Free Rad. Res., Vol. 33, pp. 1–12
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Direct NAD(P)H Hydrolysis into ADP-Ribose(P) and Nicotinamide Induced by Reactive Oxygen Species: A New Mechanism of Oxygen Radical Toxicity

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Accepted by Prof. J.V. Bannister

(Received 1 April 1999; In revised form 16 September 1999)

The effect of different oxygen radical-generating systems on NAD(P)H was determined by incubating the reduced forms of the pyridine coenzymes with either Fe^{2+}–H_2O_2 or Fe^{3+}–ascorbate and by analyzing the reaction mixtures using a HPLC separation of adenine nucleotide derivatives. The effect of the azo-initiator 2,2′-azobis(2-methylpropionamidine)dihydrochloride was also tested. Results showed that, whilst all the three free radical-producing systems induced, with different extent, the oxidation of NAD(P)H to $NAD(P)^+$, only Fe^{2+}–H_2O_2 also caused the formation of equimolar amounts of ADP-ribose(P) and nicotinamide. Dose-dependent experiments, with increasing Fe^{2+} iron (concentration range 3–180 μM) or H_2O_2 (concentration range 50–1000 μM), were carried out at pH 6.5 in 50 mM ammonium acetate. $NAD(P)^+$, ADP-ribose(P) and nicotinamide formation increased by increasing the amount of hydroxyl radicals produced in the medium. Under such incubation conditions $NAD(P)^+$/ADP-ribose(P) ratio was about 4 at any Fe^{2+} or H_2O_2 concentration. By varying pH to 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 and 7.4, $NAD(P)^+$/ADP-ribose(P) ratio changed to 5.5, 3.2, 1.8, 1.6, 2.0, 2.5, 3.0, 5.4 and 6.5, respectively. Kinetic experiments indicated that 90–95% of all compounds were generated within 5 s from the beginning of the Fenton reaction. Inhibition of ADP-ribose(P), nicotinamide and $NAD(P)^+$ production of Fe^{2+}–H_2O_2-treated NAD(P)H samples, was achieved by adding mannitol (10–50 mM) to the reaction mixture. Differently, selective and total inhibition of ADP-ribose(P) and nicotinamide formation was obtained by performing the Fenton reaction in an almost completely anhydrous medium, i.e. in HPLC-grade methanol. Experiments carried out in isolated post-ischemic rat hearts perfused with 50 mM mannitol, showed that, with respect to values of control hearts, this hydroxyl radical scavenger prevented reperfusion-associated pyridine coenzyme depletion and ADP-ribose formation. On the basis of these results, a possible mechanism of action of ADP-ribose(P) and nicotinamide generation through the interaction between NAD(P)H and hydroxyl radical (which does not involve the C-center where "conventional" oxidation occurs) is presented. The implication of this phenomenon in the pyridine coenzyme depletion observed in postischemic tissues is also discussed.

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Keywords: NAD(P)H, reactive oxygen species, ADP-ribose(P), ischemia and reperfusion, energy metabolism

Abbreviations: ADP-ribose(P), ADP-ribose-phosphate, i.e. adenosine 2′-monophospho-5′-diphosphoribose; ROS, reactive oxygen species

INTRODUCTION

Numerous *in vitro* and *in vivo* studies have evidenced the potential toxicity of reactive oxygen species (ROS) towards several molecules of biological interest. For instance, interaction with ROS is responsible for the oxidation of protein SH-groups,[1] the irreversible modification of polyunsaturated fatty acids of membrane phospholipids,[2] the modification of the base moiety of nucleic acid nucleotides,[3] etc. Due to ROS involvement in many human pathologies, including tissue ischemia and reperfusion,[4] cerebral trauma,[5] aging,[6] etc., studies are carried out for determining either mechanisms of ROS production,[7] or the type of ROS responsible for molecular damaging,[8] or new target molecules sensitive to ROS oxidizing activity.[9] Recently, it has been determined that pyridine coenzyme pool undergoes a significant depletion in different cellular and animal models of increased ROS production.[10,11] Results obtained by inducing ROS generation following a reductive stress,[11–14] as it occurs in reperfused tissues after periods of ischemia, are particularly intriguing because, at present, no satisfying explanation of this phenomenon has yet been provided. Data available in literature have shown that vanadium salts provoke NAD(P)H oxidation via the formation of the diperoxovanadate complex capable of oxidizing reduced pyridine coenzymes.[15,16] Under such experimental conditions, it has been reported that 10–15% of the starting NAD(P)H is not oxidized to $NAD(P)^+$, but it is transformed in a different compound, the chemical nature of which has never been established.[16] Furthermore, direct consequences of ROS interaction with NAD(P)H have not yet been evaluated.

By using HPLC for the quantification and characterization of adenine nucleotide derivatives, this study shows that NADP(H) is simultaneously oxidized to $NAD(P)^+$ and hydrolyzed into ADP-ribose(P) and nicotinamide, following the incubation with certain ROS-generating systems. The ROS responsible for this phenomenon, as well as the possible reaction mechanism of ADP-ribose(P) and nicotinamide formation which does not involve the reactive center where "conventional" NAD(P)H oxidation takes place, is described. The biological relevance of this finding, showing a role of NAD(P)H as novel targets of ROS toxicity (assessed by experiments in isolated postischemic rat hearts), is also presented.

MATERIALS AND METHODS

Chemicals

Ultrapure NAD(P)H and $NAD(P)^+$ were obtained from Boehringer (Mannheim, Germany), ADP-ribose(P) and nicotinamide were purchased from Sigma (St. Louis, Mo., USA) and the azo-initiator 2,2′-azobis(2-methylpropionamidine)dihydrochloride was provided from Kodak (Eastman Fine Chemicals, NY, USA). Tetrabutylammonium hydroxide was supplied from Nova Chimica (Milane, Italy) as a 55% water solution, and HPLC-grade methanol was furnished from J.T. Baker (Deventer, Holland). All other reagents were obtained at the highest purity available from commercial sources.

