BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **234,** 44–47 (1997)
ARTICLE NO. RC976577

Proliferation of Mouse Fibroblasts Induced by 1,2-Dimethylhydrazine Auto-Oxidation: Role of Iron and Free Radicals

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Received March 31, 1997

Activation of 1,2-dimethylhydrazine (DMH) by prolonged auto-oxidation (24-h) induced proliferation of mouse fibroblasts at low hydrazine concentrations (0.1-1.0 mM) as determined by [^{3}H-methyl]-thymidine uptake of confluent quiescent cells. Incubations were performed under conditions in which alkyl radicals are slowly formed by DMH auto-oxidation. The proliferative stimulus induced by DMH auto-oxidation complements that induced by insulin, PMA, and EGF. Inhibition by the iron chelators, o-phenanthroline and desferrioxamine, demonstrates that the induction of the proliferative effect is dependent on simple iron complexes. Proliferation was also inhibited by superoxide dismutase, catalase, and mannitol, implicating reactive oxygen species, although superoxide dismutase and catalase also inhibited alkyl radical formation, as determined by spin-trapping. These results suggest that cell proliferation induced by DMH auto-oxidation is mediated by reactive oxygen species, mainly the hydroxyl radical, and is dependent on simple iron complexes, possibly involving the Fenton reaction. © 1997 Academic Press

Several hydrazine derivatives have pharmacological applications such as anti-depressants (phenelzine), anti-hypertensive agents (hydralazine), anti-cancer agents (procarbazine) or anti-tuberculosis drugs (isoniazide) (1, 2). However, they also have toxic side effects, including carcinogenic and mutagenic properties (3, 4). DMH is a potent carcinogen, widely used as a model for colon cancer. Hydrazine derivatives are extensively metabolized *in vivo* and their toxicity has been ascribed to metabolism-generated reactive intermediates such as alkyldiazonium ions (5-7), carbon-centered radicals (8-11) and reactive oxygen species (12, 13).

The importance of de-regulated cell proliferation in the induction of malignant transformation is well established (14) and increased cell division has been considered to have a role in the mechanisms of carcinogenesis induction (15). On the other hand, it has been proposed that the establishment of a pro-oxidant state could have a role in chemical carcinogenesis (16, 17) and proliferative properties have been demonstrated for reactive oxygen species (18, 19). Administration of DMH to rats has been shown to first inhibit and then increase DNA synthesis in the colonic tissue with concurrent reduction of cAMP levels and increase in type I protein kinase activity (20). Activation of protein kinase C upon administration of DMH to rats has also been demonstrated (21). The induction of a pro-oxidant state by hydrazine derivatives and/or their metabolites, with the expression of proliferative properties, could constitute another mechanism which contributes to the carcinogenic properties of these compounds.

Our recent studies have demonstrated that mono and di-substituted hydrazines, activated by three different oxidation systems to generate alkyl radicals, induced increased cytotoxicity to mouse fibroblasts, an effect that correlated with alkyl radical formation, as measured by ESR/spin-trapping (22). In these studies, an apparent proliferative process occurring at low hydrazines concentrations was observed upon activation of these compounds by incubation in a 24 h auto-oxidation system. Here we further investigated the proliferative properties of DMH using a proliferation assay in which [^{3}H-methyl]-thymidine uptake of confluent quiescent cells was measured during the 24-h incubation period with the hydrazine derivative and different scavengers were used in order to identify the reactive species involved in this process.

Abbreviations used: DMH, 1,2-dimethylhydrazine.2HCl; DTPA, diethylenetriaminepentaacetic acid; POBN, α-(4-pyridyl-1-oxide)-N-tert-butylnitrone; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; Tempol, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl; SOD, superoxide dismutase; ESR, electron spin resonance; PMA, phorbol-12-myristate-13-acetate; EGF, epidermal growth factor; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; TCA, trichloroacetic acid.

