). The blood was removed from the lungs by perfusing them via the catheterized hepatic portal vein with Krebs-Ringer bicarbonate (KRB) plus 3% bovine serum albumin (BSA), KRB plus 3% Dextran 70 (Sigma Chemical Co., St. Louis, MO), or KRB alone. All perfusates contained 5.6 mM glucose. Blood was drained from the lungs via the transected abdominal aorta (Fig. 2). This route of initial perfusion was chosen because perfusion via the inferior vena cava would put the liver in a parallel perfusion circuit with the lungs. Since both the lungs and liver are low resistance beds, perfusion via the inferior vena cava would result in poor removal of blood from the lungs and would require a longer period of time for the lungs to clear. By initially perfusing the lungs via the hepatic portal vein, both the liver and lungs were perfused in series so all of the perfusate would ultimately pass through the lungs and the lungs would be more efficiently and completely cleared of blood. This rapid removal of blood would allow for a more rapid transfer of the lungs to the *in vitro* perfusion chamber and would leave us with a viable pair of lungs.

After the lungs were cleared of blood, the thoracic cavity was opened and the pulmonary artery cannulated. Perfusion of the lungs was transferred from the hepatic portal vein to the pulmonary artery. The lungs were then removed from the animal, suspended in a water-jacketed *in vitro* perfusion chamber and perfused at a flow rate of 10 ml/min with the temperature maintained at 37° C.

Once the lungs were suspended in the perfusion chamber, the perfusate was allowed to recycle through the lungs. The perfusate entered the lungs through the cannulated pulmonary artery and drained from the lungs via the cut left ventricle. Perfusate samples were taken after 15 minutes of re-circulation, frozen at -20° C, and later thawed as a group for determination of TXB2 by RIA.

Radioimmunoassay: Pulmonary effluent TXB2 concentrations were determined by radioimmunoassay using the methods of Granstrom and Kindahl (38,39). An aliquot of effluent (100 ul) was added to 200 ul radioimmunoassay buffer (RIA buffer), 100 ul gelatinized radioimmunoassay buffer (gel buffer), 100 ul labelled ligand, and 100 ul antibody. The RIA buffer consists of 1.55 g Tris 7.0 (Sigma Chemical Co., St. Louis, MO), and 9.0 g NaCl dissolved in 1.0 liter distilled water. To this was added 1.0 ml of 2.0 M MgSO₄ and 1.0 ml of 0.2 M CaCl₂. Gel buffer consists of RIA buffer to which gelatin was added (0.25 g gelatin per liter RIA buffer). The effluent-RIA buffer-Gel buffer-ligand-antibody mixture was incubated overnight at 4° C and the unbound labelled ligand precipitated by the addition of 0.9 ml dextran-coated charcoal (0.3 g Dextran 70 and 3.0 g charcoal suspended in 1.0 liter RIA buffer). The supernatant remaining after centrifugation at 1000 x g was transferred to liquid scintillation vials to which 5.0 ml fluor was added (Atomlight, New England Nuclear, Boston, MA).

The cross reactivity of the TXB2 antibody was less than 0.3% with PGA2 and less than 0.1% with PGF2a, PGE2 and 6-keto-PGF1a. The TXB2 antibody cross reacted approximately 60% with 2,3 dinor TXB2. Therefore, the TXB2 levels determined represent a mixture of TXB2 and its 2,3 dinor metabolite.

The sensitivity of the RIA was evaluated on the basis of the linearity of the standard curves. The minimum detectable values for TXB2 was 3.0 pg/ml and the maximal detectable levels of TXB2 was 1000 pg/ml. The volume of pulmonary effluent that was added to the RIA was appropriately adjusted such that the concentrations of the cyclooxygenase products were above the minimum sensitivity but below the maximal detectable levels for each cyclooxygenase product assay.

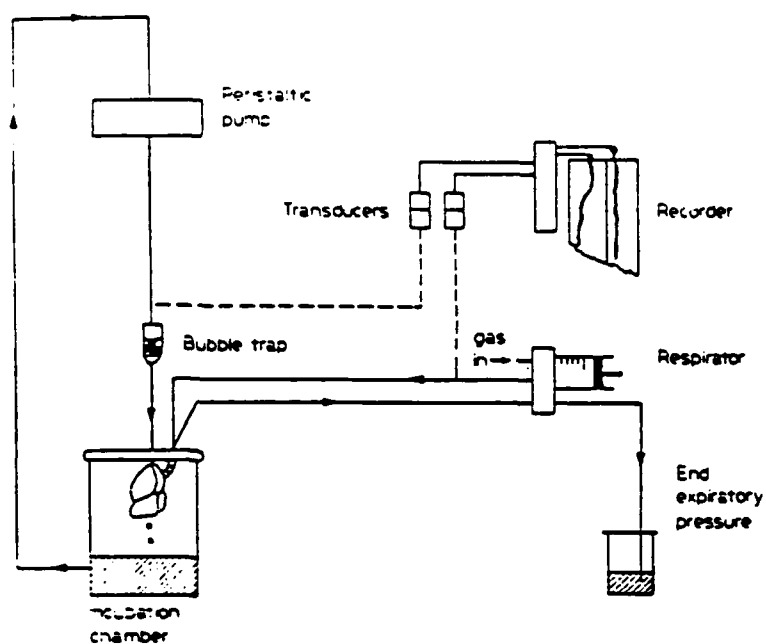


Figure 2: Diagram of apparatus used in the lung perfusion studies. Lungs from irradiated or sham irradiated animals were removed from the animal and suspended in a water-jacketed incubation chamber warmed to 37° C. The lungs were perfused with KRB, KRB-BSA, or KRB-Dextran 70 for a period of 15 minutes. Pulmonary effluent TXB2 and 6KPGFla concentrations were determined by radioimmunoassay.

Pulmonary Perfusates and the Radioimmunoassay: Three perfusion media were prepared: 1) Krebs-Ringer bicarbonate containing 5.6 mM glucose (KRB), 2) KRB plus 3% bovine serum albumin (BSA), and 3) KRB plus 3% Dextran 70 (molecular weight 70,000). To each of these three perfusion media (KRB, KRB-BSA, or KRB-Dextran 70), known concentrations of unlabelled thromboxane B₂ (TXB₂) (0.3, 0.1, 0.03, 0.01, and 0.003 ng/ml) were added.

