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Mutation Research 372 (1996) 9–15

Fundamental and Molecular Mechanisms of Mutagenesis

Both bovine and rabbit lymphocytes conditioned with hydrogen peroxide show an adaptive response to radiation damage

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Received 19 March 1996; accepted 17 April 1996

Abstract

We have carried out experiments to study the possible induction of an adaptive response in cultured bovine and rabbit lymphocytes conditioned with subtoxic doses of hydrogen peroxide after stimulation and subsequently challenged with 1 Gy of X-rays. Peroxide treatment was given at different doses 48 h after the addition of PHA to stimulate the cells. A protective effect of pre-exposure to H_2O_2 against radiation damage detected as micronuclei in binucleated cells was evident for all the animals tested regardless the dose of H_2O_2 used, although this effect was in general of greater magnitude in bovine than in rabbit cells. These results lend further support to our previous finding in human lymphocytes that DNA single strand breaks induced by H_2O_2 (most likely due to the generation of hydroxyl radicals) is the most important lesion to trigger the adaptive response.

1. Introduction

Ionizing radiation, UV light, and a multitude of chemical agents cause damage to DNA. In living cells that have suffered such damage a variety of cellular responses are triggered.

When cells are exposed to low doses of an agent that induces damage to DNA, they often become less sensitive to the effects of a higher dose administered subsequently, i.e., they exhibit the so-called Adaptive Response (AR).

Since it was first demonstrated [1] that bacteria exhibit an AR to alkylating agents via the induction of the DNA repair system, many attempts have been made to demonstrate AR in eukaryotes [2–4], and particularly in mammalian cells [5–12]. The existence of inducible DNA repair pathways in mammalian cells could be of considerable significance in the evaluation of the mutagenic and carcinogenic potential of radiation and environmental chemicals.

The existence of AR in mammalian cells has been reported for various alkylating agents [13,14] and in response to tritiated thymidine ([^{3}H]TdR) or ionizing radiation [5,6,11,15–21].

Hydrogen peroxide (H_2O_2) is cytotoxic to mammalian cells due to the generation of oxidative free radicals [22–24]. It has been shown to mimic ionizing radiation to a certain extent, and has been found to produce almost exclusively single strand breaks (ssb) in DNA, contrasting with ionizing radiations [25] that also induce double strand breaks (dsb) and base damage.

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PII S0027-5107(96)00082-6

We have reported the use of the cytokinesis block micronucleus method [26] to evaluate the AR as a good alternative to the single fixation method and scoring of chromosomal aberrations at metaphase for the study of the AR in human lymphocytes [9]. Using this method we also observed a significant decrease in the yield of micronuclei in binucleated cells conditioned with H_2O_2 or low-dose X-rays in G_0 phase [10].

The present paper describes experiments on the AR in cultured bovine and rabbit lymphocytes when they are conditioned with different doses of H_2O_2 24 h before exposure to a challenge dose with 1 Gy of X-rays.

2. Material and methods

2.1. Animals

Two healthy cows of the same race and reared in the same farm and two adult male rabbits were used in the experiments. The animals were kept with ad libitum access to standard diet and drinking water.

2.2. Cell culture

Whole blood (0.5 ml) from the animals was added to 4.5 ml of RPMI 1640 medium containing 20% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2% phytohemagglutinin (PHA) to stimulate G_0 lymphocytes.

2.3. Conditioning treatment

Conditioning treatment consisted of a single 30 min pulse with 5, 25, 50 or 75 μM H_2O_2 for bovine lymphocytes and 25, 50 or 75 μM for rabbit lymphocytes, given 48 h after setting up the cultures. The cells were then centrifuged and the medium changed. The same manipulations, except the addition of H_2O_2, were carried out with the cells receiving radiation alone.

2.4. Challenge treatment and cytokinesis block

Challenge treatment was at 24 h after conditioning treatment. The cells were exposed to 1 Gy of