0006-291X/97 $25.00

MATERIALS AND METHODS

Chemicals. The following reagents were obtained from commercial sources: DMH, DMEM, DTPA, POBN, DBNBS, DMPO, Tempol, SOD from bovine erytrhocytes, catalase from bovine liver, desferrioxamine, and o-phenanthroline were from Sigma Chemical Co (St.Louis, MD, USA). [^{3}H-methyl]-thymidine was from Amersham. FCS was from Cultilab (Campinas, Sao Paulo, Brasil). Hydrazine derivatives stock solutions were prepared immediately before use in DMEM containing 0.1 mM DTPA. Milli-Q (Millipore) grade water was used throughout.

Cells and culture conditions. The Myc 9E transfectant cell line (gently provided by Dr. Mari C. S. Armelin) was derived from a Balb/c-3T3 (clone A31) by over-expression the mouse *c-myc* proto-oncogene (23). These cells display normal cell morphology and have constitutive *c-myc* expression to levels comparable to PDGF-stimulated Balb/c-3T3 cells (23). Cells were routinely cultured from stock cells stored in liquid nitrogen. Cultures were maintained in tissue culture dishes in DMEM supplemented with 10% fetal calf serum without antibiotics, in a humidified 5% CO_2 atmosphere at 37°C and subcultured twice a week, before reaching confluence.

Treatment of mouse fibroblasts with activated hydrazine derivatives. Confluent quiescent Myc 9E cells seeded in 24-well trays, 3 days before treatment, were rinsed with PBS and incubated with increasing concentrations of DMH in 0.3 mL of DMEM containing 0.1 mM DTPA, 0.5% FCS and 0.5 μCi/mL [^{3}H-methyl]-thymidine for 24 h at 37°C in a 5% CO_2 atmosphere.

Proliferation assay. Cultures were seeded 3 days before treatment at 2.5×10^4 cells/well in 24-well trays. Upon confluence, cells were made quiescent by lowering the FCS concentration to 0.5% for 24 h. Following treatments, the reaction mixtures were immediately removed, cells were rinsed with PBS, and lysed with 0.5 N NaOH at 65°C for 15 min. The supernatants were transferred to pieces of thick filter paper and serially washed with 5% TCA, ethanol (twice) and acetone, air dried and the radioactivity incorporated into DNA was determined in a scintillation counter. Three independent experiments were performed in sixtuplicate.

Electron spin resonance studies. Standard reaction mixtures containing DMH, 1 μg/mL insulin, 0.1 mM DTPA, 0.5% FCS and 100 mM POBN in DMEM were incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h. Aliquots (100-200 μL) taken from the incubation mixtures were transferred to flat quartz cells. Spectra were recorded at room temperature on a Bruker ER 200D-SRC spectrometer. The concentration of POBN-radical adducts was calculated by double integration using Tempol as standard.

RESULTS AND DISCUSSION

Proliferation Induced by DMH Autooxidation

We had previously observed that prolonged incubation (24 h) with low concentrations of hydrazine derivatives induced an apparent proliferative effect on mouse fibroblasts (A31 or Myc 9E) and cytotoxicity became evident at higher concentrations, correlating with alkyl radical formation (22). Incubations were performed in DMEM and DTPA, as to produce alkyl radicals slowly for 24 h by auto-oxidation, and DMH auto-oxidation led to a 10-fold increase in methyl radical formation (when compared to 1 h incubations) as determined by ESR/spin-trapping (spectra as in 11)(22) Growth of sub-confluent cells was measured by [^{3}H-methyl]-thymidine uptake after treatment with the hydrazines. In order to better characterize this proliferative response, here we use an assay in which DNA synthesis of previously confluent and quiescent cells is measured upon prolonged incubation with DMH. Myc 9E cells were used because the response is more pronounced in this cell line. It can be observed that also in quiescent cells, low concentrations of DMH induce DNA synthesis measured by [^{3}H-methyl]-thymidine incorporation (Figure 1). This effect was observed between 0.1 and 1.0 mM DMH and varied between 15 and 30% in different experiments. This variation can be due to cytotoxic and proliferative processes competing in opposite directions.