The RIA, in the presence of unextracted perfusion media or RIA buffer control was performed as described by Granstrom and Kindahl (38,39). RIA buffer and KRB plus 3% BSA were prepared as previously described (29,32). Aliquots (0.1 ml) of the perfusion media or RIA buffer containing known concentrations of unlabelled cyclooxygenase product were added to 200 μ l RIA buffer, 100 μ l KRB plus 3% BSA, 100 μ l ³H labelled cyclooxygenase product, and 100 μ l specific antibody. After incubating at 4° C overnight, 0.9 ml dextran-coated charcoal (0.3 g Dextran 70 plus 3.0 g charcoal per liter RIA buffer) was added. After 20 min, the incubation tubes were centrifuged at 1000 x g and the supernatant decanted into 5.0 ml liquid scintillation vials containing 4.0 ml fluor (Atomlight, New England Nuclear, Boston, MA). The radioactivity was determined using a liquid scintillation counter (Beckman Instrument Co., Model LS-3150T, Irvine, CA). The total radioactivity added to the assays was determined by substituting 0.9 ml RIA buffer for the dextran-coated charcoal. The radioactivity of each vial was then calculated as the percent total label recovery. The percent label recovery for each perfusion media was then compared to the label recovery for the RIA buffer control.

Statistical Analysis: The developed tension at each concentration of U46619 or KCl in irradiated aortic rings was compared to the response in the paired control vessel segment by the paired Students t-test. The relationship between the agonist concentration and the developed tension was evaluated by the Pearson Product-Moment Correlation. The maximum developed tension, slope, and ED50 of the concentration-response curves for irradiated and control vessels were compared by the unpaired Students t-test. The vascular responses to WR2721 in both irradiated and sham irradiated aortic rings were analyzed in a comparable fashion.

The effect of the perfusion media on the RIA was evaluated by an Analysis of Variance and a Dunnett's test. The effect of the various perfusion media on cyclooxygenase product release from irradiated or sham irradiated isolated perfused rat lungs was assessed via Analysis of Variance and a Newman-Keul's test. The effect of ionizing radiation exposure on the pulmonary release of cyclooxygenase products was compared by an unpaired Student's t-test. In all studies and in all statistical tests performed, the confidence interval was set at 95%.

Results

The vascular responses of aortic rings challenged with U46619, four and 24 hours after 20.0 Gy whole body irradiation were not significantly different from the vascular responses of aortic rings obtained from sham irradiated controls (Fig. 3). At 48 hours post irradiation, vascular reactivity was significantly ($p < .05$) depressed compared with control (Fig. 3). The maximum contraction and the slope of the concentration-response curve to U46619 48 hours after radiation exposure was also decreased (Table 1). The U46619 concentration-response curves four and 24 hours post irradiation showed no significant change in either the maximum contraction or the slope (Table 1). The ED50 for U46619 four, 24 and 48 hours post irradiation was not significantly different than that of their respective controls.

In order to assess if the vascular smooth muscle was damaged 48 hours after irradiation, aortic rings were isolated and challenged with cumulative concentrations of KCl. KCl was chosen as the agonist for this series of studies because the contractile response elicited by KCl is receptor independent. Aortic

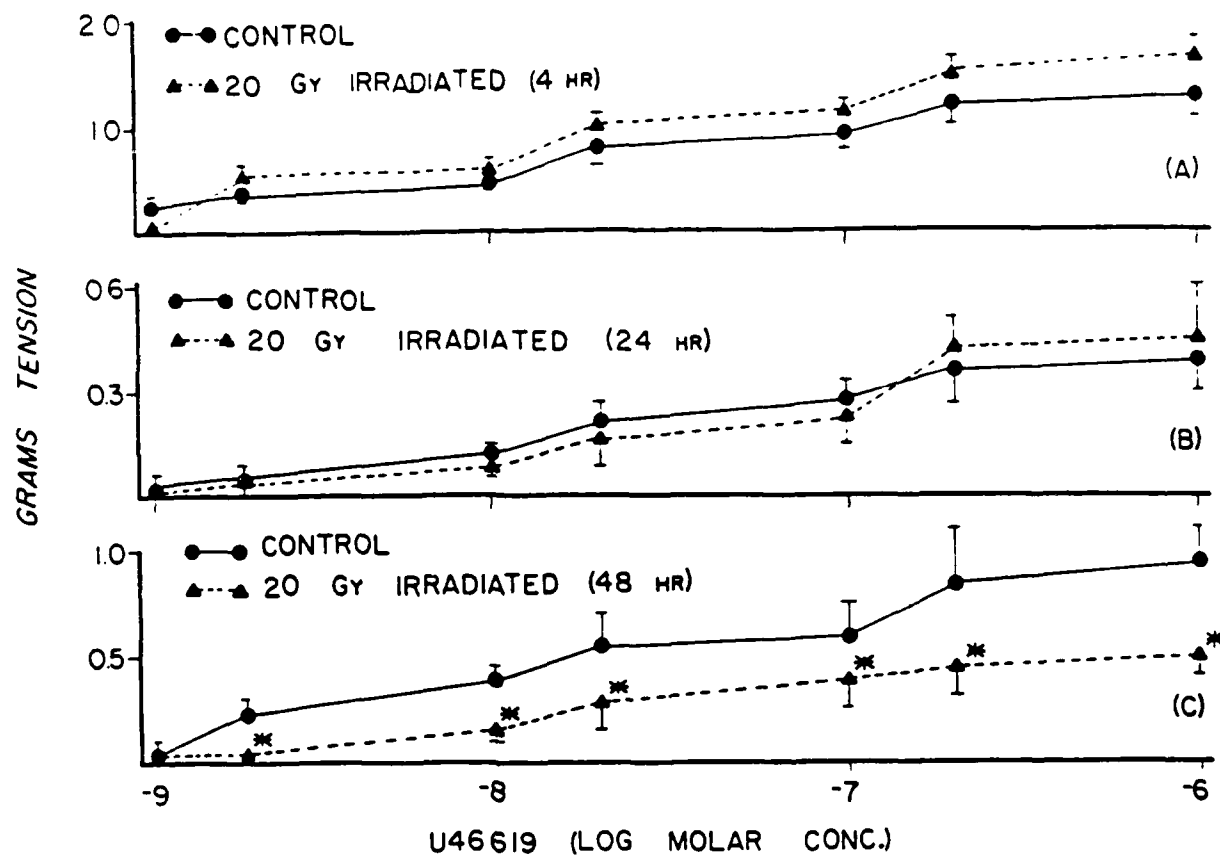


Figure 3: Vascular reactivity of aortic rings to cumulative concentrations of U46619 at various times after 20.0 Gy whole body gamma irradiation or sham irradiation. Data are expressed as mean $\pm$ standard error for 6 to 9 animals per group. * $p < .05$

Table 1

Effect of Gamma Irradiation on the Vascular Response of Rat Aortic Rings to U46619

	Maximum Contraction (Grams Tension/			Slope			ED50 (10^{-8} M Concentration)		
	Hours			Hours			Hours		
	4	24	48	4	24	48	4	24	48
Control	1.30 ^a	.40	.92	.37	.13	.30	2.40	3.70	2.50
	$\pm .18$	$\pm .09$	$\pm .28$	$\pm .07$	$\pm .03$	$\pm .12$	$\pm .67$	$\pm .76$	$\pm .85$
Irradiated (20 Gy)	1.67	.44	.50 [*]	.52	.18	.18 [*]	2.14	5.60	3.70
	$\pm .14$	$\pm .16$	$\pm .20$	$\pm .06$	$\pm .07$	$\pm .08$	$\pm .64$	$\pm .75$	± 1.20

^a Data are expressed as mean $\pm$ standard error of the mean for 6-9 animals per group^{*} $p < .05$ compared to sham irradiated controls

rings challenged with KCl 48 hours after whole body irradiation showed no depression in vascular reactivity (Fig. 4), nor was the maximum contraction, slope or ED50 of the concentration-response curve affected (Table 2).

