Stimulation of poly(ADP-ribose) polymerase activity by the anti-tumour antibiotic, streptozotocin.

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SUMMARY

Streptozotocin is a carcinogenic, anti-tumour antibiotic which lowers cell NAD^+ levels. 24 hours exposure to streptozotocin increases the biosynthetic pathway of NAD by 30%; and increases the activity of NAD glycohydrolase by 40%. By contrast removal of NAD to form the chromosomal polymer, poly(ADP-ribose) was stimulated about 200%. The drop in cell NAD level may be accounted for by increased synthesis of poly(ADP-ribose). Many alkylating agents cause a drop in NAD levels. These observations suggest a completely novel avenue for the exploration of the mode of action of some anti-cancer drugs

1. Introduction

Streptozotocin (NSC-85998) is a broad spectrum antibiotic with several distinctive features. It is both mutagenic and carcinogenic, also it is used clinically as an anti-tumour drug; finally, it causes diabetes in some species (1-3). It is chemically the 2-deoxy-D-glucose derivative of the well known alkylating mutagen and carcinogen, 1-methyl-1-nitroso-urea (4). Streptozotocin decreases the tissue content of both NAD^+ and NADH in islets of Langerhans and in liver of rodents (5-7, 37). 1-methyl-1-nitroso-urea (MNU) (MSC-23909) produces a similar, dose related depression of NAD level (6). The decrease in NAD may be a common consequence of these cell killing alkylating agents (27-29). NAD is the specific substrate for the synthesis of the chromosomal polymer, poly(ADP-ribose). A possibility that requires investigation is that the synthesis of chromosomal poly(ADP-ribose) may be involved in either the carcinogenic (mutagenic) or anti-tumour (cytotoxic) effects of alkylating agents. Poly(ADP-ribose) is synthesized from NAD by a chromosomal enzyme in nucleated cells (8-10); the enzyme is dependent upon both DNA and histones

for activity. The first ADP-ribose moiety from NAD is apparently covalently linked to chromosomal proteins; additional units are successively added to the protein-bound poly(ADP-ribose). The physiological function of this polymer is unknown; its involvement in the control of DNA replication has been suggested (11-21).

2. Materials and Methods

These experiments have been performed in the naturally synchronous plasmodial myxomycete, Physarum polycephalum, which so far appears to be a typical nucleated organism (32).

Microplasmodia of Physarum polycephalum were grown in Marmite medium; 1% (w/v) glucose, 1% (w/v) Marmite (Trent Yeast Extract Co. Ltd., Burton on Trent) and 20 mM Na_3 citrate, pH 4.6.

For the estimation of NAD microplasmodia were harvested by centrifugation at 1000 g and an equal volume of ethanol was added. The suspension was then sonicated for 20 sec at low power in an MSE 150 watt sonicator. The suspension was centrifuged at 100 Kg for 10 min. The NAD content of the supernatant was measured (22). The method was checked by adding radioactive NAD to the alcoholic suspension before sonication. The amount of radioactive NAD was measured after sonication, centrifugation and chromatography on PEI-cellulose thin layers. The recovery of the NAD was quantitative. There was also no loss of NAD from the supernatant after 24 hours at 4°C.

The specific activity of the NAD glycohydrolase was estimated in microplasmodia washed twice with cold water. The mould was sonicated for 2 x 20 sec at low power, and the crude sonicate was the enzyme preparation. The assay contained in a total volume of 650 µl, sodium phosphate buffer pH 6.8, 38 mM; ^{14}C (nicotinamide) NAD^+, 1.25 µCi, 220 nMol, 0.338 mM; and 50 µl enzyme. The mixture was incubated at 26°C and samples were taken at 5, 10, 20 and 30 min and made 50% (v/v) in ethanol. The products were separated on PEI-cellulose TLC with solvent A; butanol:methanol:ammonia:water (60:20:1:20, v/v/v/v). All the nicotinamide was found as nicotinic acid.

