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RADIATION RESEARCH 118, 581–586 (1989)

SHORT COMMUNICATION

Induction of Marrow Hypoxia by Radioprotective Agents

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The ability of thiol and non-thiol radioprotectors to induce hypoxia was determined using the binding of [^{3}H]misonidazole by bone marrow cells as a measure of hypoxia. When administered at maximally radioprotective doses, four drugs (WR-2721, cysteamine, 5-hydroxytryptamine, and 16,16-dimethyl prostaglandin E_2) significantly increased the amount of [^{3}H]misonidazole bound by marrow cells, while no significant increase in binding was observed with three other agents (endotoxin, AET, superoxide dimutase). Doses of WR-2721 previously shown to provide suboptimal radioprotection did not significantly increase ^{3}H-misonidazole binding. These results suggest that the physiological effects of some radioprotectors, that is, their ability to induce marrow hypoxia, may contribute to their efficacy *in vivo*.

Keywords: radioprotective agents, review

INTRODUCTION

Many compounds which possess sulfhydryl groups have been shown to protect bone marrow from radiation injury. The most effective thiol radioprotective agent is ethiofos [*S*-2-(3-aminopropylamino)ethylphosphorothoic acid or WR-2721] (*1*). Several mechanisms of action have been postulated. These include hydrogen atom donation, OH radical scavenging, formation of mixed disulfides, metal chelation, and the production of hypoxia (*2*). Actual radioprotection may occur through a combination of these mechanisms. Studies using model biomolecule systems show that sulfhydryls do interact with radiation-induced free radicals, but direct evaluation in animal models has been more difficult to achieve because of the short half-life of various radical species. Nonetheless, it is believed that chemical mechanisms play a major role in the radioprotective effects of these drugs in *in vivo* systems.

The possibility that the production of tissue hypoxia by radioprotectors contributed to the protective effects observed with these drugs has also been considered. Some radioprotectors produce profound physiological changes including hypertension, apnea, brachycardia, and altered blood flow (*3*). Many of these altered physiologies can be induced by WR-2721 (*4*) and also by 16,16-dimethyl prostaglandin E_2 (DiPGE$_2$) (*5, 6*). The biological responses to these radioprotectors may have an impact on marrow oxygenation. WR-2721 has been postulated to reduce the peripheral

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oxygen tension (*4*). Tissue hypoxia may also explain the protection observed with certain non-thiol compounds, including 5-hydroxytryptamine (5-HT).

Direct evaluation of the role of marrow hypoxia in the radioprotection of the hematopoietic system has been hampered by the lack of a suitable method for quantifying tissue hypoxia *in vivo*. With current microelectrode technology, the pO_2 cannot be accurately determined in the marrow of small rodents. Attempts to correlate hypoxia with other physiological parameters such as blood flow or production of lactic acid are complicated since they may be influenced by changes unrelated to hypoxia. To overcome these problems and directly evaluate the role of marrow hypoxia in radioprotection we have used the sensitizer–adduct technique to assess the extent of marrow hypoxia following treatment with radioprotective agents. This system is based on the metabolic reduction and subsequent binding of misonidazole to tissues as a function of the tissue oxygen concentration (*7, 8*). Misonidazole (Miso) is preferentially bound by hypoxic tissue. Using Miso binding as a measure of tissue oxygenation, we have demonstrated that treatment with WR-2721, $DiPGE_2$, cysteamine (Cys), or 5-HT induces significant hypoxia in mouse bone marrow.

MATERIALS AND METHODS

Mice. Six- to twelve-week-old BALB/c female mice obtained from Jackson Laboratories (Bar Harbor, ME) were used in these experiments. Mice were housed in a Canadian Council on Animal Care accredited facility on a 12-h light/dark cycle. Mice were provided with a standard rodent pellet diet and with acidified water *ad libitum*.

[^{3}H]Misonidazole. ^{3}H-labeled misonidazole ([^{3}H]Miso) (sp act 356.7 μCi/mg) was prepared by Dr. James Raleigh as described (*9*). Animals received a single intraperitoneal (ip) injection of [^{3}H]Miso in a 0.5 ml volume of phosphate-buffered saline (PBS) according to the schedule detailed below. The dose of [^{3}H]Miso was calculated to achieve a peak plasma concentration of approximately 100 μM.