Next a determination of the effect of pre-load tension on the radiation-induced alteration in vascular reactivity was performed. The pre-load tension during the equilibration period, 48 hours post irradiation, was increased from 1.0 g to 1.5 g and the vascular response to U46619 determined in control and irradiated aortic rings. Increasing the pre-load tension did not ameliorate the radiation-induced depression in vascular reactivity to U46619 (Fig. 5). The maximum contraction and slope of the U46619 concentration-response curve from irradiated vessel segments were also decreased ($p < .05$) compared to sham irradiated rings while the ED50 for both irradiated and control curves were not significantly different (Table 3). Increasing the pre-load tension did not induce a difference in the vascular reactivity of aortic rings challenged with KCl (Fig. 6), nor did it affect the maximum contraction, slope, or ED50 of the KCl concentration-response curves (Table 4).

Studies were then performed to determine if treatment of animals with WR2721 prior to irradiation would prevent the radiation-induced decrease in vascular reactivity. Aortic rings from WR2721 pre-treated, irradiated rats showed no radiation-induced decrease in vascular reactivity to U46619 when compared to untreated-sham irradiated controls (Fig. 7). WR2721 pre-treatment also prevented the radiation-induced decrease in maximum contraction and slope (Table 3).

Since the WR2721 pre-treatment prevented the radiation-induced decrease in vascular reactivity to U46619, studies were performed to determine whether this radioprotectant elicited a direct contractile response. Sham irradiated rats were pre-treated with vehicle or WR2721. Forty-eight hours later the vascular reactivity to U46619 was determined. WR2721 pre-treatment did not alter the vascular reactivity to U46619 (Fig. 8, Table 3).

Finally, the effect of WR2721 pre-treatment on the vascular response of irradiated vessels to KCl was investigated. The aortic rings from WR2721 pre-treated, irradiated rats showed the same vascular reactivity as vehicle-treated, sham irradiated controls (Fig. 9). In addition, WR2721 did not affect the maximum contraction, slope, or ED50 of the KCl concentration-response curve (Table 4).

Other studies were performed to evaluate the role of the pulmonary perfusate on cyclooxygenase product release from irradiated and control isolated perfused rat lungs. Initially, the effect of the perfusion media on the RIA for TXB2 was investigated. Addition of increasing concentrations of unlabelled TXB2 resulted in a concentration-dependent decrease in label recovery with all perfusion media. The TXB2 label recovery with each of the perfusion media was comparable to that observed for the RIA buffer control suggesting that none of the perfusion media affected the TXB2 assay (Table 5).

The effect of various perfusion media on pulmonary TXB2 release was examined. Perfusion of irradiated or control rat lungs with KRB alone resulted in a significantly higher ($p < .05$) pulmonary release of TXB2 compared to irradiated or control lungs perfused with KRB containing either 3% BSA or 3% Dextran 70. The TXB2 release from irradiated and non-irradiated rat lungs perfused with KRB plus 3% BSA was not significantly different from irradiated and control lungs perfused with KRB containing 3% Dextran 70 (Table 6).

A comparison was then made of the effects of the three perfusates on TXB2 release from irradiated compared to non-irradiated rat lungs. The radiation-induced increase in pulmonary TXB2 release could only be demonstrated in lungs perfused with

