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METABOLISM OF NAD(P)H BY BLOOD COMPONENTS

RELEVANCE TO BIOREDUCTIVELY ACTIVATED PRODRUGS IN A TARGETED ENZYME THERAPY SYSTEM

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Abstract—NADH was metabolized both by serum components and at the cell surface. The metabolism by serum was either oxidation to NAD^+, or hydrolysis of the pyrophosphate to yield nicotinamide mononucleotide (reduced) (NMNH) and AMP. NMNH was further hydrolysed to yield nicotinamide riboside (reduced) (NRH), which was stable. NAD^+ was hydrolysed (although at a slower rate than was NADH), but was also reduced to yield NADH. The reduction of NAD^+ was catalysed by the enzyme serum L(+)lactate dehydrogenase (EC 1.1.1.27) and was dependent on the concentration of L(+)lactate in the serum. NADPH was hydrolysed in a similar manner to NADH but not oxidized by serum. NADH generated from NAD^+ by serum derived from human, foetal calf and horse sources was capable of driving the bioreductive activation of CB 1954 by the enzyme DT diaphorase. Cell surfaces oxidized NADH to NAD^+, but did not oxidize NADPH or NRH. These observations suggest that NAD(P)H would be unsuitable as a source of reducing equivalents for the bioreductive activation of prodrugs by a reductase enzyme in Antibody Directed Enzyme Prodrug Therapy (ADEPT). In contrast, NAD^+ (which could act as a source of NADH) and NRH could avoid the shortcomings of NAD(P)H, and act as suitable cofactors for an enzyme in an ADEPT system.

A recent innovation in the attempt to produce a tumour-selective cytotoxic chemotherapy has been the Antibody Directed Enzyme Prodrug Therapy (ADEPT†) approach [1, 2]. A tumour-selective monoclonal antibody (or fragment) is conjugated to an enzyme that is capable of bioactivating a prodrug. Therapy proceeds along a multistage line. A conjugate of an enzyme with a monoclonal antibody is allowed to localize at the site of the tumour. The unlocalized conjugate is either given time to be eliminated from the body, or is hastened on its way by a "clearance" antibody. A non-toxic prodrug is then administered and activated at the site of the tumour by the bound conjugate [3].

This multifactorial system will require optimization in each of its components. Scope for optimization at the prodrug level will depend on the number of candidate enzyme–prodrug pairs available. The enzymes carboxypeptidase G2 [4], alkaline phosphatase [5], β-lactamase [6, 7], penicillin amidase [8] and cytosine deaminase [9] have been considered. In these cases the activating chemical event is hydrolysis.

Reductive processes are also capable of activating prodrugs. Nitro reduction can activate compounds based on 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) [10, 11]. CB 1954 is a monofunctional alkylating agent that becomes both more reactive towards DNA [12], and difunctional following bioactivation in cells of the rat Walker 256 tumour where it induces DNA interstrand crosslinks [13]. The enzyme that bioactivates CB 1954 by reducing the 4-nitro group of CB 1954 to a 4-hydroxylamino group is DT diaphorase [NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2)] [11]. The increase in cytotoxicity accompanying bioreduction of the 4-nitro group of CB 1954 is 10,000-fold on a dose basis. So large an increase in cytotoxicity makes CB 1954 an attractive ADEPT candidate prodrug.

It is a requirement of ADEPT enzymes that an equivalent activity does not exist within the host's cells. Despite being a mammalian enzyme, rat DT diaphorase may be suitable in this respect, because the human form of DT diaphorase is much less able to metabolize CB 1954 to its cytotoxic form than is the rat enzyme [14].

Nitroreduction of CB 1954 by DT diaphorase may therefore be a possible ADEPT system. However, in this case a cofactor will also have to be present to supply a source of reducing equivalents for the enzyme. The biogenic cofactors for reductases are NADH and NADPH, of which NADH is the more readily available although DT diaphorase can use both. In view of our interest therefore in the

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† Abbreviations: CB 1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; NMNH (nicotinamide mononucleotide-reduced), dihydronicotinamide ribotide; NRH (nicotinamide mononucleoside-reduced), dihydronicotinamide riboside; PBS, phosphate-buffered saline; FCS, foetal calf serum; ADEPT, Antibody Directed Enzyme Prodrug Therapy.

bioreductive aspects of ADEPT, we wished to examine the suitability of NADH and NADPH as cofactors in such a system. It was decided to investigate the effects on NAD(P)H of commercial animal sera, human serum and whole blood, and of cultured cells.