Poly(ADP-ribose) polymerase was measured in isolated nuclei (23). The assay solution contained 10 mM-$MgCl_2$; 60 mM - KCl; 4 mM - KF; 100 mM-Tris-HCl buffer, pH 8.2; 10 mM-dithiothreitol and 26.6 µM - ^3H (adenine) NAD (1842 µCi/µMol) in a total volume of 0.10 ml (19). The nuclear suspension was incubated at 15° for 15 min and the reaction was terminated with 5 ml of cold 5% (w/v) trichloroacetic acid. The acid insoluble radioactivity was measured in 0.5% PPO in toluene-Triton X100 (7:3, v/v) scintillation solution.

Treatment with streptozotocin was in every case at 1 mg/ml of the drug.

The measurement of the synthesis of nicotinic acid dinucleotide was performed by measuring the synthesis from ^{14}C-nicotinic acid in high speed supernatants (120 Kg, 60 min). The product was separated by TLC on PEI-cellulose in two solvent systems: (a) Solvent A, and (b) Solvent A followed after drying by 1.0 M acetic acid for 2 cm. then 0.3 M LiCl in 0.9 M acetic acid. No synthesis of nicotinic acid dinucleotide was detectable in crude homogenates.

DNA was estimated by the method of Burton (33) with calf thymus DNA as standard. Protein was estimated by the method of Lowry et al. (34) with bovine serum albumin as standard. Pigment was measured as absorbance of a 50% aqueous-acetone extract at 415 nm in 1.0 cm cuvettes.

Streptozotocin was estimated by the method of Forist (35). The mould was washed three times with buffer; it was shown that this effectively removed all outside drug and that no significant amount leaked out of the mould. The recovery of added streptozotocin was quantitative.

3. Results and discussion

Streptozotocin is known to inhibit DNA, RNA and protein synthesis (24, 25) in mammalian cells. We have confirmed that it inhibits both DNA and protein biosynthesis in P. polycephalum.

The inhibition of DNA biosynthesis is detectable after 1 hour treatment with 1 mg/ml streptozotocin when a 23% inhibition of incorporation of ^{3}H-thymidine is observed. After 6 hours treatment with the drug, DNA biosynthesis was inhibited by 88% but after 24 hours a recovery was beginning; the inhibition was then only 77%.

The incorporation of leucine into acid-insoluble material was also inhibited, but in this case the degree of inhibition was less than with DNA and it seemed to occur later. Perhaps the inhibition of protein biosynthesis is a consequence of the effect of the drug on the chromatin. 6 hours treatment with streptozotocin inhibited protein biosynthesis by about 40%; after 24 hours treatment the degree of inhibition was about 75%.

Measuring the total amount of DNA and protein in the cultures showed that within 10 hours of treatment the increase in DNA content had ceased and the rate of protein accumulation was much reduced. The effect on DNA content was much more marked than that on protein content of the cultures. The rate of pigment accumulation was considerably lowered from the onset of drug treatment.

Treatment of P. polycephalum with streptozotocin (1 mg/ml) lowered the NAD^{+} content of microplasmodia. In 4 hours the NAD^{+} level was down to half and at 24 hours it was down to 20% of the control (Fig. 1). This is in accord with a number of previous reports (5, 6, 7), although in this organism the time of change was somewhat different. This difference was probably due to the pH of the medium at which this organism is grown. This was between pH 4 and 5; at this pH the streptozotocin is maximally stable. Actual measurement of the concentration of streptozotocin in the culture medium at pH 5.0 revealed that at this pH, in this medium, the drug was stable. The

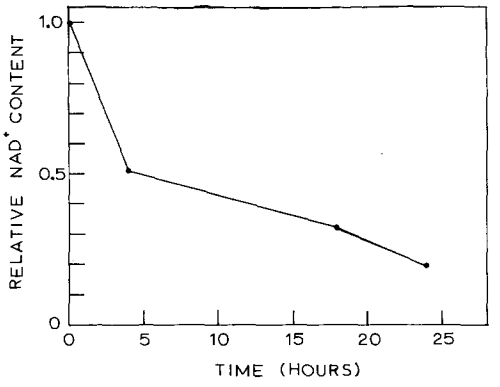

Fig. 1. Effect of streptozotocin on NAD level of P. polycephalum microplasmodia

The vertical ordinate shows the relative amount of NAD^+ in the streptozotocin treated culture compared to an identical control culture. The horizontal ordinate shows the time the culture was exposed to the streptozotocin.