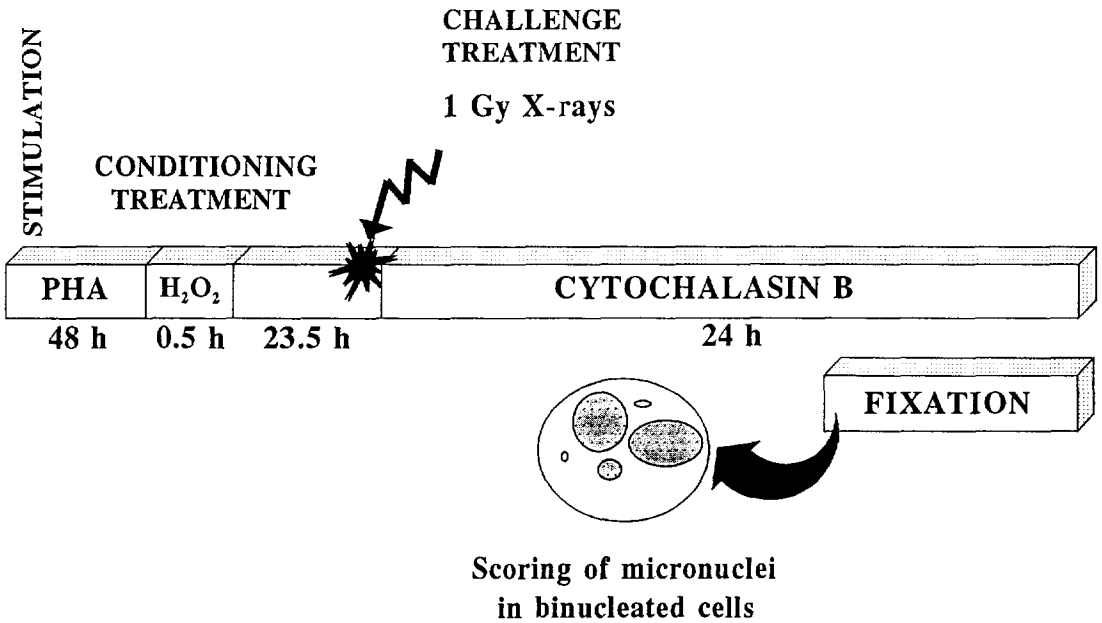

Fig. 1. Experimental procedure for conditioning pulse-treatment with H_2O_2 given at different doses (5, 25, 50 or 75 μM) before irradiation in previously stimulated bovine and rabbit lymphocytes.

X-rays from a Philips SL 75-20 linear accelerator, 8 MeV, delivered at a dose rate of 2 Gy/min.

Cytochalasin B (Cyt B, Sigma) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml, and stored at −80°C. On the day of the use, the stock solution was diluted with PBS and added to the cell cultures at a final concentration of 6 μg/ml to ensure an efficient inhibition of cytokinesis [27] immediately after the X-ray exposure. After recovery in Cyt B for 24 h, fixation was performed basically following a cytological procedure previously described [28] with several modifications. Cells exposed briefly to a 0.075 M KCl pre-warmed solution were then fixed in freshly made ice-cold Carnoy's fixative (absolute methanol/acetic acid, 3:1) for several minutes. Subsequently, they were washed in PBS and finally fixed in a freshly made methanol/acetic solution (6:1). Cytological preparations were made by dropping cells onto wet slides and staining with Giemsa.

Two thousand binucleated cells were scored blind for micronucleus frequency in each treatment by two different observers. A one-tailed *t*-test was used to determine if the number of micronuclei observed in cells conditioned before irradiation with 1 Gy of X-rays was significantly lower than the expected, i.e., if they showed the adaptive response.

3. Results

Previous experiments carried out in our laboratory [8–10] showed that conditioning either stimulated or unstimulated (G_0) human lymphocytes with low doses of H_2O_2 results in a protection against chromosome damage induced by X-rays delivered later on.

As can be seen in Fig. 1, cells were pulse-treated for 30 min with different doses of H_2O_2 (5, 25, 50, 75 μM) as a conditioning treatment 48 h after the addition of PHA to stimulate lymphocytes and irradiated 24 h later with 1 Gy of X-rays. After a recovery

Table 1
Adaptive response in bovine lymphocytes conditioned with different doses of H_2O_2 48 h after stimulation

Animal	Conditioning pretreatment (H_2O_2) (μM)	Challenge treatment X-rays (Gy)	No. of micronuclei			No. of cells scored
			Observed		Expected [a]	
			No.	%	%	
A	–	–	38	19	–	2000
	5	–	33	16.5	–	2000
	25	–	159	79.5	–	2000
	50	–	114	57	–	2000
	75	–	117	58.5	–	2000
	–	1	278	139	–	2000
	5	1	216	108	136.5 [b]	2000
	25	1	319	159.5	199.5 [b]	2000
	50	1	271	135.5	177 [b]	2000
	75	1	209	104.5	178.5 [b]	2000
B	–	–	16	8	–	2000
	5	–	23	11.5	–	2000
	25	–	25	12.5	–	2000
	50	–	30	15	–	2000
	–	1	290	145	–	2000
	5	1	178	89	148.5 [b]	2000
	25	1	172	86	149.5 [b]	2000
	50	1	187	93.5	152 [b]	2000

[a] Sum of the two individual treatments minus the control.
[b] Observed frequency significantly lower than expected ($P < 0.01$) (one-tailed *t*-test).

time of 24 h in Cyt B, micronuclei were scored in binucleated cells. An interesting feature observed was that bovine lymphocytes in general appeared as more sensitive to irradiation than human cells, and accordingly the dose of X-rays (1 Gy) was lower than that customarily used for human lymphocytes (1.5 Gy).

Table 1 shows the frequencies of micronuclei in binucleated cells in bovine lymphocytes from two animals. As can be seen, the difference between the expected and observed values when they were conditioned with peroxide before challenging with X-rays (combined treatments) was always statistically significant, i.e., they showed the adaptive response in a similar way to that observed in our previous experiments with human cells [7–10].