Drugs and treatment schedule. 16,16-Dimethyl prostaglandin E_2 was obtained in the free oil form as the generous gift of Dr. Douglas Morton (Upjohn Co., Kalamazoo, MI). Stock solutions were prepared by dissolution in ethanol to a concentration of 10 mg/ml and diluted to the indicated concentration in PBS prior to administration as a single subcutaneous injection into the nape of the neck in a 0.1 ml volume. WR-2721 was obtained from the Cancer Treatment Division of the National Cancer Institute. All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO) and were prepared in PBS immediately prior to use. Superoxide dimutase (SOD) was administered intravenously, while the remaining drugs were given as a single ip injection in a volume of 0.01 ml/g body wt. Mice were treated first with radioprotectors. Miso was injected at various intervals thereafter corresponding to the expected time of maximum radioprotective effect. Specifically, [^{3}H]Miso was administered 5 min after 5-HT (300 mg/kg); 10 min after $DiPGE_2$ (40 μg/mouse) or Cys (100 mg/kg); 15 min after 2-(2-aminoethyl)-2-thiopseudorea dihydrobromide (AET) (300 mg/kg); 30 min after WR-2721 (200–400 mg/kg); 60 min after SOD (200 mg/kg); and 24 h after endotoxin (LPS) (50 μg/mouse). In each experiment, control animals received 0.01 ml/g of PBS (ip) followed by [^{3}H]Miso at intervals equivalent to those used for the radioprotectors.

Bone marrow assay. Sixty minutes after [^{3}H]Miso, animals (three to five per group) were killed by cervical dislocation and the femurs were removed. Marrow cells were isolated by flushing femurs of individual animals with ice-cold complete medium (MEM Spinner with 5% fetal calf serum). Known numbers of cells were placed in duplicate tubes and washed four times with complete medium to remove any unbound [^{3}H]Miso. Following the last wash, the acid-soluble and acid-precipitable cellular fractions were isolated according to techniques previously described (*10*), and the total amount of [^{3}H]Miso bound to these fractions was determined by liquid scintillation counting. The results calculated for individual animals were expressed as pmol [^{3}H]Miso bound per 10^6 cells. Each drug was evaluated on two or three separate occasions. A Student's *t* test was used to determine significant differences between control and experimental groups.

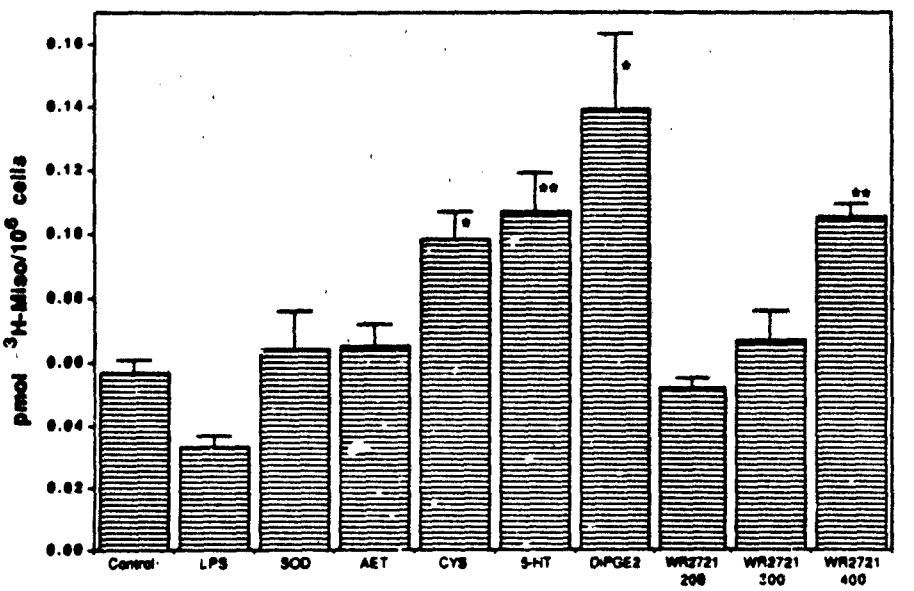

FIG. 1. Effect of *in vivo* treatment with various radioprotective agents on [^{3}H]Miso binding by bone marrow cells. Drug treatments and sample preparation are detailed under Materials and Methods. The doses of WR-2721 are provided as milligrams per kilogram body weight. Standard error bars are provided. (*) $P < 0.01$ or (**) $P < 0.001$.

RESULTS

Seven chemicals with known radioprotective properties were screened for their effects on [^{3}H]Miso binding to normal bone marrow cells *in vivo*. Cys, $DiPGE_2$, 5-HT, and WR-2721 treatment resulted in significantly increased binding to the acid-insoluble, macromolecular fraction (Fig. 1). No significant increase in marrow binding was observed following treatment with LPS, SOD, or AET.

In these studies, the dose and exposure time of each radioprotector duplicated that previously reported to produce maximum radioprotective effects (*6, 11–16*). We also determined the dose dependency of the degree of marrow hypoxia produced by increasing concentrations of WR-2721 by administering graded doses of WR-2721 (200–400 mg/kg) 30 min prior to [^{3}H]Miso. The results in Fig. 1 indicate that as the dose of WR-2721 was increased, the amount of [^{3}H]Miso bound to the marrow also increased. The response was statistically significant only at the optimal radioprotective dose of 400 mg WR-2721/kg of body weight.