Experimental Conditions for NAD(P)H Oxidation and Hydrolysis

Reduced pyridine coenzymes were freshly prepared as 5 mM stock solutions and used at a 500 μM final concentration. The effects of the following free radical-generating systems were evaluated:

(1) $FeCl_2$ (3–180 μM) + H_2O_2 (50–1000 μM);
(2) $FeCl_3$ (3–180 μM) + ascorbic acid (10–1000 μM);

(3) azo-initiator 2,2′-azobis(2-methylpropionamidine)dihydrochloride (1–10 mM);
(4) xanthine (1 mM) + xanthine oxidase (0.25 U).

As far as system (1) is concerned, dose-response experiments, time-course studies and studies on the influence of incubation conditions were carried out. Referring to systems (2) and (3), only dose-response experiments were carried out while, for system (4), ambiguous results did not allow to use this ROS-generating system. In fact, xanthine–xanthine oxidase incubation with NAD(P)H caused the unexpected hydrolysis of the phosphoric ester bond and a consequent production of AMP, which also occurred when xanthine was omitted from the reaction mixture. Such an effect, still persistent after a partial purification of the commercial xanthine oxidase, was evidenced also with other substrate usable by phosphatases (ATP, ADP, $NAD(P)^+$). Therefore, we did not perform any experiment with this superoxide-generating system.

In all the experimental conditions, except when HPLC-grade methanol was used or unless indicated, 50 mM ammonium acetate, pH 6.5, was adopted as the incubation medium. The choice of pH 6.5 was purposely done in order to perform a relevant number of experiments at pH value similar to that observable in postischemic tissues. The most effective concentrations of Fe^{2+} (180 μM) and H_2O_2 (1000 μM) were incubated with NAD(P)H either for various times (5–30 s), or for a fixed time (15 s) but at different pH values (2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 and 7.4). Incubations were carried out at 37°C and were started by adding iron to the reaction system (1) and (2), and azo-initiator to system (3); pH was checked immediately before the beginning and just before the end of each reaction carried out at pH 6.5 and lasting for 1 h (no appreciable pH variations were observed). In all the experiments, control was represented by NAD(P)H incubated in the presence of buffer only. Additional controls were represented by NAD(P)H incubated with 50 mM ammonium acetate and the highest concentrations of either Fe^{2+} only or H_2O_2 only. Incubations of systems (1) and (2) were stopped by the addition of large EDTA excess (10 mM final concentration), while reaction of system (3) was stopped by loading samples onto the HPLC column. Each reaction mixture (50 μl) was analyzed by HPLC for the determination of adenine nucleotide derivatives.[17]

Heart Perfusion and Tissue Preparation

In these experiments, male Wistar rats of 300–350 g body weight (b.w.) were used exclusively. Animals were anesthetized by intraperitoneal injection of 50 mg/kg b.w. of ketamine and 1500 IU of heparin were injected into the caudal vena cava. Hearts were then excised, quickly mounted through the aorta and perfused with an oxygen-saturated ($O_2 : CO_2$; 19 : 1; v : v) Krebs–Ringer buffer (pH 7.40) containing 2.5 mM $CaCl_2$, 10 mM glucose and 12 IU/l insulin according to the retrograde non-recirculating Langendorff mode. Buffer temperature was maintained at 37°C and perfusion pressure was kept constant at 7.85 kPa. After 30 min of normoxic preperfusion ($n = 6$), hearts were subjected to 30 min of global normothermic ischemia followed by 30 min of normoxic reperfusion ($n = 6$). An equal number of hearts was submitted to the same perfusion scheme using a Krebs–Ringer buffer supplemented with 50 mM mannitol. After preperfusion or reperfusion, all hearts were freeze-clamped, deproteinized by homogenization in ice-cold 1.2 M $HClO_4$ and centrifuged at 20,190*g* at 4°C. Supernatants were neutralized by 5 M K_2CO_3 and finally loaded (50 μl) onto a HPLC column for the determination of $NAD(P)^+$ and ADP-ribose.[17]

HPLC Apparatus and Chromatographic Conditions

A ConstaMetric 3500 HPLC dual pump system (ThermoQuest Italia, Rodano, Milane, Italy) was connected to a SpectraSystem UV-6000LP diode array detector, set up for data acquisition between

200 and 300 nm, and equipped with a Kromasil C-18, 5 μm particle size, 250 × 4.6 mm column (Eka Chemicals AB, Bohus, Sweden), provided of its own guard-column. Acquisition and analysis of chromatograms were performed by the software (ChromQuest) supported by HPLC manufacturer. Sample analysis were carried out according to a slight modification of a previous method for the separation of adenine nucleotides.[17] Assignment and purity of peaks both of NAD(P)H-treated samples and of heart tissue extracts were performed by comparing retention times and absorbance spectra of peaks of chromatographic runs of ultrapure standards.

RESULTS

System (1) for ROS Generation

Since NADH and NAD(P)H reactivity was almost identical, we grouped results obtained with both types of reduced coenzymes (Figures 3–5) and we referred throughout the text to NAD(P)H, $NAD(P)^+$ and ADP-ribose(P), unless differently specified.