MATERIALS AND METHODS

Chemicals were supplied by the Sigma Chemical Co. (Poole, U.K.) unless otherwise stated. Foetal calf and horse sera were supplied by Gibco (Uxbridge, U.K.).

MAWI (human colon carcinoma) cells were grown as monolayers in DMEM supplemented with 10% foetal calf serum (FCS). CB 1954 was supplied by Prof. M. Jarman and Dr D. Wilman (ICR).

DT diaphorase was prepared from Walker cells as before [11].

Assay of NAD(P)H and its metabolites by HPLC. The concentrations of NADPH and NADH and its metabolites were assayed by anion exchange HPLC (Partisphere 5-SAX column, eluted isocratically at 1.5 mL/min with 0.1 M (NADH) or 0.3 M (NADPH) NaH_2PO_4, monitored by UV absorbance (ABS 1000 S diode array detector), and fluorescence (Gilson 121 fluorometer with wide band glass filters; excitation centered at 350 nm; emission at 450 nm). Metabolites were identified by comparison of their elution characteristics and spectrum (as captured by a diode array detector) with those of commercial standards. Serum-containing samples were injected directly onto the column without processing.

Fractionation of FCS into high and low molecular mass components. The low molecular mass component of FCS was obtained by molecular filtration (10,000 molecular mass limit; "Centriprep 10"; Amicon, High Wycombe, U.K.). This filtrate was heated at 100° for 30 min and clarified by 0.2 μM filtration. The retentate was dialysed exhaustively against water, and the precipitate removed by centrifugation.

Assay of L(+)lactate. Concentration of lactate in serum ultrafiltrate was determined by a chromogenic lactate oxidase-based kit (Sigma).

Reduction of CB 1954 by serum-reduced NAD⁺. The NADH generated by the reducing system in serum was assayed by its ability to act as a cofactor in the reduction of CB 1954 by DT diaphorase. NAD^+ (500 μM) was incubated with DT diaphorase (100 μg/mL), CB 1954 (100 μM) and serum (10%) in phosphate-buffered saline (PBS). The reaction was monitored by loss of CB 1954 and the formation of its metabolite, 5-(aziridin-1-yl)-4-hydroxylamino-2-benzamide. Their concentrations were assayed by HPLC as previously described [14].

Preparation of NRH from NMNH. NMNH (1.5 mL; 10 mM) in PBS was treated with 1000 U of alkaline phosphatase (type VII-S, 50 μL; 20,000 U/mL) at 37° for 1 hr. The alkaline phosphatase was removed by molecular filtration as above. The solutions were analysed before and after digestion with alkaline phosphatase by anion exchange HPLC as above. Post digestion, a complete conversion to a novel compound was observed. Its spectral, ion-exchange and fluorescence characteristics indicated that dephosphorylation to yield the riboside had occurred. Identity was confirmed by mass spectrometry carried out on a TSQ 700 triple quadrupole system (Finnigan Mat) equipped with an electrospray ion source (Analytica): m/z 257 $[M+H]^+$.

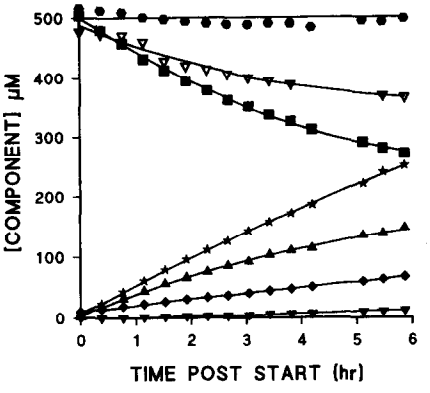

Fig. 1. The effect of FCS on NADH. NADH (500 μM) was added to 10% FCS in PBS (1 mL), and incubated at 37°. At intervals, aliquots (10 μL) were analysed by anion exchange HPLC. Reduced pyridinium compounds were quantitated by fluorescence, and other compounds by 260 nm absorbance. NADH (■); AMP (★); NMNH (▲); NAD^+ (◆); NRH (▼); NADH + AMP + NAD^+ + NRH (●). Also shown is the concentration of NAD^+ during a similar experiment (▽).