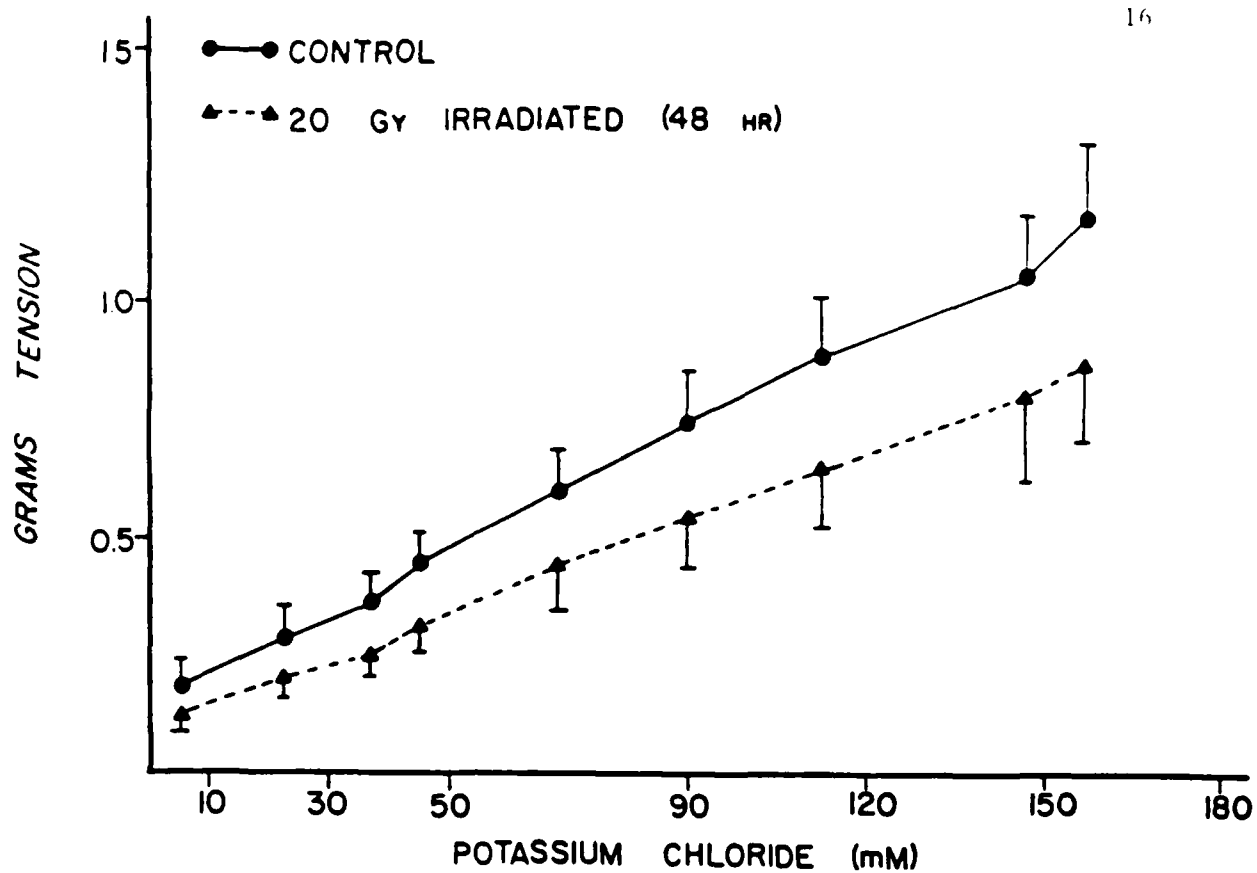


Figure 4: Vascular reactivity of aortic rings from 20.0 Gy whole body irradiated or sham irradiated animals. Data are expressed as mean $\pm$ standard error for 6 animals per group.

Table 2

Effect of Gamma Irradiation on the Vascular Response
of Rat Aortic Rings to KCl

	Maximum Contraction (Grams Tension)	Slope	ED50 (mMolar Concentration)
Control	1.25 ^a + .16	.007 + .0008	53.4 + 7.1
Irradiated (20 Gy)	.98 + .11	.005 + .001	45.6 + 7.8

^a Data are expressed as mean + standard error of the mean for 6 animals per group

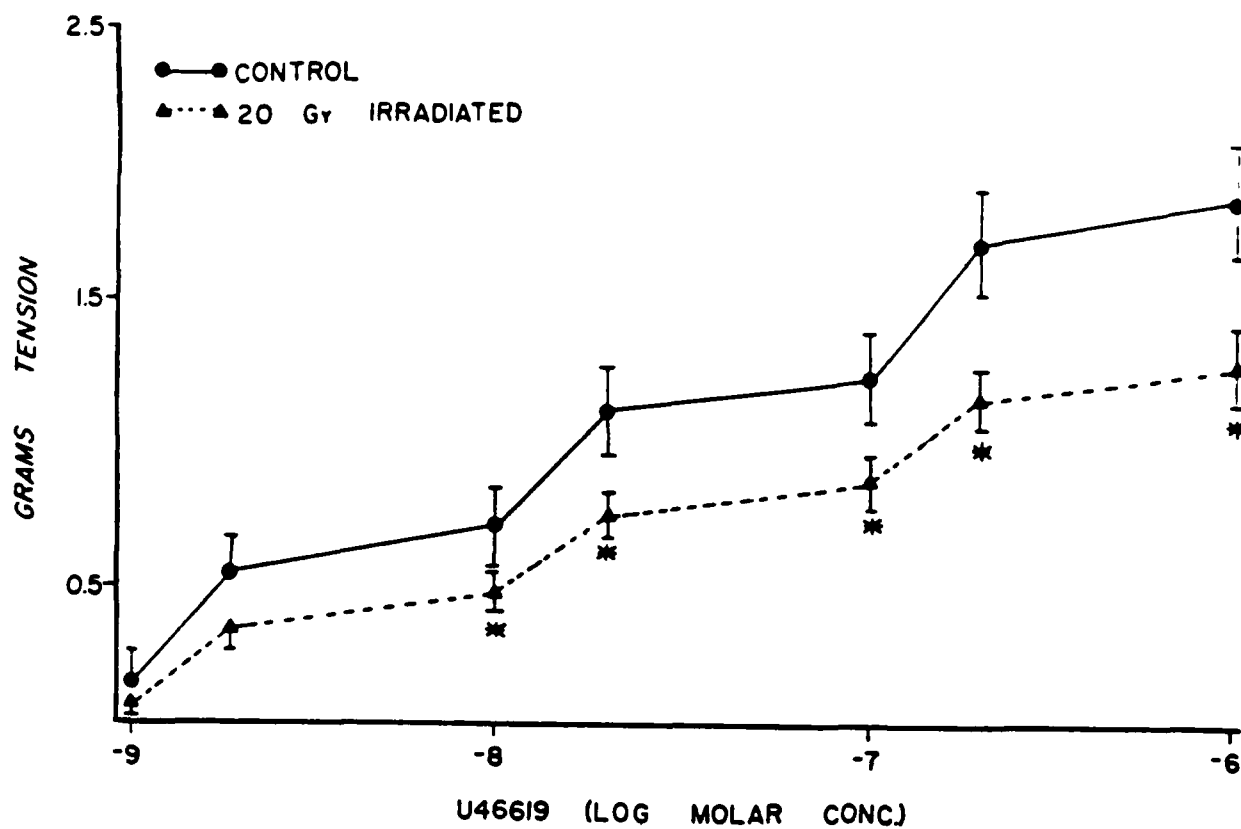


Figure 5: Vascular reactivity of aortic rings from 20.0 Gy whole body irradiated or sham irradiated animals. Pre-load tension was set at 1.5 g. Data are expressed as mean $\pm$ standard error for 6 animals per group. *p < .05

Table 3

Effect of Pre-irradiation WR2721 on the Vascular Reactivity
of Isolated Rat Abdominal Aortic Rings to U46619

	Maximum Contraction (Grams Tension)	Slope	ED50 (mMolar Concentration)
Control	1.84 ± .32 ^a	.62 ± .12	3.03 ± .59
WR2721	2.05 ± .29	.70 ± .11	2.90 ± .49
Control	2.11 ± .26	.73 ± .07	3.93 ± .96
20 Gy Irradiated	1.36 ± .17 [*]	.51 ± .06 [*]	4.41 ± .85
Control	2.08 ± .25	.67 ± .12	2.56 ± .82
20 Gy Irradiated After WR2721	1.99 ± .17	.67 ± .09	3.44 ± .84

^a Data are expressed as mean ± standard error of the mean for 6 animals per group.