DISCUSSION

The oxygen dependence of the metabolic reduction and covalent binding of misonidazole has been well described (*7, 8*). Because the K_m for the oxygen inhibition of the binding is similar to the K_m of the oxygen effect on radiosensitivity (*8*), misonidazole binding can be used as a reliable marker of radiobiological hypoxia. In this study, the binding of Miso to the macromolecular fraction of bone marrow cells was used

to indirectly assess the extent of hypoxia produced by treatment with radioprotective agents. Previous studies showed that increased binding of Miso occurs to both the small molecular weight species found in the acid-soluble fractions and to macromolecules such as DNA which are associated with the acid-precipitable fraction (*10*). The ratio of bound products between the acid-soluble and -precipitable fractions is dependent upon cell line and handling procedures, but the binding kinetics is similar for both fractions (*10, 17*). In this study, only the binding to the macromolecular fraction was considered to avoid possible problems in interpretation arising from an increased concentration of small molecular weight thiol species to which [^{3}H]Miso is capable of binding, although this would not be a problem for non-thiol containing compounds such as 5-HT or $DiPGE_2$.

Four of seven agents tested (WR-2721, $DiPGE_2$, Cys, 5-HT) produced significant increases in [^{3}H]Miso binding, suggesting that the marrow of animals treated with these drugs would be less oxygenated at times associated with peak radioprotective effects. In support of this hypothesis is evidence that various physiological and chemical properties of these drugs are such that they may contribute to the production of relative marrow hypoxia. For example, the largest uptake of [^{3}H]Miso occurred in bone marrow cells from animals receiving $DiPGE_2$. This concentration of $DiPGE_2$ produces a 16% increase in hematocrit (*6*) and a 30% reduction in breathing rate (*18*) 15 min after administration. A dose-dependent reduction in peripheral blood flow with blood pooling in the spleen also has been noted for WR-2721 (*4*). Similarly, the hypotensive and vasoactive effects of 5-HT (*15*) and Cys (*19*) have been described. Changes in blood flow distribution and respiration rate might reasonably be expected to have indirect effects on the oxygen supply of the marrow. In addition, a chemical reduction in the amount of oxygen available to the marrow may directly contribute to hypoxia. WR-1065, the dephosphorylated product of WR-2721, consumes oxygen in an *in vitro* system (*20*). Our preliminary results measuring the oxygen consumption of marrow cells treated *in vivo* with WR-2721 suggest that increased oxygen consumption also occurs in the host animal. Other thiol containing compounds such as Cys have been shown to undergo autooxygenation *in vitro* (*21*); however, their ability to produce the same effects *in vivo* have yet to be evaluated. These physiological/chemical drug effects coupled with the known dependence of Miso binding on oxygen concentration are consistent with the hypothesis that the observed increase in [^{3}H]-Miso binding was a consequence of a shift of marrow oxygen tension to lower values. A counterargument to this proposal is the possibility that these drugs affect the *in vivo* reduction of Miso which is independent of any changes in marrow pO_2. However, this latter possibility is unlikely because (1) the addition of exogeneous thiols has been shown to decrease, not increase, the binding of misonidazole to cellular macromolecules (*7*), and (2) even if these drugs were to increase the metabolism of Miso (through the stimulation of nitroreductase enzymes, for example), the resultant reactive species would be oxidized back to the parent compound under conditions of normal marrow pO_2.

In the case of WR-2721, the increase in marrow binding was found to be dose dependent, with only the optimally protective dose (400 mg/kg) producing significantly increased binding. However, other studies have demonstrated that smaller doses of WR-2721 (75–300 mg/kg) afford some marrow protection (*22*). This suggests

that several mechanisms contribute to the radioprotective effects of WR-2721, and that at low drug doses, chemical rather than physiological mechanisms predominate. Similarly, the failure of AET or SOD to alter [^{3}H]Miso binding suggests that any physiological changes produced by these drugs were insufficient to render the marrow relatively hypoxic and that chemical and/or alternative physiological changes must be postulated to account for their radioprotective properties. In the case of LPS, less [^{3}H]Miso binding was observed in drug-treated animals than in controls. The reason for this has yet to be determined. However, the possibility that LPS treatment altered the pharmacokinetics of Miso or resulted in increased oxygen delivery to the marrow should be considered.

Several investigators have drawn attention to the fact that protection with WR-2721 appears to be most effective for cells or tissues that are at intermediate oxygen tensions (*23, 24*). In such cases, it has been postulated that small reductions in oxygen tension are sufficient to render the tissues hypoxic. Previous studies by Meyn and Jenkins (*25*) which measured the induction of DNA strand breaks *in vivo* and *in vitro* have suggested that marrow resides at relatively low oxygen tensions *in vivo*. In such a case, small shifts in the availability of oxygen to critical hematopoietic targets may exert protective effects. Confirmation of the hypothesis that small changes in physiological oxygen concentrations produce radiobiological hypoxia awaits techniques for accurately measuring the *in vivo* pO_2 of cell populations.

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