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Effects of Nicotinamide on NAD and Poly (ADP-Ribose) Metabolism in DNA-Damaged Human Lymphocytes

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The effect of nicotinamide on unscheduled DNA synthesis was studied in resting human lymphocytes. In cells treated with UV irradiation or with MNNG, nicotinamide caused a two-fold stimulation of unscheduled DNA synthesis and retarded the rate of NAD^+ lowering caused by these treatments. Nicotinamide also reduced the burst of poly(ADP-ribose) synthesis caused by MNNG treatment. Thus under conditions that it enhances unscheduled DNA synthesis, nicotinamide causes marked effects on the metabolism of NAD^+ and poly(ADP-ribose). The effect of nicotinamide on unscheduled DNA synthesis was shown to be independent of protein or polyamine synthesis.

Key words: DNA repair synthesis, normal human lymphocytes, nicotinamide, nicotinamide adenine dinucleotide (NAD), ornithine decarboxylase, poly(ADP-ribose)

Poly(ADP-ribose) polymerase catalyzes the conversion of NAD^+ to poly(ADP-ribose) with the liberation of one nicotinamide ring from each molecule of NAD^+ hydrolyzed [1]. When the activity of this chromatin-bound enzyme is stimulated by DNA-damaging treatments, there is an associated lowering of intracellular NAD^+ pools [2]. While the specific function of poly(ADP-ribose) are unknown, it has been suggested that chromatin proteins are modified by the polymer to facilitate transient alteration of chromatin structure [3]. We have recently reported that addition of nicotinamide to resting normal human lymphocytes that have been treated with UV irradiation, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), or dimethyl sulfate results in a dose-dependent increase in unscheduled DNA synthesis. At a concentration of 2 mM nicotinamide, the highest concentra-

Abbreviations used: NAD^+, nicotinamide adenine dinucleotide (oxidized form); MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; αMO, alpha-methyl-L-ornithine; αDFMO, alpha-difluoromethyl-DL-ornithine; DMSO, dimethylsulphoxide; HPLC, high pressure liquid chromatography; poly(ADP-ribose), poly(adenosine diphosphoribose); Hepes, N-2-hydroxyethylpiperazine-N′-2-ethansulfonic acid; MGBG, methylglyoxal bis(guanylhydrazone).

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tion lymphocytes will tolerate without cytotoxic effects, there is a two-fold increase in unscheduled DNA synthesis compared to that which occurs in cells receiving only the damaging treatments [4]. In the present study we examined several different aspects of NAD^+ metabolism to determine what part they might play in the nicotinamide stimulation of unscheduled DNA synthesis. Since nicotinamide is a precursor for NAD^+ synthesis as well as a potent poly(ADP-ribose) polymerase inhibitor [5], its effects on unscheduled DNA synthesis may be due to its ability to stimulate NAD^+ synthesis or to its ability to inhibit NAD^+ consumption reactions such as that catalyzed by poly(ADP-ribose) polymerase. In addition, nicotinamide has been reported to induce the synthesis of several enzymes including ornithine decarboxylase [6] which is the rate-limiting enzyme in polyamine biosynthesis. Since UV irradiation has been reported to induce ornithine decarboxylase [7] and since polyamines have been reported to be involved in control of DNA synthesis and transformation [8–10], we also examined the possibility that nicotinamide stimulation of unscheduled DNA synthesis might be mediated through induction of polyamine or protein synthesis.

MATERIALS

Methyl-L-ornithine (αMO) was purchased from Calbiochem (La Jolla, CA) and methylglyoxal bis(guanylhydrazone) (MGBG) was from Sigma Chemical Co. (St. Louis, MO). αDifluoromethyl-DL-ornithine (αDFMO) was a gift from the Merrel Research Center, Merrel-National Laboratories (Cincinnati, OH). Sources of reagents for poly(ADP-ribose) assays may be found in reference [11].