^{*} p < .05 compared to control animals

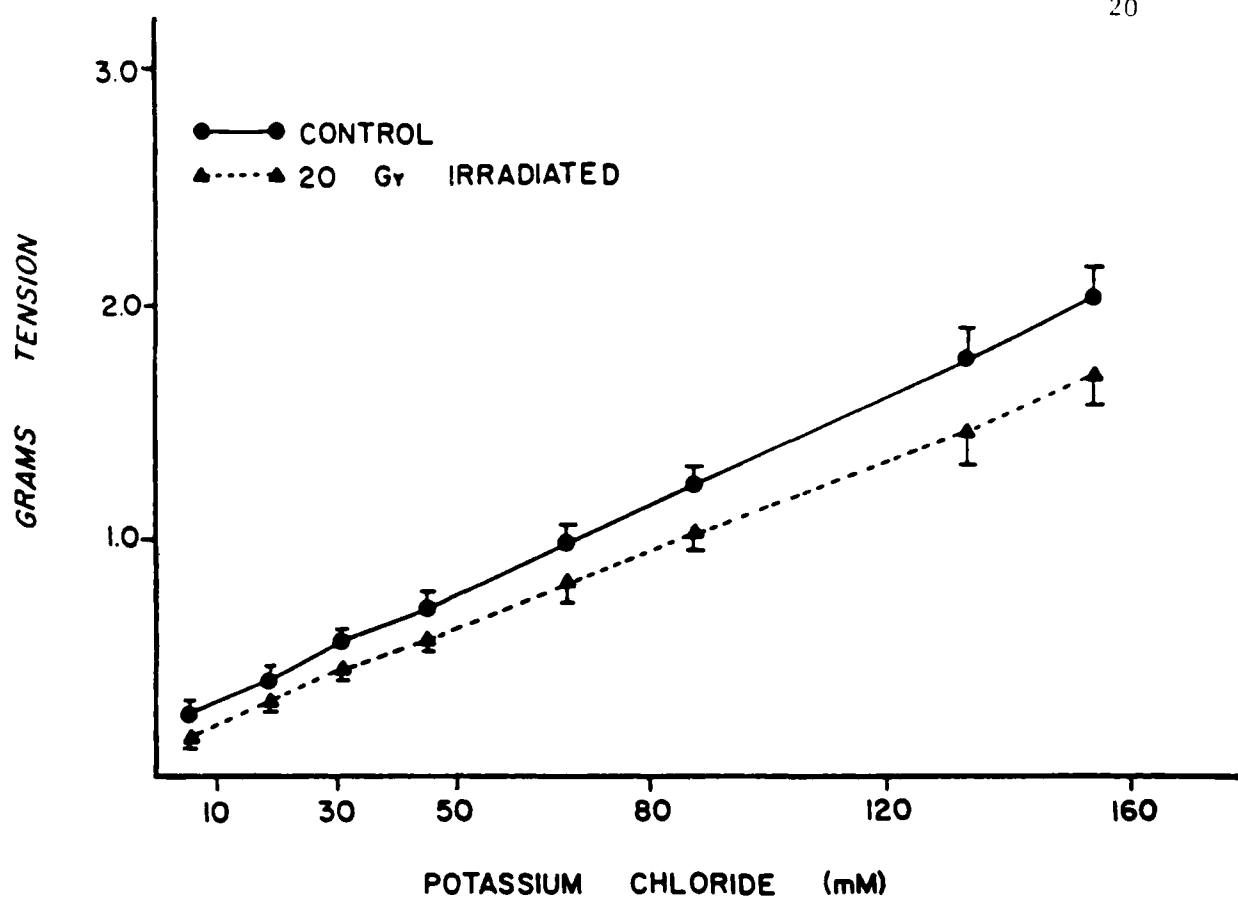


Figure 6: Vascular reactivity of aortic rings from 20.0 Gy whole body irradiated or sham irradiated animals. Pre-load tension was set at 1.5 g. Data are expressed as mean $\pm$ standard error for 6 animals per group.

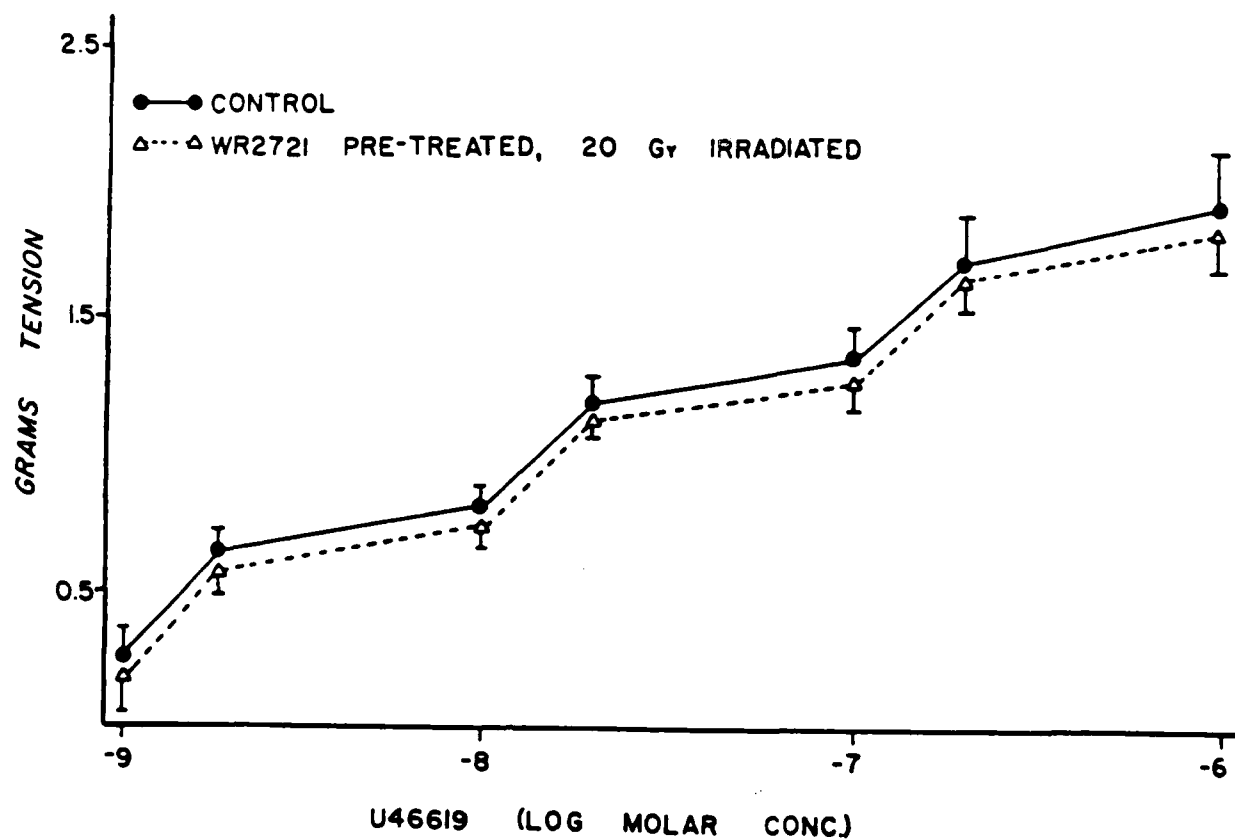


Figure 7: Vascular reactivity curves of aortic rings from sham irradiated or WR2721 pre-treated, 20.0 Gy irradiated animals. Data are expressed as mean $\pm$ standard error for 6 animals per group.