Preparation of human serum. Blood (10 mL) obtained by venipuncture was allowed to clot. The serum was obtained by gentle centrifugation. Experiments were also conducted with heparinized fresh whole blood.

RESULTS

The effect of serum on NAD(P)H

NADH (500 μM) was lost from 10% FCS in PBS, with about half disappearing in 6 hr (Fig. 1). The two principle products were NMNH and AMP. Another product was NRH, although this hydrolysis product arose more slowly than the first two. It was more clearly illustrated by use of 100% FCS (Fig. 2). A further product was identified as NAD^+. When the molarities of NADH, NAD^+, AMP and NRH were summated, the sum was a constant 500 μM (Fig. 1). A separate experiment showed that when AMP and NMNH (both 500 μM) were incubated in 10% FCS in PBS, no reconstitution to NADH occurred. NADH could also be cleaved to NMNH and AMP by snake venom phosphodiesterase.

NAD^+ (500 μM) was lost more slowly than NADH, the only identifiable hydrolysis product being AMP. Additionally, small quantities of NADH were formed (see below). NADP(H) was hydrolysed in the same way and at the same rate as NAD(H) but was neither oxidized nor reduced.

The ability of serum to reduce NAD⁺

When NAD^+ was incubated with 10% FCS in

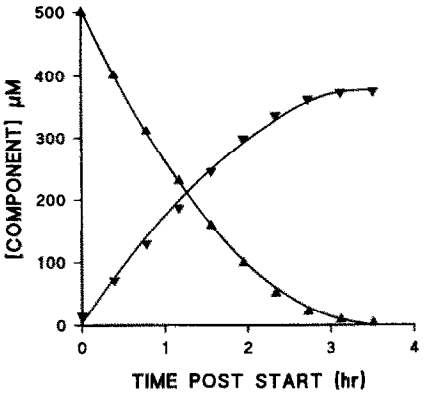

Fig. 2. The effect of FCS on NMNH. NMNH (500 μM) was added to 100% FCS, and incubated at 37°. At intervals, aliquots (10 μL) were analysed by anion exchange HPLC. Reduced pyridinium compounds were quantitated by fluorescence. NMNH (▲); NRH (▼).

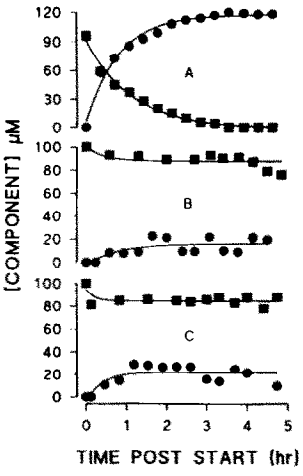

Fig. 3. Reduction of CB 1954 by of foetal calf (A), horse (B) and human (C) serum. NAD^+ (500 μM) was incubated with DT-diaphorase (100 μg/mL), CB 1954 (100 μM) and serum (10%) in PBS at 37° and aliquots analysed by reverse-phase HPLC at intervals. The reaction was monitored by loss of CB 1954 (■) and the formation of its metabolite, 5-(aziridin-1-yl)-4-hydroxylamino-2-benzamide (●).

PBS, a proportion was reduced to NADH. This reduction occurred as soon as the NAD^+ was added, and constituted 2–4% of the applied NAD^+. The resultant concentration of NADH in the mixture was relatively constant, (and proportional to the dose of NAD^+). Traces of the hydrolysis products of NADH were also seen. A separate experiment showed that by contrast there was no reduction of NMN^+ to NMNH or of $NADP^+$ to NADPH by FCS (data not shown).

Levels of L(+)lactate in foetal calf, human and horse sera

The concentrations of L(+)lactate in foetal calf, human and horse sera were 17.6, 4.4 and 3.9 mM respectively.