METHODS

Determination of Unscheduled DNA Synthesis

Normal human lymphocytes were isolated from peripheral blood [12] and were suspended at 2×10^6 cells/ml in complete medium composed of alpha-modified Eagle's medium buffered with 25 mM Hepes, pH 7.2, and supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. This medium contained 8 μM nicotinamide. For UV irradiation, cells were collected by centrifugation at 800g for 5 min, resuspended at 2×10^6/ml in phosphate-buffered saline, and UV irradiated as previously described [13]. Control cells were subjected to the same procedure except that they were not irradiated. Cells were removed from phosphate-buffered saline by centrifugation at 800g for 5 min and resuspended in complete medium at 1×10^6/ml. For chemical treatment, MNNG was dissolved in DMSO at a concentration of 10 mg/ml just prior to addition to cell cultures. Cells were treated with MNNG at a final concentration of 136 μM and 0.2% DMSO. Control cells were treated with 0.2% DMSO. For the determination of unscheduled DNA synthesis, in the presence of inhibitors of polyamine synthesis or protein synthesis, MNNG treated and control cells were immediately adjusted to contain either 50 mM αMO, 80 μM MGBG, 50 mM αMO, and 80 μM MGBG, 11 mM DFMO, 500 μg/ml cycloheximide, or an equivalent amount of saline in the case of controls. Two-milliter samples of the cell cultures were immediately distributed into sterile culture tubes and adjusted to 10 mM in hydroxyurea and to 2 mM nicotinamide or to contain an equivalent amount of sa-

line. Cells were incubated for 30 min at 37°C, and then [^{3}H]dThd (specific activity 73.6 Ci/mmol) was added to a final concentration of 10 μCi/ml and incubations were continued for 6 hours at 37°C. Incorporation of radioactivity into trichloroacetic acid precipitates was determined as previously described [13].

Determination of NAD$^+$ Lowering

Cells were subjected to different culture conditions and to DNA damaging treatments described previously. Two-milliliter aliquots of cell suspensions were distributed into sterile plastic culture tubes and incubated at 37°C. At selected times, cells were collected by centrifugation at 2250g for 3 min at 4°C, medium was removed by aspiration, and the cell pellets were acidified with 200 μl 0.35 N perchloric acid. After 15 min at 4°C, NAD$^+$ extracts were neutralized with 25 μl of a solution containing 1 M Imid base, 2.1 M KOH, and 0.5 M KCl. NAD$^+$ was measured by enzymatic cycling techniques as previously described [14].

Determination of Poly(ADP-Ribose) Levels

Freshly isolated lymphocytes were tested with DNA-damaging agents as described previously. Samples of the cultures were transferred to sterile 50 ml plastic centrifuge tubes and at various times cells were collected by centrifugation at 1990g for 5 min at 4°C. The medium was quickly aspirated and the cell pellets were precipitated by immediately adding 5 ml ice cold 20% trichloroacetic acid. After 30 min at 4°C, precipitates were collected by centrifugation at 1990g for 5 min at 4°C. Pellets were washed with an additional 5 ml ice cold 20% trichloracetic acid and the precipitates collected as above. Cell precipitates were dissolved in 6 M guanidine hydrochloride, 0.1 M potassium phosphate buffer, pH 8.6, and poly(ADP-ribose) was extracted utilizing dihydroxyboryl-sepharose affinity chromatography [11]. The unique compound, 1,N^6-ethenoribosyladenosine, derived only from poly(ADP-ribose), was obtained by digesting the polymer to nucleosides with snake venom phosphodiesterase and bacterial alkaline phosphatase followed by reaction of the nucleosides with chloroactaldehyde [11]. Ethenoribosyladenosine was separated from other fluorescent compounds by reversed phase high pressure liquid chromatography on a Varian 4-mm ID × 300-mm MCH-10 reversed phase column coupled to a 4-mm ID × 50-mm guard column packed with 40-μM Vydac reversed phase packing material by isocratic elution with 7 mM ammonium formate, pH 5.8, methanol 88/12 (v/v) utilizing a Varian Model 5000 HPLC equipped with a heating block maintained at 25°C. Ethenoribosyladenosine was quantified by fluorescence detection as previously described [11] using a Varian Fluorichrom Fluorometer equipped with a 220-nm interference filter for excitation and a Corning 3-75, 370-nm, cut-off filter and a Corning 5-58, 410-nm, bandpass filter for emission. The recovery of the polymer was routinely 70 to 80%. The limit of sensitivity was 1 pmol of ethenoribosyladenosine.