In Figure 1, a representative chromatogram of control NADH (Panel A) and of NADH incubated with the highest concentrations of Fe^{2+} (180 μM) and H_2O_2 (1000 μM) is reported. In chromatogram of treated NADH (Panel B), in addition to the peak of NADH itself, three peaks were attributed to nicotinamide ($k' = 6.77$) NAD^+ ($k' = 8.34$) and ADP-ribose ($k' = 17.50$), on the basis of comparison with k' values and absorbance spectra of standard nicotinamide, NAD^+ and ADP-ribose, as well as of cochromatograms of treated NADH supplemented with external nicotinamide and ADP-ribose. To show the similar reactivity of NAD(P)H, we reported in Figure 2 a representative chromatogram of control NAD(P)H (Panel A) and of NAD(P)H incubated with 180 μM Fe^{2+} and 1000 μM H_2O_2 (Panel B). On the basis of comparisons of retention factors, absorbance spectra and cochromatograms, the three main peaks

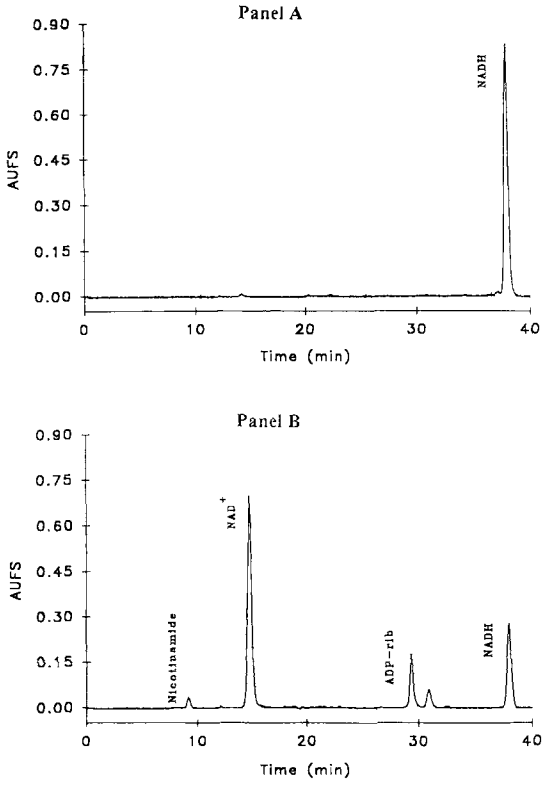

FIGURE 1 Representative chromatogram of control (Panel A) and $Fe^{2+} + H_2O_2$-treated NADH (Panel B). NADH (500 μM) was incubated in the presence either of buffer only (50 mM CH_3COONH_4, pH 6.5) or of 180 μM $FeCl_2$ + 1000 μM H_2O_2, for 1 h at 37°C. Samples (50 μl) were analyzed by HPLC using a method for the separation of adenine nucleotide derivatives. $\lambda = 260$ nm.

were attributed to nicotinamide, $NADP^+$ and ADP-ribose(P), respectively. Figure 3 reports NAD-(P)H, $NAD(P)^+$ and ADP-ribose(P) concentrations determined after incubation of NAD(P)H either with increasing H_2O_2 and fixed Fe^{2+} (Panel A) or with increasing Fe^{2+} and fixed H_2O_2 (Panel B). Since variations of nicotinamide were practically identical to those of ADP-ribose(P) we omitted them from figures, just for the sake of clarity. In both conditions, production of $NAD(P)^+$, nicotinamide and ADP-ribose(P) correlated with the dose of H_2O_2 or Fe^{2+} and $NAD(P)^+$/ADP-ribose(P) ratio ranged between 3.7 and 4.6 (mean value = 4.05; SD = 0.52), for any concentration of the Fenton reagents used.

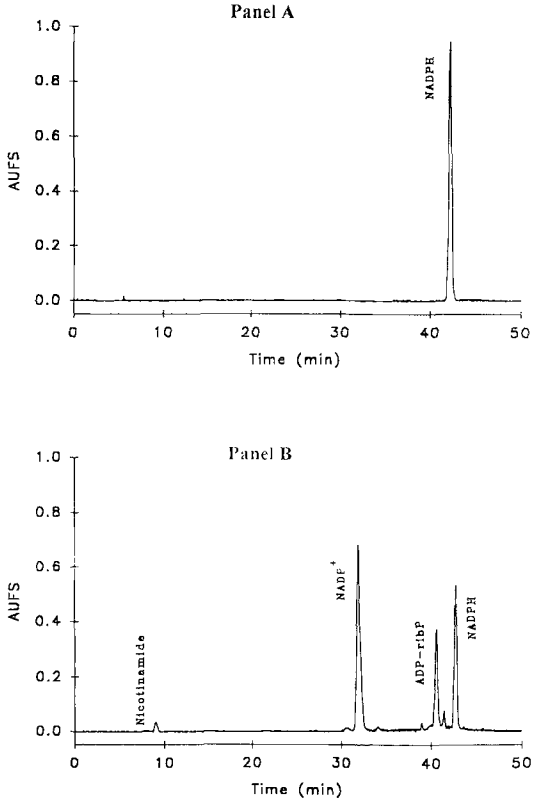

FIGURE 2 Representative chromatogram of control (Panel A) and $Fe^{2+} + H_2O_2$-treated NAD(P)H (Panel B). NAD(P)H (500 μM) was incubated in the presence either of buffer only (50 mM CH_3COONH_4, pH 6.5) or of 180 μM $FeCl_2$ + 1000 μM H_2O_2, for 1 h at 37°C. $\lambda = 260$ nm.