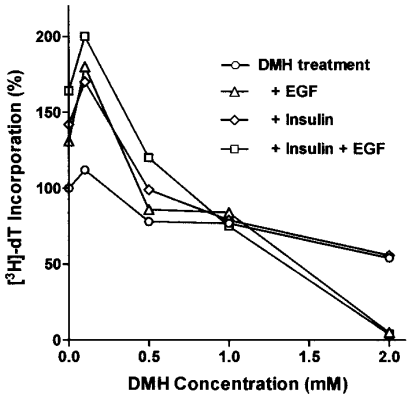

FIG. 1. DNA synthesis stimulation of mouse fibroblasts induced by prolonged treatment (24 h) with DMH: Effect of proliferation supplements. Confluent quiescent Myc 9E cells were incubated with increasing concentrations of DMH in DMEM containing 0.1 mM DTPA, 0.5% FCS, and the following hormones and/or growth factors: 1 μg/mL insulin and/or 10 ng/mL EGF and 0.5 μCi/mL [^{3}H-methyl]-thymidine for 24 h at 37°C in a 5% CO_2 atmosphere. The radioactivity incorporated into DNA was determined as described in Materials and Methods. Standard deviations were between 10 and 15%.

Effect of Proliferation Complements

Since the induction of proliferation or the triggering of cell division involves the establishment of competence and progression, we evaluated the effect of DMH in the presence of insulin, EGF and PMA. Both progression factors, like insulin and EGF (Figure 1), and the competence factor, PMA (not shown), all stimulated the proliferative process induced by DMH auto-oxidation. These results indicate that DMH auto-oxidation could be acting at the level of both competence and progression.

Effect of Free Radical Scavengers

In order to investigate the possible involvement of carbon-centered or oxygen free radicals in the induction of the proliferative process by DMH auto-oxidation, the proliferation assays were carried out in the presence of spin-traps. No inhibition of the proliferative effect was observed when incubations were performed

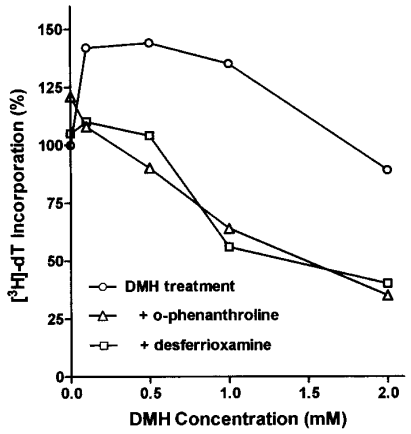

FIG. 2. DNA synthesis stimulation of mouse fibroblasts induced by prolonged treatment (14 h) with DMH: Effect of iron chelators. Confluent, quiescent Myc 9E cells were incubated with increasing concentrations of DMH in DMEM containing 0.1 mM DTPA, 0.5% FCS, and the following iron chelators: 2 μM o-phenanthroline or 0.1 mM desferrioxamine and 0.5 μCi/mL [^{3}H-methyl]-thymidine for 14 h at 37°C in a 5% CO_2 atmosphere. Incubations were maintained for 14 h due to toxicity of the chelators. The radioactivity incorporated into DNA was determined as described in Materials and Methods. Standard deviations were between 10 and 15%.

TABLE I

Relative Methyl-POBN Radical Adduct Formation upon DMH Auto-Oxidation

System	Methyl-POBN radical adduct[a] (%)
DMH 24 h[b]	100
+o-Phenanthroline (2 μM)	79
+Desferrioxamine (0.1 mM)	89
+SOD (20 μg/mL)	34
+Albumin (20 μg/mL)[c]	80
+Catalase (0.5 μg/mL)	52
+Albumin (0.5 μg/mL)[d]	80
+Mannitol (300 μM)	100

[a] Values are means of two independent determinations. Differences between determinations were below 10%. Radical concentrations were calculated as described in Materials and Methods.

[b] The standard reaction mixtures containing 5 mM DMH, 0,1 mM DTPA, 1 μg/mL insulin, and 100 mM POBN were incubated in DMEM with 0.5% FCS for 24 h at 37°C in a 0.5% CO_2 atmosphere.

[c] Albumin concentration was the same as SOD to evaluate the protein effect.

