Oxidant Injury of Cells

DNA Strand-breaks Activate Polyadenosine Diphosphate-ribose Polymerase and Lead to Depletion of Nicotinamide Adenine Dinucleotide

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Abstract

To determine the biochemical basis of the oxidant-induced injury of cells, we have studied early changes after exposure of P388D$_1$ murine macrophages to hydrogen peroxide. Total intracellular NAD$^+$ levels in P388D$_1$ cells decreased with H_2O_2 concentrations of 40 μM or higher. Doses of H_2O_2 between 0.1 and 2.5 mM led to an 80% depletion of NAD within 20 min. With doses of H_2O_2 of 250 μM or lower, the fall in NAD and, as shown previously, ATP, was reversible. Higher doses of H_2O_2 that cause ultimate lysis of the cells, induced an irreversible depletion of NAD and ATP.

Poly-ADP-ribose polymerase, a nuclear enzyme associated with DNA damage and repair, which catalyzes conversion of NAD to nicotinamide and protein-bound poly-ADP-ribose, was activated by exposure of the cells to concentrations of 40 μM H_2O_2 or higher. Activation of poly-ADP-ribose polymerase was also observed in peripheral lymphocytes incubated in the presence of phorbol myristate acetate-stimulated polymorphonuclear neutrophils. Examination of the possibility that DNA alteration was involved was performed by measurement of thymidine incorporation and determination of DNA single-strand breaks (SSB) in cells exposed to H_2O_2. H_2O_2 at 40 μM or higher inhibited DNA synthesis, and induced SSB within less than 30 s.

These results suggest that DNA damage induced within seconds after addition of oxidant may lead to stimulation of poly-ADP-ribose polymerase, and a consequent fall in NAD. Excessive stimulation of poly-ADP-ribose polymerase leads to a fall in NAD sufficient to interfere with ATP synthesis.

Introduction

H_2O_2 in concentrations achieved in the proximity of stimulated neutrophils and macrophages, induces cell lysis of target cells over a period of several hours, as was shown for a number of different cell types (1–6). Various biochemical changes occur long before the loss of cell integrity. In sequential order these are the activation of the hexose monophosphate shunt (HMPS),[1] which provides reducing equivalents to oxidized glutathione (2, 7), inhibition of the glycolytic pathway (8) with loss of intracellular ATP (1, 9), increase in intracellular Ca^{++} and Na^+ (Hyslop, P. A., D. B. Hinshaw, I. U. Schraufstatter, L. A. Sklar, R. G. Spragg, and C. G. Cochrane, manuscript submitted for publication; and ref. 10), aggregation of actin-rich filaments in the cytoskeleton (12), cell blebbing (11, 12), and cell lysis (2–4).

Since ATP is essential to many cell functions, we sought to determine the basis of its depletion in P388D$_1$ murine macrophages. Stimulation of the HMPS was calculated to account for ~15% of the fall in ATP, but the remainder of the depletion was unexplained. The earlier, preliminary observation that NAD levels were also depleted in P388D$_1$ cells exposed to oxidant (2) was of potential importance since low NAD levels have been associated for many years with an inhibition of the glycolytic pathway and ATP formation (13, 14).

The catabolism of NAD is known to be regulated by two enzymes, nuclear poly-ADP-ribose polymerase and cytoplasmic NAD glycohydrolase (15). NAD glycohydrolase forms free ADP-ribose, which we did not detect in H_2O_2 injured cells.

We therefore examined the possibility that activation of poly-ADP-ribose polymerase was responsible for the loss of NAD and ATP in P388D$_1$ cells exposed to H_2O_2.

Methods

Cell culture. P388D$_1$ murine macrophages were grown to confluence in RPMI 1640 (Irvine Scientific, Santa Ana, CA), supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, and 50 μg/ml gentamycin sulfate (MA Bioproducts, Walkersville, MD). Just before the experiment they were removed with a rubber policeman, centrifuged at 400 *g* for 5 min, and resuspended in full medium or modified Gey's buffer (MGB) containing 147 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM $Na_2SO_4 \cdot 7H_2$, 5.5 mM glucose, 1.5 mM $CaCl_2$, 0.3 mM $MgSO_4 \cdot 7H_2O$, and 1 mM $MgCl_2 \cdot 6H_2O$, and 10 mM Hepes, pH 7.4.