RESULTS

Table I shows that treatment of normal human lymphocytes with either UV irradiation or MNNG caused a marked stimulation of unscheduled DNA synthesis. The level of this unscheduled DNA synthesis increased in a dose-dependent manner when nicotinamide was added to the culture medium; it was essentially

TABLE I. Stimulation of Unscheduled DNA Synthesis by Nicotinamide*

Treatment	Unscheduled DNA synthesis[a]		Nicotinamide stimulation (B/A)
	Additions to medium		
	None (A)	2 mM Nicotinamide (B)	
Control	2,730	2,350	0.86
20 J/m² UV	26,900	55,300	2.06
Control	2,750	2,530	0.92
136 μM MNNG	10,700	19,700	1.84
136 μM MNNG + αMO	8,780	17,000	1.92
136 μM MNNG + MGBG	9,070	18,200	2.01
136 μM MNNG + αMO + MGBG	9,400	18,100	1.91
136 μM MNNG + αDFMO	6,480	14,000	2.16
136 μM MNNG + Cycloheximide	7,000	13,600	1.94

*All measurements were performed in the presence of 10 mM hydroxyurea as described under Methods. Where indicated, cells were incubated with either 50 mM αMO, 80 μM MGBG, 50 mM αMO and 80 μM MGBG, 11 mM αDFMO, 500 μg/ml cycloheximide, or equivalent amounts of saline in the case of controls. Values reported are the averages of triplicate determinations which varied less than 10%.

[a]Unscheduled DNA synthesis (DPM [^{3}H]dTMP incorporated/10^6 cells/6 hr).

doubled by incubating the cells in 2 mM nicotinamide. We have previously shown that this unscheduled DNA synthesis was of the repair type [4]. This doubling of DNA synthesis was not seen when 2 mM nicotinamide was added to control, undamaged lymphocytes. Since nicotinamide has previously been shown to induce the synthesis of several enzymes including ornithine decarboxylase [6], we considered the possibility that such an induction might account for its ability to enhance the DNA repair process. Table I shows the effects of three different inhibitors of polyamine biosynthesis on the ability of nicotinamide to stimulate unscheduled DNA synthesis in DNA-damaged lymphocytes. αMO is a competitive inhibitor of ornithine decarboxylase and MGBG is an inhibitor of S-adenosylmethionine decarboxylase, another important enzyme required for polyamine biosynthesis. αDFMO has recently been shown to be an irreversible suicide inactivator of ornithine decarboxylase in many different tissues and cell types [15,16]. Although these compounds produced a slight reduction in the total amount unscheduled DNA synthesis, they had no effect on the nicotinamide stimulation. Thus in all cases the presence of nicotinamide caused a two-fold increase in the level of unscheduled DNA synthesis. Although we did not measure uptake of these compounds nor their effect on ornithine decarboxylase activity in the lymphocytes, previous studies have demostrated that αMO and MGBG are effective polyamine synthesis inhibitors in lymphocytes [17,18] at concentrations one-tenth of those used in this study. In addition, αDFMO has been shown to penetrate a wide variety of cells and tissues and rapidly inhibits polyamine biosynthesis [15,16]. Thus these results suggest that ornithine decarboxylase activity or polyamine biosynthesis is not necessary for nicotinamide stimulation or unscheduled DNA synthesis in resting human lymphocytes. To examine further the possibility that the nicotinamide effect was mediated by induction of protein

synthesis, we incubated the nicotinamide treated DNA-damaged cells in cycloheximide at concentrations up to 500 μg/ml to preclude any protein synthesis. We have previously shown that incubation of lymphocytes in 50 μg/ml cycloheximide inhibits protein synthesis by greater than 96% [13]. As shown in Table I, even at the concentration of 500 μg/ml, cycloheximide was unable to prevent the two-fold stimulation of unscheduled DNA synthesis induced by nicotinamide.