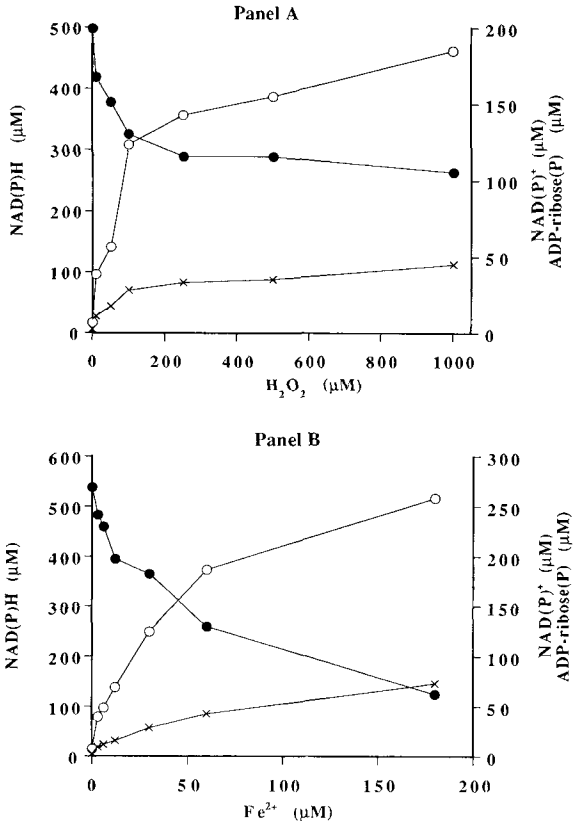

FIGURE 3 ADP-ribose(P) (×), $NAD(P)^+$ (○) and NAD(P)H (●) as a function of increasing ROS generation. NAD(P)H (500 μM) was incubated for 1 h at 37°C (pH 6.5) either with increasing H_2O_2 (50–1000 μM) and fixed $FeCl_2$ (180 μM) (Panel A), or with increasing $FeCl_2$ (3–180 μM) and fixed H_2O_2 (1000 μM) (Panel B). Each point is the mean of six experiments (three of which were carried out with NADH and three with NAD(P)H). Values of nicotinamide and standard deviations have been omitted for the sake of clarity.

Maximal generation of $NAD(P)^+$, nicotinamide and ADP-ribose(P) was observed at the highest concentrations of H_2O_2 and Fe^{2+}. The kinetic of nicotinamide, ADP ribose(P) and $NAD(P)^+$ formation was studied by incubating 500 μM NAD(P)H with the most effective dose of H_2O_2 (1000 μM) + Fe^{2+} (180 μM) at 37°C, pH 6.5, for different times. For practical reasons, the minimal incubation time was no shorter than 5 s. More than 90% of oxidation and hydrolysis of NAD(P)H occurred within 5 s from the beginning of the Fenton reaction (Figure 4). To test the involvement of hydroxyl radicals in NAD(P)H oxidation and hydrolysis, we performed experiments in which increasing concentrations of mannitol (10–50 mM) were added to NAD(P)H before the challenge with the highest concentrations of both iron and hydrogen peroxide. Data summarized in Table I show that mannitol decreased $NAD(P)^+$, nicotinamide and ADP-ribose(P) production, thus strongly suggesting that $^{\bullet}OH$ plays a central role in the formation of these compounds from NAD(P)H.

We also evaluated in detail the effects of changing the pH of the incubation medium on the reaction of NAD(P)H with ROS, produced by Fe^{2+} (180 μM) + H_2O_2 (1000 μM). At pH lower than 5.5 ammonium acetate was replaced by

sodium acetate. Since incubations were also carried out at low pH values, where acidic NAD(P)H hydrolysis might have occurred, their duration never exceeded 15 s, i.e. for a sufficient time to complete the reaction (see Figure 4). Data reported in Figure 5 show that the minimal value of $NAD(P)^+$/ADP-ribose(P) ratio (1.6) occurred at pH 4.5. Both higher and lower pH induced an increase of the ratio which, at pH 2.0 and 7.4, had similar values (5.5 and 6.5, respectively). Since the minimal value of the ratio was very close to the pK_a of acetic acid, it raised the possibility that acetate might participate in the reaction. For this purpose, same experiments of Figure 5 were carried out by replacing sodium acetate with 50 mM sodium malonate ($pK_{a2} = 5.7$). Results demonstrated that minimal value of the ratio was again obtained at pH 4.5, thus excluding participation in the reaction with NAD(P)H of chemical species other than hydroxyl radicals (data not shown). It should be accentuated that changes of $NAD(P)^+$/ADP-ribose(P) ratio (both

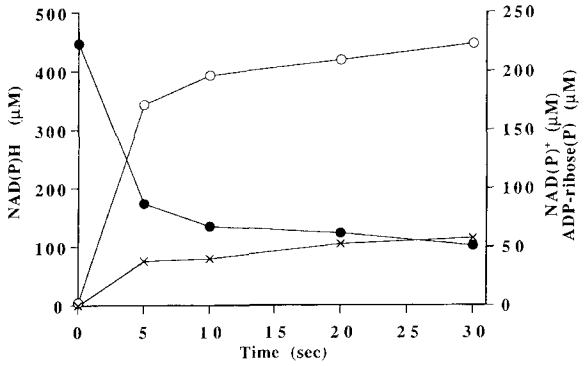

FIGURE 4 Time-course changes of ADP-ribose(P) (×), $NAD(P)^+$ (○) and NAD(P)H (●) during incubation with $Fe^{2+} + H_2O_2$. NAD(P)H (500 μM) was challenged with 180 μM $FeCl_2$ and 1000 μM H_2O_2 for different times (minimum 5 s). Reaction was stopped by adding large EDTA excess (10 mM). Each point is the mean of six experiments (three of which were carried out with NADH and three with NADPH). Values of nicotinamide and standard deviations have been omitted for the sake of clarity.