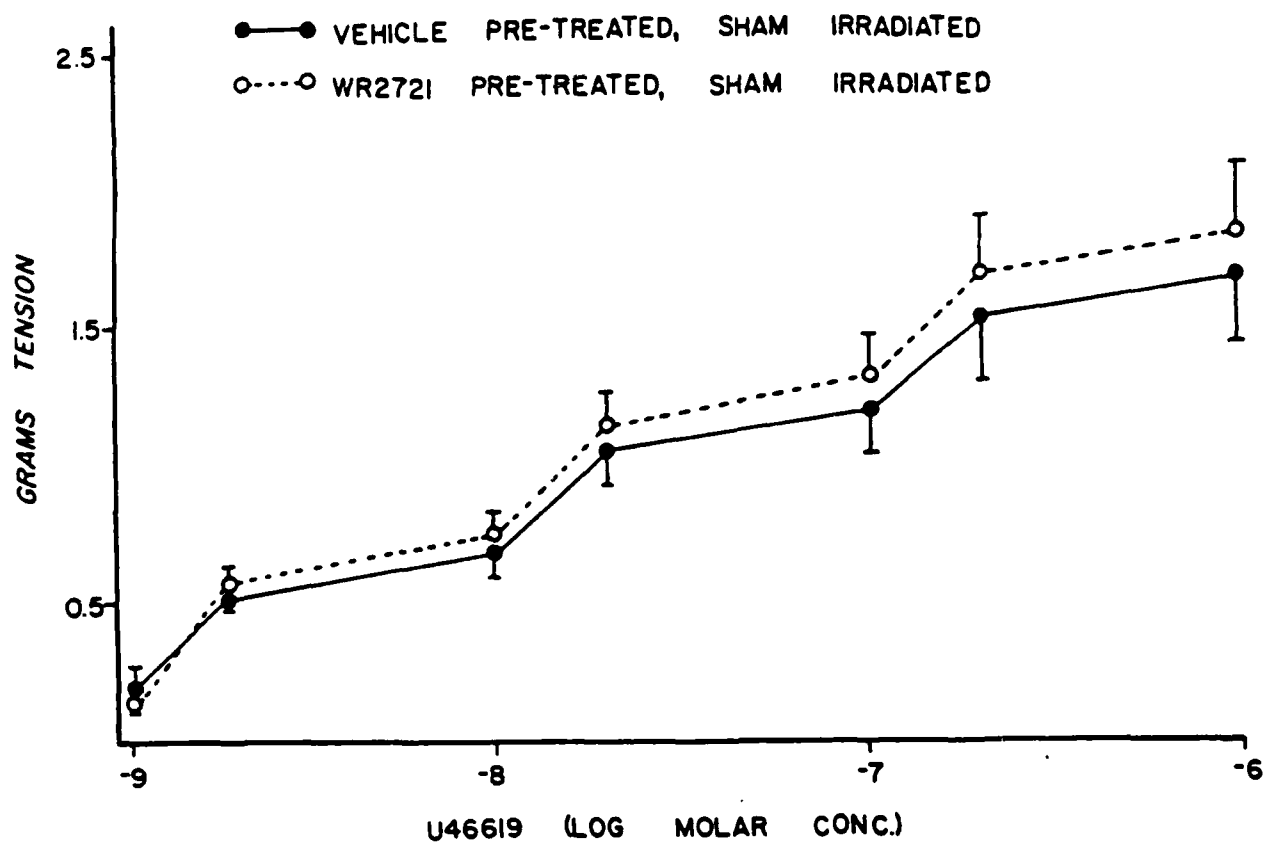


Figure 8: Vascular reactivity curves of aortic rings from sham irradiated animals injected with either WR2721 or vehicle 48 hours before challenge with U46619. Data are expressed as mean $\pm$ standard error for 6 animals per group.

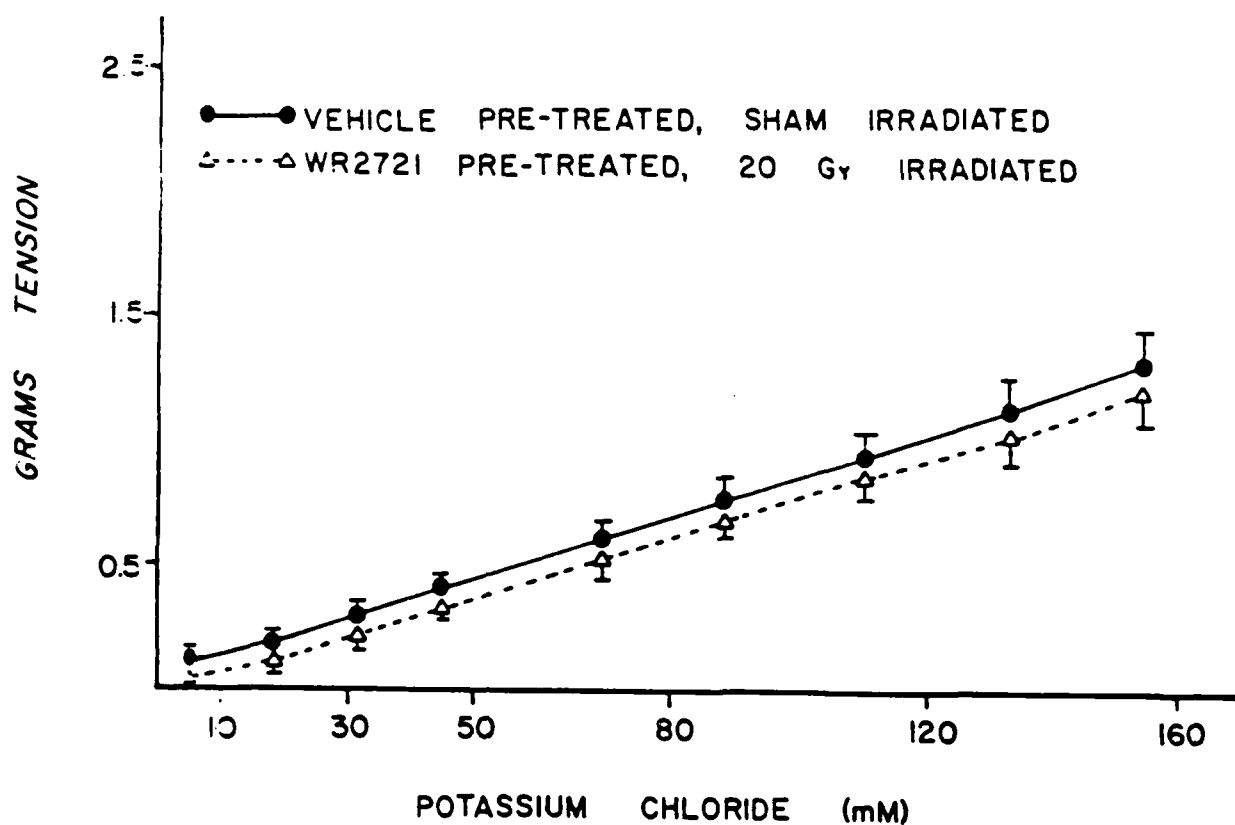


Figure 9: Vascular reactivity curves of aortic rings from vehicle pre-treated, sham irradiated rats compared to WR2721 pre-treated, 20.0 Gy irradiated animals. Vascular rings were challenged with cumulative concentrations of KCl as the agonist. Data are expressed as mean $\pm$ standard error for 6 animals per group.