[d] Albumin concentration was the same as catalase to evaluate the protein effect.

in the presence of POBN and DBNBS, which are efficient in detecting alkyl radicals (24) (not shown). Unfortunately, DMPO, which is very efficient in detecting oxygen radicals, was too toxic under these conditions. However, we cannot rule out the involvement of free radicals in DMH-induced proliferation due to possible inaccessibility of the spin-traps to the site of free radical formation.

Effect of Iron Chelators

In view of the possible catalysis of DMH oxidation by transition metals, incubations were carried out in the presence of o-phenanthroline, a compound known to complex and remove iron from DNA, or desferrioxamine, a peptide which binds iron with a very high constant but does not penetrate all types of cells (25). Neither compound removes iron from the active sites of enzymes. Iron chelation with either compound eliminates or greatly inhibits the proliferative process induced by DMH auto-oxidation (Figure 2), although they neither inhibit substantially methyl radical formation (Table I) nor alter DMH cytotoxicity (Figure 2). These results indicate that, in this system, iron is not important in the oxidation of DMH to methyl radicals (the reaction mixture already contains DTPA), but acts mainly in the subsequent steps leading to proliferation. In addition, these results suggest that methyl radicals are not likely to be involved in the induction of proliferation, but are in agreement with our previous results implicating alkyl radicals in the cytotoxicity of activated hydrazine derivatives.

Effect of Reactive Oxygen Species Scavengers

Reactive oxygen species have been implicated in the toxicity of hydrazine derivatives and have also been shown to induce cell proliferation at low doses (18,19). The reactive oxygen species scavengers, SOD (superoxide anion), catalase (H_2O_2) or mannitol (hydroxyl radical), all inhibited the proliferative effect induced by DMH auto-oxidation (Figure 3). SOD and catalase, but

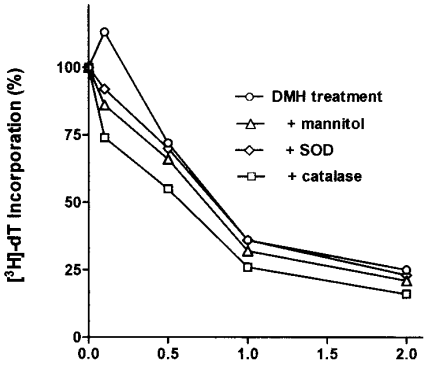

FIG. 3. Proliferation of mouse fibroblasts induced by prolonged treatment (24 h) with DMH: Effect of reactive oxygen species scavengers. Confluent, quiescent Myc 9E cells were incubated with increasing concentrations of DMH in DMEM containing 0.1 mM DTPA, 0.5% FCS, and the following scavengers: 20 μg/mL SOD (3750 U/mg) or 0.5 μg/mL catalase (10,000-25,000 U/mg) or 300 μM mannitol and 0.5 μCi/mL [^{3}H-methyl]-thymidine for 24 h at 37°C in a 5% CO_2 atmosphere. The radioactivity incorporated into DNA was determined as described in Materials and Methods. Standard deviations were between 10 and 15%.

not mannitol, also substantially inhibited methyl radical formation in this system (Table I), but the experiments with the iron chelators have ruled out the involvement of methyl radicals in this proliferative process (Figure 2, Table I). These results strongly suggest the involvement of an iron catalyzed reduction of H_2O_2 to hydroxyl radicals (Fenton reaction (26)), since both mannitol and iron chelators inhibit the proliferative process.

Our results implicate reactive oxygen species, mainly the hydroxyl radical, in the induction of cell proliferation, which may be generated by the iron-catalyzed reduction of H_2O_2 formed during DMH auto-oxidation. Thus, hydrazine-induced proliferation may be mediated by reactive oxygen species and could constitute another mechanism in the carcinogenic properties of these derivatives.

ACKNOWLEDGMENTS

We are grateful to Dr. Ohara Augusto for use of ESR facilities and fruitful discussions; Dr. Paulo Lee Ho and Dr. Mari C. S. Armelin for careful revision of the manuscript and fruitful discussions; and Dr. Isaias Raw for continuing support. This work was supported by FAPESP, CNPq, and Fundação Butantan.

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