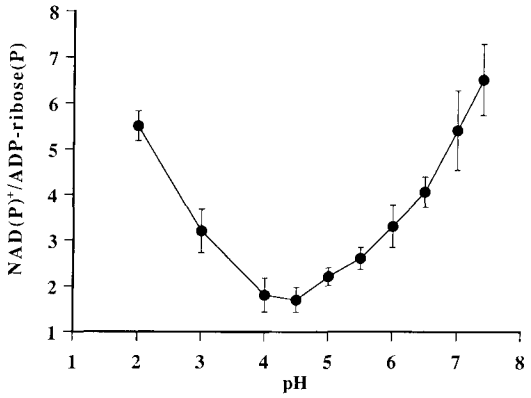

FIGURE 5 Influence of pH on hydroxyl radical-induced NAD(P)H oxidation and hydrolysis. Incubations of 500 μM NAD(P)H with 180 μM $FeCl_2$ and 1000 μM H_2O_2, for 15 s at 37°C, were carried out in 50 mM CH_3COONa (from pH 2.0 to 5.5) or 50 mM CH_3COONH_4 (from pH 5.5 to 7.4). Samples were analyzed by HPLC to determine ADP-ribose(P), nicotinamide, $NAD(P)^+$ and NAD(P)H. Results are reported as variations of $NAD(P)^+$/ADP-ribose(P) ratio as a function of pH. Each point is the mean of six experiments (three of which were carried out with NADH and three with NAD(P)H).

TABLE I Effect of increasing mannitol concentrations on ADP-ribose(P), nicotinamide and $NAD(P)^+$ production induced by challenging 500 μM NAD(P)H with Fe^{2+} 180 μM and H_2O_2 1000 μM, for 1 h at 37°C in 50 mM ammonium acetate, pH 6.5

Added mannitol (mM)	ADP-ribose(P) (μM)	Nicotinamide (μM)	$NAD(P)^+$ (μM)	NAD(P)H (μM)
0	75.7 (6.85)	79.4 (7.55)	301.2 (22.76)	154.9 (10.65)
10	64.1 (4.32)	69.6 (5.16)	273.3 (18.91)	182.3 (9.98)
20	58.3 (5.34)	56.9 (5.48)	253.8 (15.37)	211.4 (11.56)
30	50.9 (4.01)	48.2 (3.77)	215.4 (13.88)	269.8 (13.00)
40	46.0 (2.95)	41.1 (3.62)	189.6 (14.71)	291.5 (17.63)
50	39.1 (3.15)	31.3 (1.94)	166.3 (12.03)	324.7 (21.80)

Values are the mean (SD) of six experiments (three of which were carried out with NADH and three with NAD(P)H).

with acetate and malonate) were due to modifications of ADP-ribose(P) values, since $NAD(P)^+$ concentrations ranged from about 190 to about 250 μM, at any pH used (mean $NAD(P)^+$ value = 217.6 μM; SD = 18.32). This demonstrates that the efficiency of the Fenton reaction was not significantly influenced by pH changes and strongly indicates the existence of two distinct mechanisms for ADP-ribose(P), nicotinamide and $NAD(P)^+$ production. A further corroboration of this hypothesis was obtained by performing the Fenton reaction, in presence of NAD(P)H, in an almost completely non-aqueous, polar medium (100% HPLC-grade methanol; water content = 0.02%). Under such experimental conditions, 75.9 μM (SD = 5.16) of $NAD(P)^+$ were produced after 5 min incubation at 37°C, whilst neither ADP-ribose(P) nor nicotinamide were formed. It is worth noting that control NAD(P)H did not undergo degradation at any pH tested, as well as that the highest concentrations of H_2O_2 only (1000 μM) or Fe^{2+} only (180 μM) did not cause any nicotinamide and ADP-ribose(P) formation. Furthermore, these last products were not detected when $NAD(P)^+$ was challenged with any $Fe^{2+} + H_2O_2$ concentration.

Systems (2) and (3) for ROS and Free Radical Generation

The effects of incubation of NAD(P)H with the highest doses of ascorbate (1000 μM) and Fe^{3+} (180 μM) for 1 h at 37°C, in CH_3COONH_4 pH 6.5 (system 2), were minimal in terms of $NAD(P)^+$ production (mean value = 15.3 μM; SD = 2.18) and did not generate any nicotinamide and ADP-ribose(P). Azo-initiator (system 3) oxidized NAD(P)H to $NAD(P)^+$ in a dose-dependent manner (Table II) but did not induce any detectable nicotinamide and ADP-ribose(P) production.

Pyridine Coenzyme Depletion in Postischemic Rat Heart

HPLC determination of pyridine coenzymes (NAD^+ and $NADP^+$) and ADP-ribose of perchloric acid tissue extracts showed that, at the end of preperfusion, control and mannitol-treated hearts did not significantly differ for their NAD^+, $NADP^+$ and ADP-ribose levels (Table III). At the end of 30 min of reperfusion following 30 min of global normothermic ischemia, NAD^+ and $NADP^+$ concentrations of control hearts were markedly decreased by 44.3% and 48.1%, respectively, whilst that of ADP-ribose showed a 5.9 times increment. Heart perfusion with a 50 mM mannitol-supplemented medium diminished either nicotinic coenzyme depletion or ADP-ribose increase. In fact, in this group of hearts, NAD^+ and $NADP^+$ were 25.8% and 28.2% lower

TABLE II Effect of increasing concentrations of the azo-initiator 2,2′-azobis(2-methylpropionamidine)dihydrochloride on ADP-ribose(P) and $NAD(P)^+$ production after incubation of 500 μM NAD(P)H for 1 h at 37°C in 50 mM ammonium acetate, pH 6.5

Added azo-initiator (mM)	$NAD(P)^+$ (μM)	NAD(P)H (μM)
0	1.4 (0.28)	482.6 (30.47)
1	29.4 (1.96)	442.6 (32.35)
5	80.5 (6.11)	400.4 (31.29)
10	136.2 (9.05)	335.6 (23.40)

Values are the mean (SD) of six experiments (three of which were carried out with NADH and three with NAD(P)H). ADP-ribose(P) and nicotinamide were undetectable in all samples.