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rate of loss of the drug was about 10% in 7 days in either fresh complete medium, conditioned medium, or medium in which there were growing plasmodia. This contrasts with the situation with mammalian cells; in tissue culture the half-life of the drug appears to be between 1 and 2 hours (25) and in mice most of the drug has disappeared by 4 hours (36). Thus, the effects in mammalian cells may be due to a transient exposure to the drug. Because P. polycephalum is grown at an acidic pH it is exposed to a constant concentration of the drug for an extended period. This may result in a more intense response to the drug.

This supposition was confirmed by measuring the amount of drug inside the mould. The drug concentration rose rapidly, during 1 hour, to a stable level of 5.5 μg of streptozotocin/mg protein with a stable outside concentration of 1000 μg of streptozotocin/ml of medium.

The depression of the NAD^+ level may have been due to decreased biosynthesis and/or to increased degradation. In particular, it may have been due to increased synthesis of poly(ADP-ribose) from NAD.

We have shown that the biosynthesis of NAD^+ in P. polycephalum proceeds

only via nicotinic acid, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide and NAD^+. Synthesis from tryptophan or from nicotinamide does not occur in this organism (to be reported in detail elsewhere). Degradation is by NAD glycohydrolase, phosphodiesterase and poly(ADP-ribose) polymerase.

It was observed that streptozotocin caused a slight increase in the in vitro rate of the biosynthesis of nicotinic acid adenine dinucleotide. In the extracts from the control cultures the rate of formation of nicotinic acid adenine dinucleotide was 317 ± 21 pMol nicotinic acid adenine dinucleotide formed/min per mg protein (average of eight independent experiments). After treatment with streptozotocin (1 mg/ml) for 24 hours the rate of formation was 404 ± 35 pMol nicotinic acid adenine dinucleotide formed/min per mg protein (8 experiments). In these eight independent experiments the ratio of the rates of formation in the drug treated to the control averaged 1.270 ± 0.056. There was an increase in the specific activity of this pathway. We conclude that in these experiments the depression of the NAD^+ level was not caused by inhibition of the rate of formation.

Treatment of P. polycephalum with streptozotocin (1 mg/ml) leads to an increase in the specific activity of NAD glycohydrolase over 24 hours (Fig. 2). The control specific activity varied between 1.7 and 2.1 nMol of NAD hydrolyzed/min per mg of protein. After 4 hours of drug treatment, the specific activity had risen 11% and after 24 hours it had risen 44%. Initially, it seems unlikely that an apparent increase of 11% after 4 hours accounts for a 50% decrease in NAD^+ content (Fig. 1).

The degradation of NAD occurs also via the synthesis of poly(ADP-ribose). The decrease in NAD levels may be caused by increased formation of poly(ADP-ribose). We have measured the activity of poly(ADP-ribose) polymerase in nuclei isolated from cultures treated with streptozotocin. The ratio of the activity in treated cultures to the activity in control cultures is shown in Fig. 3. After 4 hours of exposure to the drug the enzyme activity was already 50% higher than the control. 24 hours exposure to the drug resulted in a

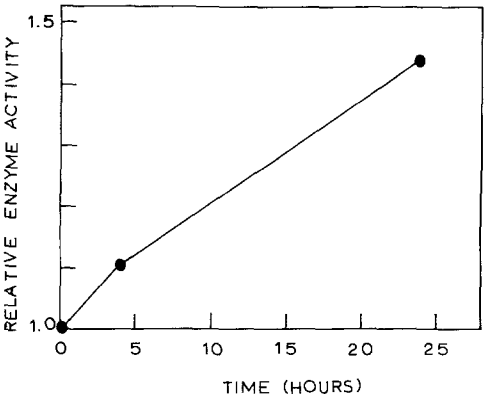

Fig. 2. Effect of streptozotocin on the specific activity of NAD glycohydrolase in P. polycephalum.