Human peripheral lymphocytes were prepared from fresh acid citrate dextrose (ACD) blood after removal of platelet-rich plasma on lymphocyte separation medium (Litton Bionetics, Kensington, MD) (16). Contaminating erythrocytes were lysed in 154 mM NH_4Cl, 12 mM NaH_2CO_3, 0.1 mM Na_2EDTA, pH 7.4, and the cells were washed two times in MGB. They were 95% mononuclear cells, and 95% viable.

Human polymorphonuclear neutrophils (PMN) were prepared from fresh ACD blood by counterflow centrifugal elutriation as previously described (18). The cell preparation was 98% pure, and at least 98% viable.

Modification of cell enzyme systems. P388D$_1$ cells were depleted of glutathione by incubation with 0.2 mM buthionine sulfoximine (BSO) (Chemalog, Chemical Dynamics Corp., S. Plainfield, NJ) for 18 h (18). Inhibition of glutathione reductase was achieved by incubation with 75 μM (1,3-bis)2-chloroethyl *l*-nitroso-urea (BCNU) (Bristol Laboratories, Syracuse, NY) (19) for 20 min at 37°C, which reduced glutathione reductase activity by >98%.

This article is publication No. 3978-IMM from the Research Institute of Scripps Clinic.
Received for publication 22 July 1985.

1. *Abbreviations used in this paper:* ACD, acid citrate dextrose; BCNU, (1,3-bis)2-chlorethyl *l*-nitroso-urea; BSO, buthionine sulfoximine, HMPS, hexose monophosphate; HPLC, high pressure liquid chromatography; MGB, modified Gey's buffer; SSB, single-strand breaks; TLC, thin layer chromatography.

J. Clin. Invest.

0021-9738/86/04/1312/09 $1.00
Volume 77, April 1986, 1312–1320

Poly-ADP-ribose polymerase activity was inhibited by incubating $P388D_1$ cells with 2.5 mM 3-aminobenzamide (Sigma Chemical Co., St. Louis, MO) (21), which was added 10 min before the addition of oxidant.

Determination of nucleotides and nucleosides by HPLC. 2.5×10^7 $P388D_1$ cells were incubated in 12.5 ml MGB or full RPMI 1640 medium for various times with the addition of H_2O_2 as indicated in the legends. The exposure of H_2O_2 was stopped by addition of 15,000 U catalase, and the cells were centrifuged at 1,000 *g* for 90 s at 4°C. The supernatant was removed and the cell pellet was resuspended in 500 μl 8% perchloric acid. The pH was adjusted to ~5.5 by removal of the perchloric acid with Freon/alamine (21). NADH and NADPH were completely degraded under these conditions (22). Cell extracts corresponding to 5×10^6 cells were applied to an anion exchange high pressure liquid chromatography (HPLC) column (Ultrasil AX anion exchange column, Altex Scientific, Inc., Berkeley, CA) and eluted with a gradient from 7 mM $NH_4H_2PO_4$, pH 3.8 to 250 mM $NH_4H_2PO_4$, 500 mM KCl, pH 4.5 (22).

500 μl of supernatant from 5×10^6 cells/ml were precipitated with 50 μl of 70% perchloric acid, the perchloric acid was removed, and 200 μl of the sample were applied to a C18 reverse-phase HPLC column (DuPont, Wilmington, DE). Nucleosides were separated with a linear gradient from 88 to 99% buffer A (buffer A, 10 mM KH_2PO_4, pH 3.3; buffer B, 10 mM KH_2PO_4, pH 4.0 in 60% acetonitrile) at a flow rate of 1.5 ml/min for 25 min (23).

Determination of ^{14}C-nicotinamide moiety in $P388D_1$ cells. 5×10^6 cells/ml in the presence or absence of H_2O_2 were incubated with 1 μCi ^{14}C-nicotinamide (55 mCi/mmol, Amersham Corp., Arlington Heights, IL) at 37°C for 30 s to 1 h. At the conclusion of experiments as noted in Results, 200 μl of cell suspension were layered on top of 150 μl silicone oil (four parts Dow Corning 550, one part Dow Corning 200; William Nye, New Bedford, MA) and centrifuged for 10 s at 10,000 *g*. The cell pellets were solubilized in 750 μl 2% SDS in 0.1 M NaOH overnight, sonicated, and counted in a liquid scintillation counter.