TABLE III Effect of the ROS scavenger mannitol on nicotinic coenzyme depletion and ADP-ribose production induced by ischemia and reperfusion in isolated postischemic rat hearts. Compounds were determined by HPLC on 50 μl of neutralized perchloric acid tissue extracts

	NAD^+	$NADP^+$	ADP-ribose
Controls			
Preperfusion	9.21 (1.18)	0.72 (0.08)	0.28 (0.02)
Reperfusion	5.13 [a] (0.97)	0.37 [a] (0.05)	1.66 [a] (0.33)
Mannitol (50 mM)			
Preperfusion	9.43 (1.02)	0.68 (0.05)	0.26 (0.01)
Reperfusion	7.00 [a,b] (0.88)	0.49 [a,b] (0.06)	0.59 [a,b] (0.11)

Each value is the mean (SD) of six hearts and is expressed as μmol/g dry weight. [a]Significantly different from preperfusion ($p < 0.05$). [b]Significantly different from reperfusion of control hearts ($p < 0.05$).

than the respective values determined at the end of preperfusion, and ADP-ribose was 2.3 times higher than the concentration recorded in control hearts (Table III), thereby suggesting that ROS produced in postischemic hearts are directly involved in the phenomenon of nicotinic coenzyme depletion. It is significant recalling that, under the chromatographic conditions used to analyze heart tissue extracts, ADP-ribose(P) was not resolved as a single, pure peak and was not therefore possible to calculate its concentrations.

DISCUSSION

Data reported in the present study show, for the first time to the best of our knowledge, the capacity of $^{\bullet}OH$-generating system to form ADP-ribose(P) and nicotinamide from NAD(P)H, contemporaneously to its oxidation into $NAD(P)^+$ At present, several reports showed that vanadium salts, through the formation and action of diperoxovanadate complex, are able to oxidize NAD(P)H, probably via the abstraction of a $^{\bullet}H$ from the C4 of the pyridine ring with the consequent formation of the transient $^{\bullet}NAD(P)$ radical.[15,16] Since in our experiments with system (1) hydroxyl radicals are the main ROS formed in the reaction mixture, the aforementioned mechanism might be involved in the observed $NAD(P)^+$ formation from NAD(P)H, with $^{\bullet}OH$ functioning as, and in place of, the diperoxovanadate complex. In our opinion, generation of ADP-ribose(P) and nicotinamide from NAD(P)H should follow a different reaction mechanism. As shown in Figure 6, the first reaction with $^{\bullet}OH$ should produce the $^{\bullet}H$ abstraction from the protonated N atom of the pyridine ring thus producing an unusual nitrogen-centered $^{\bullet}NAD(P)H$ radical (totally different from the carbon-centered $^{\bullet}NAD(P)H$ radical formed during NADH photoexcitation[18]) and a water molecule. In this unstable and peculiar form, the $^{\bullet}N$ might capture one electron of the C atom of the N–C bond. This should cause the scission of the N-glycosidic bond with the consequent formation of ADP-ribose$(P)^+$ (having a transient carbocation C^+ on the upper ribose moiety) and reduced $^{\bullet}$nicotinamide (having the $^{\bullet}N$ atom with its lone pair). A second molecule of a generic free radical (either $^{\bullet}OH$ or some secondary free radical such as $^{\bullet}NAD(P)H$ radical) should interact with reduced $^{\bullet}$nicotinamide, causing a $^{\bullet}H$ abstraction from the C4 and the immediate aromatization to nicotinamide. The nucleophilic attack of a water molecule to the carbocation of ADP-ribose$(P)^+$ should ultimately terminate the reaction originating the other end products, ADP-ribose(P) (Figure 7).

Our experimental data strongly support this proposed mechanism, for the following reasons: (i) the scission of the N–C bond of NAD(P)H is promoted by $^{\bullet}OH$, since no ROS-generating system but $Fe^{2+} + H_2O_2$ produced ADP-ribose(P) and nicotinamide from NAD(P)H. Furthermore, the direct involvement of hydroxyl radicals in NAD(P)H hydrolysis was confirmed by the dose-dependent inhibition of ADP-ribose(P) and nicotinamide production exerted by mannitol, although remarkable at relatively high mannitol concentrations; (ii) by lowering the pH of the medium up to 4.5, only the increase of ADP-ribose(P) and nicotinamide concentrations was observed, whilst $NAD(P)^+$ was comprised in a very narrow concentration range at any pH tested. This suggests that protonation of the N atom of the pyridine ring is a prerequisite for the occurrence of the reaction with $^{\bullet}OH$ and reinforces the idea of two distinct mechanisms leading to $NAD(P)^+$ or to ADP-ribose(P) and nicotinamide formation. The efficiency decrease of ADP-ribose(P) and nicotinamide production, at pH lower than 4.5, might be attributed to conformational changes of NAD(P)H induced by protonation of other groups (phosphates, N atom of adenine ring) that might result in a decrease of accessibility towards $^{\bullet}OH$; (iii) reaction of NAD(P)H with $Fe^{2+} + H_2O_2$ performed in 100% HPLC-grade methanol, i.e. in a medium with a water content of 0.02%, induced the formation of $NAD(P)^+$ only, again supporting that H_3O^+ and