The horizontal axis shows the duration of treatment with streptozotocin. The vertical axis shows the ratios of the specific activity of the enzyme in the streptozotocin cultures to the control culture. The NAD glycohydrolase four hour point is the average of five separate experiments; the 24 hour point is the average of four separate experiments.

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dramatic increase - of nearly 3 times the control activity. It would seem that the increase in the activity of poly(ADP-ribose) polymerase may be the main reason for the decrease in NAD levels in streptozotocin treated cells. This conclusion is supported by the observation of Dulin and Wyse (30) that pyrazinamid blocked the formation of streptozotocin diabetes, because we have shwon that pyrazinamid is a strong inhibitor of the poly(ADP-ribose) polymerase in P. polycephalum (M.G. Khan and S. Shall, unpublished work). Also, in enucleated cells the turnover of NAD is much reduced, implying that the nucleus is the main site for the degradation of NAD in intact cells (31).

It is possible that the increase in activity of poly(ADP-ribose) polymerase may be quite specific. The very rapid response to streptozotocin in animal cells (15-20 min) supports this idea. Also, the activities of DNA polymerase, thymidylate synthetase and thymidine kinase are not increased after 1.5 mg/ml for 18 hours (25). The increase in relative activity of this particular enzyme may be partly caused by a very low rate of turnover compared

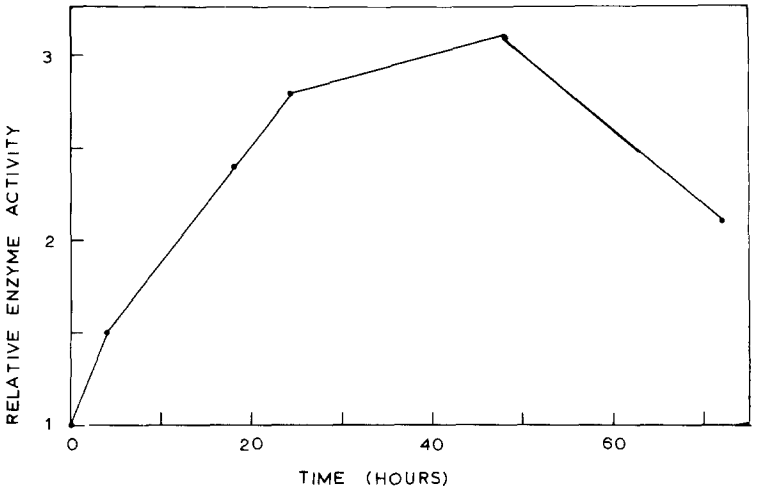

Fig. 3. Effect of streptozotocin on poly(ADP-ribose) polymerase in P. polycephalum

The horizontal axis shows the duration of treatment with streptozotocin. The vertical axis shows the ratio of the specific activities of the poly(ADP-ribose) polymerase in isolated nuclei from streptozotocin-treated cells to nuclei from control cells.

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to the majority of cellular proteins. The behaviour of P. polycephalum may be different to mammalian cells in intact organisms or in cell culture.

The overall results showed that 24 hours exposure to streptozotocin yielded a 30% increase in the biosynthetic pathway of NAD and a 40% increase in hydrolysis. By contrast removal of NAD to form poly(ADP-ribose) was stimulated about 200%. The physiological consequences of increased poly(ADP-ribose) synthesis are unknown, but its function is almost certainly chromosomal in location. It is likely, therefore, that some of the consequences of treatment with anti-tumour or carcinogenic agents such as streptozotocin may be via poly(ADP-ribose). These observations suggest a completely novel avenue for the exploration of the mode of action of some anti-cancer drugs. These observations may help to explain the excretion of 1-methyl-nicotinamide after treatment with alkylating agents (38, 39) because increase of poly(ADP-ribose) synthesis will raise the free nicotinamide level. The excess nicotinamide would then be methylated and excreted.

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