Since NAD^+ and poly(ADP-ribose) metabolism undergo major alterations during the cellular response to DNA damage [19, 20], we evaluated the effect of nicotinamide on these pathways. We have observed that the activity of poly(ADP-ribose) polymerase measured in nucleotide permeable lymphocytes was stimulated six-fold by 50 J/m² UV irradiation [13] and 15-fold by treatment with MNNG. In aggreement with the recent studies of Ogata et al [21], the maximum increase in the enzyme activity occurred between 2 and 4 hr after treatment with UV irradiation or MNNG.

Figure 1 shows that UV irradiation and MNNG treatment each caused the rapid lowering of intracellular NAD^+. MNNG, at the dose given, lowered NAD^+ levels more rapidly than did the dose of UV irradiation. MNNG treatment lowered NAD^+ to 7% of controls within 30 min but in the presence of nicotinamide this rate of lowering was less rapid such that NAD^+ levels at 30 min were 37% of controls. Likewise, UV irradiation resulted in lowering of NAD^+ levels to 4% of controls within 2 hr but in the presence of nicotinamide, NAD^+ levels at 2 hr were 35% of controls.

Concomitant with the fall of NAD^+ levels in MNNG-treated lymphocytes, we also demonstrated a rapid increase in the cellular levels of poly(ADP-ribose)

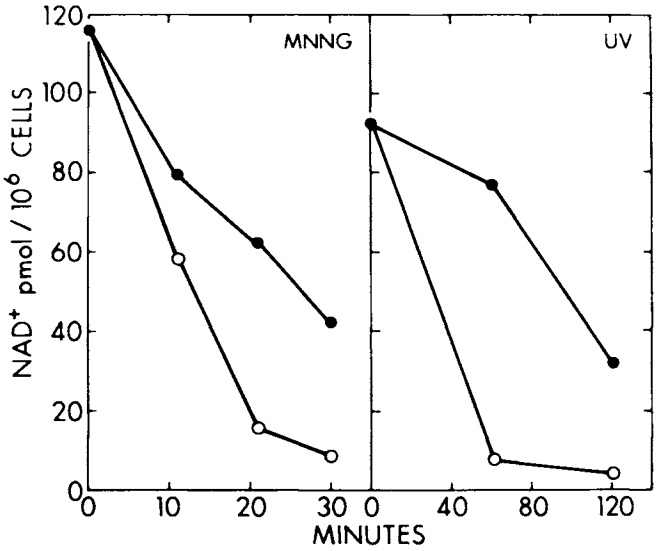

Fig. 1. NAD^+ lowering in resting human lymphocytes. Cells were treated at time zero as described under Methods with either 136 μM MNNG or 50 J/m² UV irradiation and then incubated in the presence (—●—) or absences (—○—) of added 2 mM nicotinamide. At various times duplicate samples of the cultures were removed, NAD^+ was extracted, and each extract was analyzed in duplicate as described under Methods.