The abilities of high and low molecular mass serum components to metabolize NADH and NAD^+

FCS ultrafiltrate had no effect on either NADH or NAD^+. The high molecular mass (protein) fraction was able to oxidize NADH (but less so than was full FCS). It was also able to cleave it to AMP and NMNH, but was unable to cleave the NMNH to NRH. NAD^+ was also cleaved to yield AMP. However, NAD^+ was not reduced to NADH by this fraction. When the high and low molecular mass fractions were recombined, the full range of properties described for intact FCS was restored. L(+)Lactate at the same concentration as found in serum could completely replace serum ultrafiltrate in the reduction of NAD^+ by the protein fraction. This reduction of NAD^+ was greatly enhanced by the inclusion of hydrazine (0.5 M) (which would trap the pyruvate formed) in the digestion.

The ability of NAD^+ to act as a cofactor for the reduction of CB 1954 by DT diaphorase in the presence of various sera

Mixtures were prepared containing NAD^+, DT diaphorase, CB 1954 and foetal calf, horse or human serum. The concentration of CB 1954 and of its active metabolite, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide were followed with time. FCS produced a dramatic bioreduction of CB 1954, with a total conversion to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide within about 4 hr (Fig. 3A). Both horse (Fig. 3B) and human (Fig. 3C) sera were less efficient at reducing CB 1954 to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. Only a small drop in concentration of CB 1954 was observed during 5 hr. In these cases, however, the presence of NADH, leading to reduction of the CB 1954, is more readily observed in the formation of the product. No reduction of CB 1954 was observed in the absence of NAD^+ with any of the sera. Likewise, in the absence of serum, NAD^+ cannot act as a cofactor in the reduction of CB 1954 by DT diaphorase (data not shown). The abilities of these sera to reduce NAD^+ correspond to their L(+)lactate contents.

The effect of cultured cells and whole blood on external concentrations of reduced nicotinamide compounds

Confluent monolayers of MAWI cells were extensively washed with PBS, and exposed to NADH, NADPH or NRH. At intervals up to 2 hr, samples were removed, centrifuged to remove debris, and the concentrations of the reduced nicotinamide analogues determined by HPLC. NADH (50 μM) was very rapidly lost to oxidation, NAD^+ being the only product. Since NADH does not penetrate cells, this activity may be attributed to cell surface enzymes. NADPH and NRH were by contrast relatively stable in this environment (Fig.

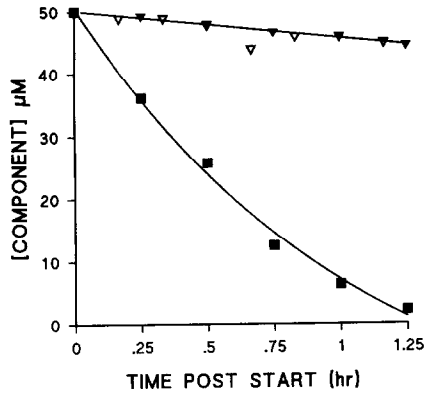

Fig. 4. Effect of a washed monolayer of MAWI cells on NADH, NADPH and NRH. Replicate flasks of confluent MAWI cells were exposed to NADH, NADPH and NRH in PBS (50 μM), and incubated at 37°. At intervals, aliquots of the PBS were removed, and analysed for NADH (■), NADPH (▽) and NRH (▲) content by HPLC.

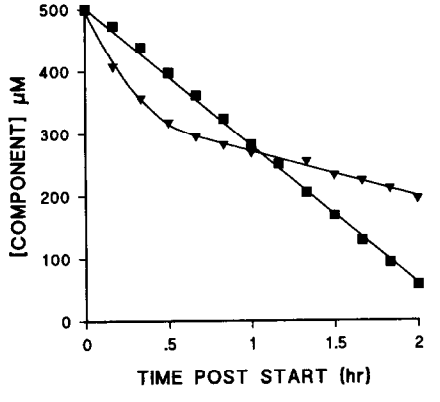

Fig. 5. Effect of whole human blood on NADH and NRH. Volumes of heparinized blood were exposed to NADH (■) and NRH (▼) (500 μM), and incubated at 37°. At times, as indicated, aliquots were removed, centrifuged and the plasma injected directly onto the anion exchange HPLC.

4). None of these agents is toxic at this concentration to these cells.

This experiment was repeated in whole human blood with NADH and NRH at concentrations of 500 μM. Over 90% of the NADH was lost within 2 hr (Fig. 5). NRH was lost in a biphasic manner, initially more rapidly than was NADH, but subsequently more slowly.