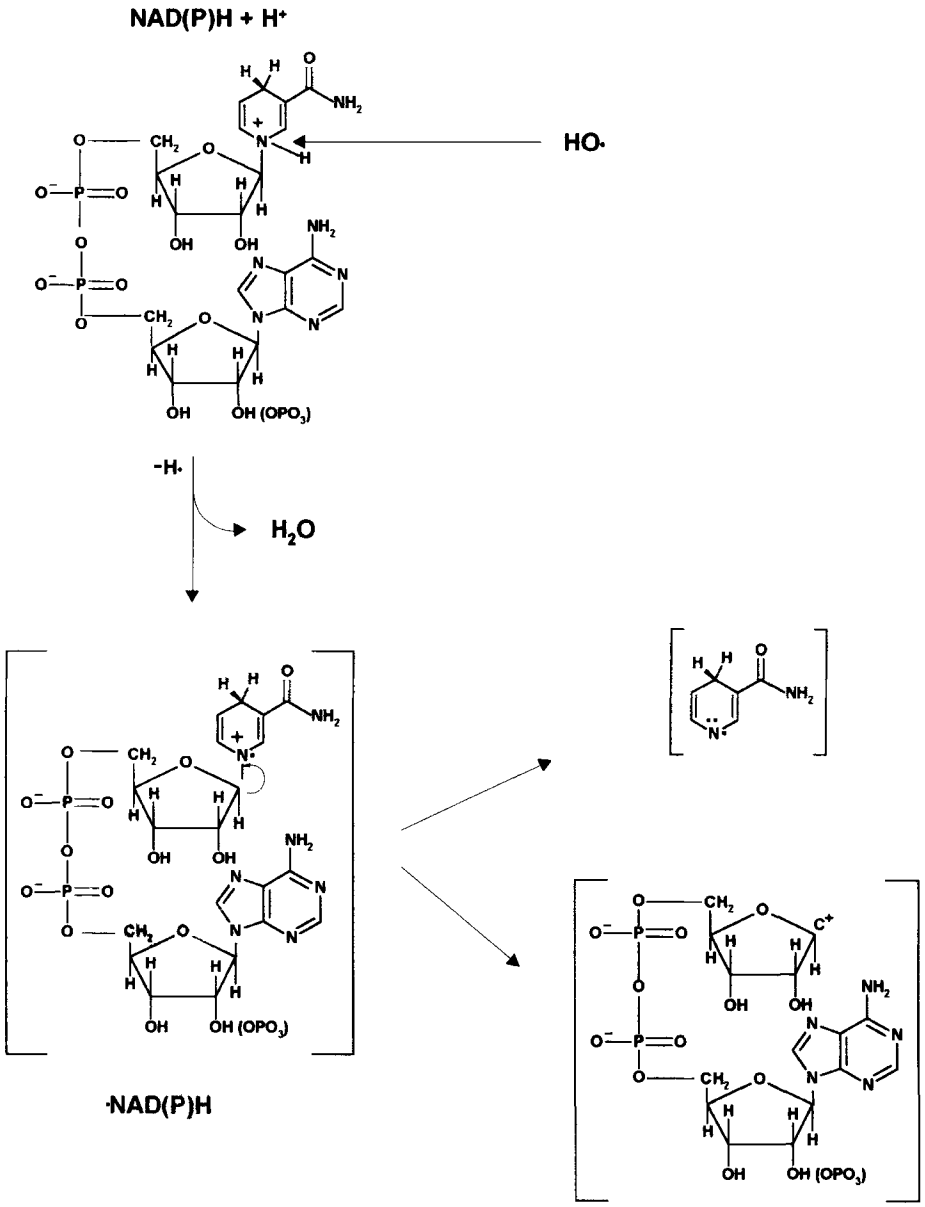

FIGURE 6 Proposed scheme of NAD(P)H reaction with hydroxyl radical. Hydroxyl radical should produce the abstraction of •H from the protonated N atom of the pyridine ring with the formation of the N-centered •NAD(P)H radical and of a water molecule. As indicated by the arrow in the second part of the reaction, one electron of the N–C bond of •NAD(P)H radical might be attracted by •N. This should cause the scission of the N–C bond, generating as intermediates ADP-ribose(P)$^+$ (with a carbocation on the ribose ring moiety) and the reduced form of nicotinamide (with the N atom presenting the lone pair and one additional electron, temporarily not engaged in any bond).

OH^- are fundamental for allowing generation of ADP-ribose(P) and nicotinamide.

Independently from the mechanism of ADP-ribose(P) and nicotinamide generation, it is important to underline that the phenomenon described in the present study *in vitro* might occur *in vivo*, particularly in postischemic tissues. In this regard, it has been reported that