For separation of different pyridine compounds the radiolabeled cells were centrifuged through silicone oil into a layer of 50 μl of 250 mM mannitol, 2.5 mM EDTA, and 17 mM MOPS, pH 7.4; the cell pellet was incubated in 200 μl of 70% ethanol in 10 mM sodium phosphate buffer, pH 7.0 for 30 min at room temperature (24). The cell extract after centrifugation was applied to thin layer chromatography (TLC) silica plates (HPTLC silica 60; Merck, Darmstadt, W. Germany), and pyridine compounds were separated as described (2, 25). Pyridines were quantified by scintillation counting.

Determination of DNA and protein synthesis. 2×10^6 $P388D_1$ cells in full culture medium containing 0.38 mM leucine were incubated for 1 h with concentrations of H_2O_2 between 10 and 1,000 μM in the presence of 1 μCi [^{3}H]leucine (120 Ci/mol, ICN) and 0.25 μCi ^{14}C-thymidine (57 mCi/mmol, ICN). The cells were centrifuged, the supernatant was removed, and the cell pellets were resuspended in 500 μl 50% TCA, followed by three washes with TCA. The TCA precipitate was solubilized as described for pyridine nucleotides, and radioactivity was measured in a liquid scintillation counter.

Determination of uptake of leucine and aminoisobutyric acid. 5×10^6 $P388D_1$ cells/ml were incubated in full medium with concentrations of H_2O_2 between 100 and 2,000 μM in the presence of 1 μCi [^{3}H]leucine and 2 μCi [^{14}C]aminoisobutyric acid (53 mCi/mmol, New England Nuclear, Boston, MA). The final concentration of leucine or amino isobutyric acid was 0.38 mM. Uptake of radiolabel was determined by centrifuging 200 μl of cell suspension through 150 μl silicone oil as described for radiolabeled nicotinamide moiety, and counting the radioactivity of the cell pellet.

Determination of DNA single-strand breaks (SSB). The formation of SSB in DNA was measured by alkaline unwinding and determination of ethidium bromide fluorescence on an SLM 8000 fluorometer with excitation at 520 nm and emission at 590 nm according to the method of Birnboim and Jevcak (26). Under the conditions employed, ethidium bromide binds preferentially to double-stranded DNA. H_2O_2 was added to 2×10^6 $P388D_1$ cells/ml and the cells were incubated at 37°C for 5 min, centrifuged for 10 s in a microfuge, resuspended in 250 mM meso-inositol, 10 mM sodium phosphate, 1 mM $MgCl_2$ (pH 7.2), and processed as described except that the alkaline lysates were incubated for 45 min at 15°C. Results are expressed as D (percent double-stranded DNA) $= (F - F_{min})/(F_{max} - F_{min}) \times 100$, where F is the fluorescence of the sample, F_{min} the background fluorescence determined in samples that were sonicated at the beginning of the unwinding period in order to induce maximal unwinding, and F_{max} is the fluorescence of samples kept at pH 11.0, which is below the pH needed to induce unwinding of single stranded DNA.

Determination of HMPS activity. HMPS activity was determined by subtracting $^{14}CO_2$ formed from C_6-labeled glucose from $^{14}CO_2$ formed from C_1-labeled glucose as previously described (2).

Determination of poly-ADP-ribose-polymerase activity. 2×10^6 $P388D_1$ cells or 5×10^6 human peripheral lymphocytes were incubated at 37°C for various periods of time in the presence or absence of H_2O_2. To stop the reaction, we centrifuged the cells at 10,000 *g* for 10 s, removed the supernatant, and resuspended the cell pellet in 500 μl 56 mM Hepes buffer, pH 7.5, containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 125 nmol NAD spikes with 0.25 μCi ^{3}H-NAD (22 μCi/mmol, ICN Pharmaceuticals, Irvine, CA) essentially as described (27). The amount of digitonin used (28) permeabilized plasma membranes leading to release of apoplasmic enzymes, but not to loss of intracellular organelles. The permeabilized cells were incubated for 5 min at 37°C as noted in Results, and the protein that was ribosylated with [^{3}H]NAD was precipitated with 200 μl 50% TCA. After two washes with TCA, the protein pellet was solubilized in 2% SDS in 0.1 M NaOH, incubated at 37°C overnight, and the radioactivity was determined by scintillation counting.

In experiments that determined the influence of PMA-stimulated PMN on TCA-precipitable ADP-ribose incorporation into lymphocytes, 5×10^6 lymphocytes were incubated in MGB in the presence or absence of 5×10^6 PMN±100 ng/ml PMA (Sigma Chemical Co.) for 30 min at 37°C. Poly-ADP-ribose polymerase was determined as described above.