(Table II). We employed a recently developed technique to extract and convert poly(ADP-ribose) to a unique fluorescent monomeric unit, ethenoribosyladenosine, which can be separated from other fluorescent compounds by reversed phase high pressure liquid chromatography and quantified by fluorescence detection [11]. While previous studies have shown changes in activity of poly(ADP-ribose) polymerase or NAD^+ levels in DNA-damaged lymphocytes [13, 19, 21], this is the first report in which intracellular polymer levels were actually measured in normal human cells by direct chemical analysis. In terms of monomeric units derived from poly(ADP-ribose), lymphocytes from some donors had between 1.5 and 3 pmol ethenoribosyladenosine/10^8 cells, whereas other donors had levels of polymer which were undetectable. Table II shows that MNNG treatment of lymphocytes from two different donors caused polymer levels to increase from undetectable levels to 68 and 44 pmol ethenoribosyladenosine/10^8 cells by 10 min. The assays performed on untreated lymphocytes would have detected 5 pmol of ethenoribosyladenosine/10^8 cells. This rapid rise in polymer levels therefore represents at least a 20-fold increase over control values established in resting lymphocytes from multiple donors. Polymer levels rapidly declined after 10 min (data not shown) indicating a very short half-life in vivo. The presence of 2 mM nicotinamide in the culture medium largely blocked the increase in polymer levels seen at 10 min such that the levels are only 25–35% of those found in cells treated with MNNG alone.

DISCUSSION

These studies suggest that the ability of nicotinamide to increase the level of unscheduled DNA synthesis in resting human lymphocytes may be mediated through its effects on the size of the NAD^+ pools by reducing the rate of NAD^+ utilization for poly(ADP-ribose) synthesis. Thus we have clearly shown that at the concentration at which nicotinamide stimulated unscheduled DNA synthesis, it also interfered with the synthesis of poly(ADP-ribose) and retarded the lowering of cellular NAD^+ levels. The capacity of nicotinamide to stimulate unscheduled DNA synthesis does not appear to be mediated through induction of ornithine decarboxylase or any other protein synthesis since it is not affected by potent inhibitors of these processes. Nicotinamide stimulation of unscheduled DNA synthesis has recently been reported to occur in primary cultures of rat hepatocytes [22].

TABLE II. Effect of Nicotinamide on Poly(ADP-Ribose) Synthesis In Vivo*

	Poly(ADP-ribose) ethenoribosyladenosine (pmol/10^8 cells)	
Treatment	Donor 1	Donor 2
Untreated	0	0
136 μM MNNG	68	44
136 μM MNNG + 2mM nicotinamide	17	15

*Freshly isolated lymphocytes were treated with the indicated compounds for 10 min at 37°C then collected by centrifugation and precipitated with trichloroacetic acid and analyzed for ethenoribosyladenosine as described under Methods.

Thus the nicotinamide effect is not species specific, since it occurs in rat liver cells and resting human lymphocytes.

Several studies have now shown that NAD^+ and poly(ADP-ribose) metabolism are altered in DNA-damaged cells [2, 19, 20]. Since ADP-ribosylation of chromatin proteins may serve to alter chromatin structure [3], the synthesis and degradation of poly(ADP-ribose) after DNA damage might provide the means of altering chromatin structure to facilitate the DNA repair process. In this regard, the drastic alterations that occur in NAD^+ and poly(ADP-ribose) metabolism following the doses of UV irradiation or MNNG used in these experiments could further damage cells by depleting their NAD^+ pools or by irreversibly altering their chromatic structure. Our studies in resting lymphocytes show that reduced rates of NAD^+ utilization and poly(ADP-ribose) synthesis are correlated with increased unscheduled DNA synthesis induced by nicotinamide. Our preliminary results with other poly(ADP-ribose) polymerase inhibitors, among them benzamide and pyrazinamide, suggest that these compounds exert similar effects on NAD^+ and poly(ADP-ribose) metabolism and also stimulate unscheduled DNA synthesis. Furthermore, these effects appear to be related to their potency as polymerase inhibitors. These results suggest at least two possibilities for the mechanism by which nicotinamide stimulates unscheduled DNA synthesis. First, by reducing the rate of NAD^+ depletion, nicotinamide may preserve the pool size of this nucleotide and help maintain the redox potential of the cell. Second, by reducing the burst of poly(ADP-ribose) synthesis that occurs in DNA-damaged cells, nicotinamide may modulate the alteration and restoration of chromatin structure to facilitate the orderly repair of DNA.

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