Table 4

Effect of Pre-irradiation WR2721 on the Vascular Reactivity
of Isolated Rat Abdominal Aortic Rings to KCl

	Maximum Contraction (Grams Tension)	Slope	ED50 (mMolar Concentration)
Control	$1.98 \pm .12^a$	$.01 \pm .0007$	48.81 ± 4.63
20 Gy Irradiated	$1.66 \pm .15$	$.01 \pm .001$	45.77 ± 3.63
Control	$1.27 \pm .15$	$.008 \pm .0009$	57.50 ± 4.37
20 Gy Irradiated after WR2721	$1.20 \pm .13$	$.007 \pm .0007$	53.11 ± 12.40

^a Data are expressed as mean $\pm$ standard error of the mean for 6-9 animals per group.

TABLE 5
Effect of Perfusion Media on the Radioimmunoassay for Thromboxane B2

	ng/ml					Slope
	.003	.01	.03	.1	.3	
RIA	61.77 ^a	57.77	53.97	50.33	41.77	-58.08
Buffer (C.C. = -0.94) ^b	± 1.45	± 2.22	± 3.68	± 5.44	± 3.49	± 11.44
KRB	57.80	51.43	52.50	48.93	39.53	-55.27
(C.C. = -0.95) ^b	± 2.72	± 5.20	± 3.97	± 4.86	± 0.88	± 13.24
KRB	69.69	66.30	63.63	61.57	47.37	-67.77
+ 3% BSA (C.C. = -0.94) ^b	± 3.90	± 5.22	± 7.24	± 9.88	± 2.80	± 18.78
KRB	60.07	56.83	53.63	49.57	44.00	-46.71
+ 3% Dextran 70 (C.C. = -0.92) ^b	± 3.38	± 3.63	± 4.04	± 3.45	± 1.59	± 8.20

^a Data are expressed as mean $\pm$ standard error of the mean for 6 samples per data point

^b $p < .05$ for the correlation

C.C. = Correlation Coefficient

KRB containing 3% BSA as the oncotic agent. Lungs from irradiated rats perfused with KRB alone or with KRB plus 3% Dextran 70, exhibited no change in the pulmonary release of TXB2 compared to lungs from control animals perfused with the same medium (Table 6).

Discussion

Whole body gamma irradiation causes a time-dependent decrease in the contractile response of rat abdominal aortic rings to the thromboxane agonist, U46619. No change in vascular response was observed prior to 48 hours post irradiation. This latent period suggests that the response is not simply the result of acute cell death. Narayan and Cliff have also demonstrated a gradually developing abnormality in both structure and function of the rabbit ear vasculature 4 to 24 hours following 75 Gy B ray exposure (40). These studies indicate that ultrastructural damage occurs throughout the observation period used in the present investigation. Nevertheless, no functional evidence of vascular tissue injury as judged by measurements of developed tension to U46619 occurred before 48 hours post irradiation.

Since whole body radiation exposure depresses the vascular response to U46619, cumulative dose-response curves to KCl were obtained from aortic rings isolated from irradiated and control animals. Irradiation did not decrease the vascular response to KCl suggesting that although radiation may cause structural damage to the vascular tissue (40-45), the effect on smooth muscle was insufficient to decrease the aortic response to KCl.

Direct damage to the vascular endothelium could play a role in the observed alteration in vascular reactivity, as this structure is particularly vulnerable to radiation injury (41,44). It is doubtful, however, that prevention of endothelial cell destruction is responsible for the observed effect of WR2721. All vessels tested, whether pre-treated with this radioprotectant or vehicle, exhibited vasodilation when exposed to acetylcholine. As Furchgott et. al. showed (46-48), acetylcholine-induced relaxation of vascular rings depended on a functioning endothelium. Both the endothelium and vascular smooth muscle appear to be functionally intact at the time period and radiation dose examined in this study. These data suggest that the protection afforded to the vascular rings by WR2721 is not due solely to a prevention of direct radiation injury to the endothelium or the vascular smooth muscle.

Another possible mechanism by which irradiation causes vascular damage is related to the radiation-induced production of free radicals. This can produce chemical modifications or damage to cellular lipids, proteins and carbohydrates which can seriously affect cellular function (49). It has been hypothesized that thiol compounds such as WR2721 compete with damaging O_2 radicals and minimize the O_2 dependent destruction of irradiated tissue (50). Other investigators have proposed that WR2721 may promote repair of free radical damage by hydrogen donation (51). Regardless of the exact mechanism, WR2721 appears to interfere with free radical-induced alteration of irradiated cells thereby ameliorating the radiation-induced injury.

The radiation-induced formation of free radicals may alter the production of vasoconstrictor and vasodilator eicosanoids. Ham et. al. found prostacyclin synthase was very sensitive to inactivation by oxidation while thromboxane A2

TABLE 6

Effect of Perfusion Media on Immunoassayable Thromboxane B₂
Release from Irradiated Isolated Perfused Rat Lungs

	<u>KRB</u> TXB ₂ (pM)	<u>KRB + 3% BSA</u> TXB ₂ (pM)	<u>KRB + 3% Dextran 70</u> TXB ₂ (pM)
Sham	2,799 ^a	142 ^c	292 ^c
Irradiated	$\pm$ 746	$\pm$ 27	$\pm$ 60
20 Gy	1,546	283 ^{b,c}	362 ^c
Irradiated	$\pm$ 193	$\pm$ 46	$\pm$ 65

^a Data are expressed as mean $\pm$ standard error of the mean for 8 - 10 animals per group

^b $p < .05$ compared to sham irradiated controls by unpaired Student's t-test

^c $p < .05$ compared to KRB perfused lungs by Analysis of Variance plus a Newman-Keul's test

synthase was more resistant (52). A radiation-induced increase in TXA2 release may play a role in the decreased vascular responsiveness to U46619 (25,28,29,31,32). The elevated levels of TXA2 may down regulate the TXA2 receptors via a mechanism analogous to other prostaglandin receptor systems (53,54). Since WR2721 treatment prior to irradiation reduced the excretion rate of TXA2 (33), this attenuation of the radiation-induced increase in TXA2 synthesis could prevent this hypothesized down regulation of the TXA2 receptors and thereby eliminate the altered vascular response to U46619. Alternatively, free radicals may directly damage cell membranes by covalently binding to membrane enzymes and/or receptors. WR2721 protection against this free radical damage could prevent the radiation-induced decrease in vascular reactivity. Further studies are necessary to elucidate the relationship between free radical formation and the altered vascular reactivity to U46619.