Separation of ADP-ribosylated proteins by HPLC. $\sim 5 \times 10^7$ $P388D_1$ cells were labeled with 0.1 μCi [^{14}C]thymidine/ml (57 mCi/mmol, ICN) in 75 ml culture medium overnight. 5×10^6 washed thymidine-labeled $P388D_1$ cells were incubated at 37°C for 30 min in the presence or absence of 1 mM H_2O_2, and processed as for poly-ADP-ribose polymerase activity except that 10 μCi [^{3}H]NAD were used. After 5 min incubation the cells were centrifuged at 10,000 *g* for 4 min at 4°C. The cell pellet was resuspended in 400 μl 100 mM Na_2SO_4, 20 mM NaH_2PO_4, pH 6.8 (buffer G) and sonicated for 30 s. 200 μl of the filtered cell sonicate were applied on a TSK 250 gel filtration HPLC column (Bio-Rad Laboratories, Richmond, CA), and eluted isocratically with buffer G with a flow rate of 1 ml/min. Radioactivity in 20-s fractions was determined by scintillation counting.

Results

Effect of H_2O_2 on NAD levels of $P388D_1$ cells. $P388D_1$ cells (2×10^6/ml MGB) were exposed to varying concentrations of H_2O_2 for the time periods shown in Fig. 1. The initial fall in NAD was maximal with a concentration of ~100 μM H_2O_2 and led to a depletion of >80% (from 1.35 to 0.18 nmol/10^6 cells) of the intracellular NAD within 20 min (Fig. 1 *A*). Doses as high as 5 mM H_2O_2 showed a similar degree of NAD depletion. The only difference was that in cells injured with 250 μM H_2O_2 or less the low NAD levels were not permanent, but NAD levels slowly recovered over a period of ~2 h. As little as 30–40 μM H_2O_2 could induce a decrease in cellular NAD levels (see Fig. 5). Half-maximal depletion of NAD was achieved at 30 min with a dose of ~50 μM H_2O_2.

If $P388D_1$ cells were exposed to H_2O_2 in full medium containing 7.4 μM nicotinamide, there was an initial drop in cellular NAD levels comparable to that seen in MGB (Fig. 1 *B*). Cellular NAD levels recovered considerably faster in the presence of nic-

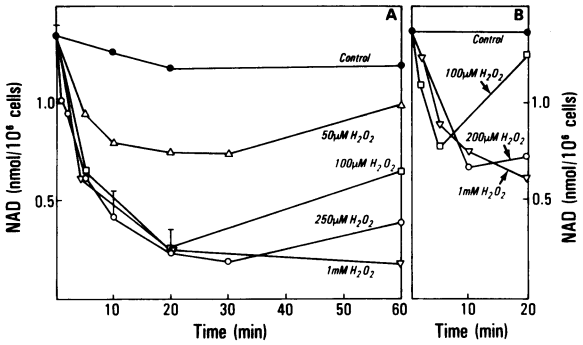

Figure 1. The effect of H_2O_2 on intracellular NAD. 2×10^6 cells/ml were incubated in 12.5 ml MGB (*A*) or full medium RPMI 1640 (*B*) in the absence of H_2O_2 (●) or in the presence of H_2O_2 in the concentrations indicated in the figure. At the time-points indicated, catalase was added to stop the reaction and samples were prepared for HPLC nucleotide determinations as described in Methods. Each point represents the mean of two experiments. Error bars represent standard deviations for $n = 4$.

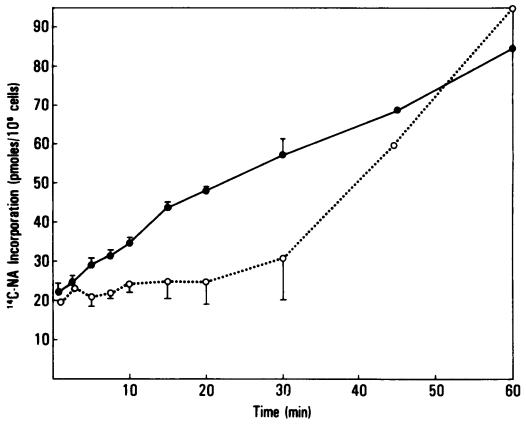

Figure 2. Uptake of ^{14}C-nicotinamide in the presence and absence of H_2O_2. 5×10^6 cells/ml were incubated at 37°C in MGB in the presence (○) or absence (●) of 500 μM H_2O_2. 10 s after addition of the H_2O_2, 1 μCi [^{14}C]nicotinamide/ml (50 mCi/mmol) was added. At the time-points indicated, 200-μl samples were layered onto silicone oil and centrifuged for 10 s in a microfuge. Mean and SD were calculated from three experiments performed in duplicates.