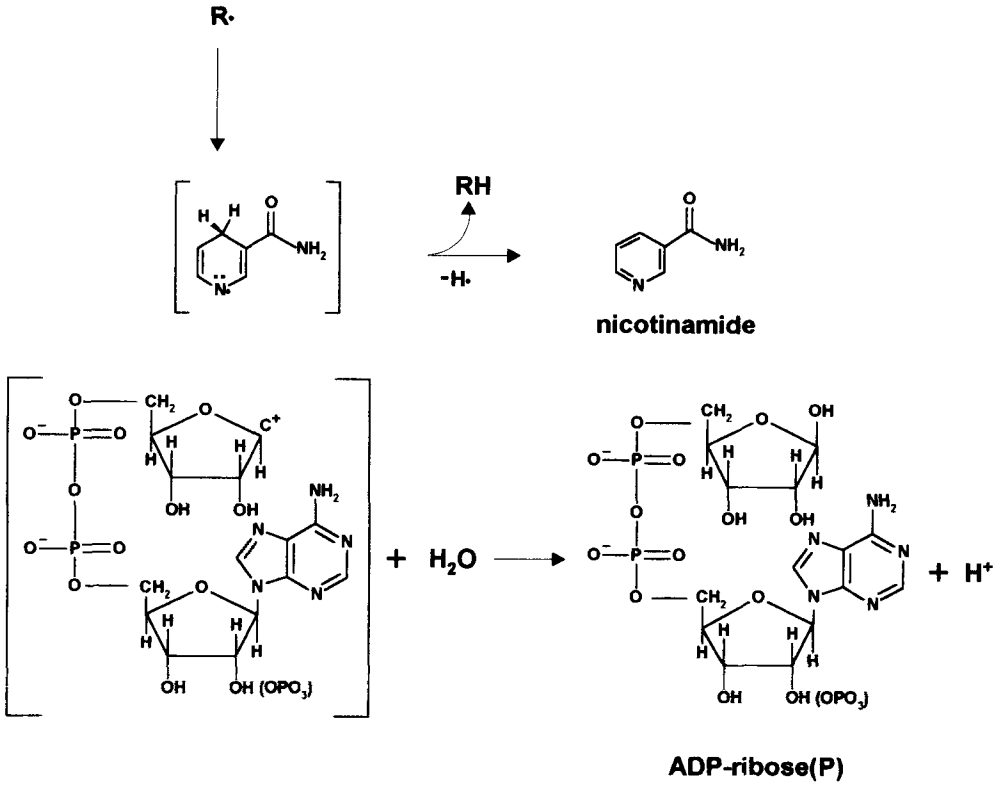

FIGURE 7 Formation of ADP-ribose(P) and nicotinamide as the end products of hydroxyl radical-mediated NAD(P)H hydrolysis. A generic free radical should cause the abstraction of a new •H from the C4 of the nicotinic ring, thus inducing aromatization and production of nicotinamide. The nucleophilic addition of a water molecule to ADP-ribose(P)$^+$ should terminate the reaction of ADP-ribose(P) formation.

myocardial[11–14,19] and cerebral[20,21] ischemia and reperfusion induce an unexplained marked depletion of pyridine coenzyme pool that might occur through the ROS-induced NAD(P)H degradation into ADP-ribose(P) and nicotinamide. Data obtained in the present study, by perfusing isolated postischemic rat hearts with the hydroxyl radical scavenger mannitol (Table III), strongly suggest that the ROS-mediated chemical mechanism of NAD(P)H hydrolysis is operative not only *in vitro*, but also *in vivo* in postischemic tissues. These results seem particularly interesting since, at present, prior studies[10–14,19–22] did not take into consideration that depletion of pyridine coenzyme pool, occurring in conditions of increased oxidative stress, might be produced through the direct interaction of ROS with NAD(P)H. It is worth underlining that ischemia and reperfusion produce some simultaneous and peculiar cell events that may foster the occurrence of hydroxyl radical-mediated NAD(P)H hydrolysis: (i) reperfusion-associated ROS overproduction;[23] (ii) the presence of adequate NAD(P)H concentration at the time of reperfusion produced by previous ischemia through the occurrence of the so-called "reductive stress";[24] (iii) acidic pH values (ranging between 5.9 and 6.4) reported in ischemic tissues[25,26] (that is reasonable to imagine are not immediately normalized during reperfusion) which should certainly favor the eventual ROS-mediated ADP-ribose(P) and nicotinamide formation from NAD(P)H. On the other hand, the eventual enzymatic depletion of pyridine coenzymes should not be possible in these conditions

either because NAD(P)H are not substrates for NAD(P)-glycohydrolase[27] or because the catalytic activity of this enzyme (which has been involved in $NAD(P)^+$ consumption as a consequence of augmented ROS production without preceding reductive stress[28]) has been reported to decrease in reperfused myocardium.[29]

Although the biological significance of cellular ADP-ribose(P) overproduction is not immediately predictable, its meaning as NAD(P)H degradation product is evident. In fact, ADP-ribose(P) metabolic fate, at least in red blood cell, does not seem related to pyridine coenzyme resynthesis;[30] in fact, in conditions of low ATP levels (as it also occurs in postischemic tissues), ADP-ribose(P) is predominantly transformed into AMP and, to a lesser extent, into ADP.[30] In the myocardium, part of ADP-ribose(P) generated from ROS-mediated NAD(P)H degradation, might be transformed into its corresponding cyclic form. However, results of experiments that we carried out in isolated postischemic rat hearts strongly indicate that the net depletion of myocardial pyridine coenzyme pool, certainly compromising the correct functioning of both energy and oxidative–reductive cell metabolism, and jeopardizing reperfusion efficacy, is very probably the ultimate consequence of NAD(P)H interaction with hydroxyl radicals.

In conclusion, results reported in this study demonstrate, for the first time to the best of our knowledge, the existence of a new reaction center in the NAD(P)H molecule localized on the N atom of the pyridine ring. The interaction of NAD(P)H with hydroxyl radicals produces, at the same time, oxidation to $NAD(P)^+$ (through the reaction with the "conventional" reactive center) and hydrolysis into ADP-ribose(P) and nicotinamide (through the reaction with the "new" reactive center). Data obtained in isolated postischemic rat hearts indicate that pyridine coenzymes can be considered new target molecules of ROS, the severe cytotoxicity of which might also be mediated (at least in postischemic tissues) by the direct ROS-induced NAD(P)H depletion and consequent ADP-ribose(P) and nicotinamide production. Further studies to validate the proposed mechanism of hydroxyl radical-mediated NAD(P)H hydrolysis are in progress.

Acknowledgments

This work has been supported in part by research funds of Catania and Rome "Tor Vergata" Universities and by a grant "ex-quota 40%: Nuovi approcci valutativi in tossicologia", from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

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