The final possible mechanism by which radiation exposure may decrease the vascular reactivity to U46619 may be related to the availability of calcium for contraction. Studies have shown that the vasoconstrictor activity of U46619 is, in part, mediated by the mobilization of intracellular calcium (55,56). Miller and Stoclet (57) showed that the endothelium releases a factor which modulates the mobilization of intracellular calcium. One can postulate that, following irradiation, an endothelial derived substance is released that decreases intracellular calcium mobilization. The alteration in intracellular calcium availability would have little effect on the vascular reactivity to KCl because the KCl-induced responses, for the most part, are mediated via the influx of extracellular calcium (58). On the other hand, the U46619-induced vasoconstriction, which is apparently more dependent on the availability of intracellular calcium, would be markedly affected by a reduction in intracellular calcium availability.

In summary, these data show that whole body ionizing radiation exposure results in a depressed vascular reactivity to the TXA2 agonist, U46619, but had no effect on the vasoconstrictor activity of KCl. Pretreatment of animals with the radioprotectant WR2721 prior to irradiation, abolishes this decreased vascular reactivity to U46619. The amelioration of the depressed vascular reactivity to U46619 is not related to any direct vasoconstrictor properties of either WR2721 or any of its metabolites in the tissues 48 hours after irradiation. These studies provide a potentially useful and rapid means of evaluating the efficacy of radioprotectants *in vivo*. Additionally, the ability of WR2721 to prevent radiation-induced alteration in vascular reactivity may be an important factor in its radioprotectant action.

In a second series of studies, the effect of altered perfusion media on TXB2 release from isolated perfused rat lungs was examined. Initially, the effect of the different perfusion media on the RIA was assessed.

The RIA for TXB2 was unaffected by any of the perfusion media. Cyclooxygenase products bind weakly to albumin but this binding will not affect the RIA if an antibody with a high avidity for the ligand is used (39,59). The fact that the albumin did not affect the RIA for TXB2, suggests that the TXB2 antibody had a high avidity for the ligand.

Cyclooxygenase product release from lungs perfused with KRB-BSA was significantly less than that released from lungs perfused with KRB. Lungs perfused with KRB-Dextran 70 also released significantly less TXB2 than lungs perfused with KRB. The TXB2 release from lungs perfused with KRB containing either Dextran 70 or

BSA were comparable. These data suggest that the omission of an oncotic agent from the perfusate will increase the release of cyclooxygenase products from the pulmonary circulation.

The greater release of cyclooxygenase products from lungs perfused with KRB compared to lungs perfused with KRB containing either oncotic agent may be due to an increased accumulation of fluid in the pulmonary interstitium. This proposed increase in interstitial fluid, although insufficient to alter pulmonary perfusion pressure or the wet to dry ratio of the lungs, may promote cyclooxygenase product formation. The presence of an oncotic agent in the perfusate would oppose this interstitial fluid accumulation and result in less cyclooxygenase product release.

Alternatively, the decreased release of cyclooxygenase products from lungs perfused with KRB-BSA or KRB-Dextran 70 may be a result of an interaction between these macromolecules and the membrane. Price et al (60) suggested that macromolecules such as albumin or Dextran 70 can bind to cell membranes and decrease the lateral mobility of exposed cell surface components. This would reduce arachidonic acid release from membrane phospholipids and decrease cyclooxygenase product formation. Therefore, the observed decrease in the TXB2 release from lungs perfused with KRB-BSA or KRB-Dextran 70 may be due to macromolecular interactions. Further studies are necessary to clarify the mechanism(s) by which the perfusates alter cyclooxygenase product release.

The increased release of cyclooxygenase products from lungs perfused with KRB-BSA after irradiation may be explained by studies of Hahn et al (61). They showed that arachidonic acid release from irradiated pulmonary endothelial cells challenged with bradykinin was significantly greater than when sham irradiated cells were challenged (61). Other investigators have shown that radiation exposure increases free radical and lipid peroxide formation (1). Low concentrations of free radicals and lipid peroxides activate the cyclooxygenase pathway (2,4). Thus, the increase in cyclooxygenase products observed with irradiated lungs perfused with KRB-BSA may be due to increased precursor release from membrane phospholipids and/or activation of cyclooxygenase.

The radiation-induced increase in cyclooxygenase products could not be demonstrated when the lungs were perfused with KRB. Absence of an oncotic agent in the perfusate would enhance fluid accumulation in the interstitium and activate the cyclooxygenase pathway. This effect may mask the radiation-induced increase in arachidonic acid metabolism.

The radiation-induced increase in cyclooxygenase products was also absent when the lungs were perfused with KRB-Dextran 70. Ross and Peeke (62) suggested that Dextran is a weak radioprotectant and probably a free radical scavenger. Since the radiation-induced increase in cyclooxygenase products may be due to an increase in free radical and lipid peroxide formation, perfusion of the lungs with Dextran 70 might reduce peroxide levels and diminish cyclooxygenase activity. This, in turn, could reduce or abolish the radiation-induced increase in pulmonary cyclooxygenase product release.

In summary, the data presented in the 1986 annual report show that whole body radiation exposure will depress vascular reactivity to a thromboxane A2 mimic in a time-dependent manner. This decrease in vascular reactivity is independent of smooth muscle damage and can be prevented by pretreatment of rats with the radioprotectant WR2721. In addition, data presented in this report confirm the

radiation-induced increase in pulmonary cyclooxygenase product release presented in the 1985 annual report. These data also show that the presence of an observable radiation-induced alteration in pulmonary cyclooxygenase product release is dependent on the perfusion media used. These data also suggest that perfusion of the lungs with a free radical scavenger could ameliorate this radiation-induced increase in cyclooxygenase product release. In both series of studies described here, the mechanisms involved in the observed alterations in the measured parameters need to be determined by further studies.

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1. Warfield, M.E., Schneidkraut, M.J., Cunard, C.M., Ramwell, P.W., and Kot, P.A. Vascular response of rat abdominal aorta to U46619 following whole body gamma irradiation. Submitted, *American Journal of Physiology*
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