DISCUSSION

In order for ADEPT to function successfully, the conjugate, the prodrug and the active drug must possess favourable pharmacological properties, as discussed by Bagshawe [1]. This will also apply to the cofactor in the case of ADEPT employing reductively activated prodrugs. Thus, the cofactor will need to be durable in the blood, will need to be able to penetrate the tumour to the site of drug activation, and will need to remain in its reduced state during this process.

This study highlights the shortcomings of NAD(P)H in these respects. NAD(P)H is hydrolysed by serum in two stages, the first to form NMNH and AMP, and the second to form NRH. This process is almost certainly enzymic in that the low molecular mass fraction of serum is unable to promote this reaction whilst the high molecular mass (protein) fraction can. The identities of the enzymes have not been established. However, an enzyme (EC 3.6.1.9) which cleaves the pyrophosphate linkages of dinucleotides (including NAD) exists in *Neurospora crassa* and potato. We have also shown that this reaction can also be catalysed by phosphodiesterase I (EC 3.1.4.1) from *Crotalus* venum. 5′-Nucleotidase (EC 3.1.3.5) which will cleave the 5′-phosphate from a wide variety of mononucleotides including NMN is a widespread mammalian enzyme, as are acid and alkaline phosphatases. Mammalian 5′-nucleotidase would, however, have been expected to dephosphorylate AMP as well.

NADH (but not NADPH) is also oxidized both by enzymes in serum and on the cell surface. If NAD(P)H was used in conjunction with a reductase enzyme for ADEPT it would, upon intravenous injection, be exposed both to cell surfaces and to serum proteins. It would be anticipated that NADH would be both rapidly oxidized, and hydrolysed, as we have shown to be the case in whole human blood. NADPH would not be oxidized, but would be hydrolysed as rapidly as NADH. NAD(P)H would therefore be unsuitable as a source of reducing equivalents in an ADEPT context.

In contrast, NRH is much more stable than NAD(P)H with respect to biological oxidation both by serum, and by cell surfaces. Although in whole human blood this compound was seen to initially disappear at a rapid rate from the plasma, it was subsequently more stable than NAD(P)H. This is presumably because, being an uncharged molecule, the NRH can penetrate the cells. Thus, the initial step is an equilibration phase with the cell contents. We have recently shown that a series of compounds based on quarternized nicotinate/amide (reduced), including NRH, can act as cofactors for a candidate reductive ADEPT enzyme, rat DT diaphorase [15]. These new reduced pyridinium compounds would, like NRH, be resistant to the biological degradation to which NAD(P)H is subject. Use of these novel cofactors will be limited to those enzymes that can utilize them. It is therefore of interest that NAD^+ can be reduced by serum. The enzyme serum lactate dehydrogenase (EC 1.1.1.27) was taken to be responsible for the observed reduction of NAD^+. This was indicated by the ability of L(+)lactate to supplant serum ultrafiltrate, the enhancement of the extent of reduction of NAD^+ by serum by inclusion of hydrazine, and the lack of reduction of $NADP^+$. In the ADEPT context a reductase could possibly be powered by administration of NAD^+, with a small but constant concentration of NADH being generated by endogenous serum lactate dehydro-

genase. The extent of NAD^+ reduction would depend on L(+)lactate concentration, which is seen to be elevated in a number of morbid conditions including cancer [16]. It is believed that the L(+)lactate derives from the tumour, and would therefore be elevated in this locale. There would be a number of advantages to such a system. NAD^+ is more resistant to hydrolysis than NADH, is more stable as a chemical and therefore much cheaper than NADH, carries only one counter ion compared with NADH's two, and is already used as a pharmaceutical [17–20].

Future work will need to establish whether this innate ability of serum to reduce NAD^+ can indeed be practically exploited in a reductive ADEPT system, or whether the novel reduced quaternary pyridinium compounds (similar to NRH) would offer a better prospect of success. These observations suggest that the pharmacological limitations of NAD(P)H need not be an obstacle to the use of bioreductive enzymes for ADEPT therapy and that the dramatic increase in cytotoxicity accompanying bioactivation of prodrugs such as CB 1954 may be exploited.

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