As can be seen in Table 2, when we carried out a similar experimental protocol using rabbit lymphocytes they showed as more sensitive to X-rays than bovine lymphocytes but also showed the AR. These cells had for all doses of peroxide significantly fewer micronuclei than expected. In general, however, rabbit lymphocytes showed a protection that was apparently of less magnitude than the response observed in bovine lymphocytes (Tables 1 and 2).

4. Discussion

The mechanism underlying the phenomenon of increased resistance to exposure to radiation is not yet fully understood. In yeast it has been reported [3] that DNA ssb, such as those induced by hydroxyl radicals, may be an important lesion that signals the induction of the recombinational repair system, which is believed to be the major mechanism that confers resistence to ionizing radiation in these cells.

Different reports have shown the existence of an adaptive response not only in procaryotic cells [1,29] but also in eucaryotic cells as have been reported for yeast [3], plant cells [2], Drosophila [4], chicken embryos [30] and different mammalian cells [5,7–12,18,20,31,32].

X-ray damage is partly attributed to the production of free radicals that are also produced by H_2O_2 [33]. Oxidation products of DNA bases are present

Table 2
Adaptive response in rabbit lymphocytes conditioned with different doses of H_2O_2 48 h after stimulation

Animal	Conditioning pretreatment (H_2O_2)	Challenge treatment X-rays	No. of micronuclei			No. of cells scored
	(μM)	(Gy)	Observed		Expected [a]	
			No.	‰	‰	
A	–	–	9	4.5	–	2000
	25	–	9	4.5	–	2000
	50	–	9	4.5	–	2000
	75	–	13	6.5	–	2000
	–	1	556	278	–	2000
	25	1	443	221.5	278 [b]	2000
	50	1	301	150.5	278 [b]	2000
	75	1	486	243	280 [b]	2000
B	–	–	12	6	–	2000
	25	–	6	3	–	2000
	50	–	10	5	–	2000
	75	–	10	5	–	2000
	–	1	592	296	–	2000
	25	1	427	213.5	293 [b]	2000
	50	1	409	204.5	295 [b]	2000
	75	1	468	234	295 [b]	2000

[a] Sum of the two individual treatments minus the control.
[b] Observed frequency significantly lower than expected ($P < 0.01$) (one-tailed t-test).

within the mammalian cell at relatively high levels caused by endogenous oxidants [34], i.e., through a mechanism involving hydrogen peroxide. H_2O_2 is well documented for its ability to induce almost exclusively DNA ssb [35] most likely due to the generation of hydroxyl radicals [36], although it can induce, to a minor extent, other types of damage such as double-strand breaks, base destruction, base liberation and cross-linking [37,38].

In this work we have found that conditioning bovine lymphocytes with H_2O_2, in agreement with our earlier results on AR in cycling or G_0 human lymphocytes [8–10], seems to protect them from radiation damage detected as micronuclei in binucleated cells. This protection seems to be of greater magnitude in one of the animals (animal B) with reductions in the yield of micronuclei that reaches values of about 40%. This is in agreement with different reports on the AR to radiation damage where interindividual differences have been observed [17,39,40].

With regards to the AR in rabbit lymphocytes, we have seen it in both animals in good agreement with that previously reported in cultured cells or in vivo with low doses of radiation instead of peroxide as conditioning treatment [18,20]. In comparison with the bovine cells, rabbit lymphocytes showed a weaker AR, since this protection reached average values of only 26% reduction of the number of micronuclei.

In conclusion, our results clearly show that the AR is induced by H_2O_2 in both bovine and rabbit lymphocytes and the cells are protected against subsequent exposure to 1 Gy of X-rays.

Although the molecular basis for this apparent protective effect of low doses of H_2O_2 against oxidative damage remains to be elucidated, it has been proposed that this enhanced resistance to ionizing radiation could result from the induction of an error-free repair pathway in response to certain signals. The abolition of the adaptation by the poly(ADP-ribose)polymerase inhibitor 3-aminobenzamide reported in human lymphocytes [15] and CHO cells [41] seems to support the involvement of DNA repair processes.

Our results on the efficiency of a conditioning with H_2O_2 in stimulated bovine and rabbit lymphocytes, in good agreement with our previous reports on the adaptive response induced by peroxide in stimulated or G_0 human lymphocytes [8–10], seems to support the relative importance of ssb for the induction of a repair mechanism acting on subsequent radiation damage.

The use of the cytokinesis-block method in the present investigation to score micronuclei in binucleated cells, as compared with the scoring of chromosomal aberrations at metaphase to study the possible adaptation in lymphocytes employed in previous investigations [18,42,43], offers the advantage of being able to harvest the cells exposed to the challenge high dose of X-rays at different stages of the cell cycle and allows them to undergo any delay needed to repair before entering mitosis and showing up as binucleated cells.

Acknowledgements

We thank Prof. E. Tello for his support and encouragement and M.A. Ledesma for expert technical assistance. One of the authors (M.J.F.) is the recipient of a fellowship from the Spanish Ministry of Education and Science. This work has been partly supported by Grants from the Commission of the European Communities (FI3P-CT92-0031) and DGICYT (PB93-0734 and CE93-0006), Spain.

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