otinamide, indicating that synthesis of NAD from nicotinamide was possible in the presence of H_2O_2. With doses of H_2O_2 of 500 μM or higher, the drop in NAD was irreversible. The threshold dose of H_2O_2 that induced cell lysis as determined by Trypan Blue exclusion over a period of 8 h after addition of the oxidant was between 250 and 500 μM in four experiments and thus correlates with the dose of H_2O_2 that led to irreversible NAD depletion.

NADH levels were affected to a lesser extent. With a dose of 1 mM H_2O_2, NADH, as measured by chemiluminescence assay (21, Calbiochem-Behring Corp., La Jolla, CA), decreased from 1.7 to 0.9 nmol/10^6 cells (mean values of four experiments) over a period of 20 min.

Human peripheral lymphocytes exposed to 250 μM H_2O_2 under the same conditions as described for P388D_1 cells lost 90% of their NAD during the first 30 min of injury: NAD concentrations decreased from 0.9 nmol/10^6 cells to 0.09 nmol/10^6 cells.

Results obtained previously in this laboratory (1), indicated that P388D_1 cells exposed to as little as 50 μM H_2O_2 show a loss of ATP content. With a dose of 250 μM H_2O_2, the ATP levels dropped from control levels of 7.2 nmol/10^6 cells to 1.9 nmol/10^6 cells 30 min after exposure of 2×10^6/ml P388D_1 cells to H_2O_2. Higher doses of H_2O_2 induced a much faster and complete reduction of ATP levels. The threshold dose of H_2O_2 required to induce a fall of ATP was slightly higher (50 μM) than that for NAD (30 μM) (as will be shown in Fig. 5). The changes in cellular NAD and ATP over time, were parallel (see ref. 1 for comparison).

Incorporation of [^{14}C]nicotinamide into pyridine moiety in the presence and absence of H_2O_2. Since the fall in NAD in H_2O_2-induced injury could be partially reduced in the presence of nicotinamide, we added [^{14}C]nicotinamide in the concentration as present in culture medium (1 mg/liter) to the incubation medium, and followed its uptake and incorporation into pyridine moiety. A linear uptake of nicotinamide during the 1 h of the experiment was detected in control cells (Fig. 2). The distribution of radiolabel in various pyridine moieties was measured by TLC. In control cells at 60 min, ~25 pmol were present as nicotinamide, which is the amount that would be expected for any freely diffusible compound, assuming a cell volume of 1 μl/10^6 cells. 50 pmol of NAD had been synthesized during this period of time, and ~5 pmol were present as NADPH and NMN. No incorporation into NADH was observed.

In cells (5×10^6/ml) exposed to 500 μM H_2O_2, no uptake of [^{14}C]nicotinamide above the amount due to diffusion was seen until 30 min after the addition of H_2O_2. All the radioactivity incorporated up to this point was present as nicotinamide. At later time-points an increase in incorporation of [^{14}C]nicotinamide into pyridine moiety above control levels was observed in oxidant injured cells (Fig. 2). The distribution of radiolabel at 1 h was identical to that seen in control cells. Therefore, the decrease in intracellular NAD concentration caused by H_2O_2 (950 pmol/10 min per 10^6 cells) would probably not be accounted for by inhibition of synthesis of NAD, unless the NAD turnover was considerably increased at the same time. We thus looked for nicotinamide formation from NAD as an indication for catabolism of NAD.

Detection of nicotinamide in the extracellular medium. If the catabolism of NAD were increased when P388D_1 cells were exposed to H_2O_2, there should be an increase in the metabolic products, nicotinamide (NA) and ADP-ribose. We therefore assayed for the presence of these products.

Incubation of P388D_1 cells in the presence of H_2O_2 led to the appearance of nicotinamide in the extracellular medium (Fig. 3). The amount of nicotinamide found in the extracellular medium of P388D_1 cells incubated in the presence of H_2O_2 was similar to the concentration of the sum of NAD and NADH lost from the cell. After 20-min exposure to 500 μM H_2O_2, a difference of 2.2 nmol nicotinamide was found in the extracellular medium of H_2O_2 injured and uninjured cells. At this time the injured cells had lost 1.2 nmol NAD and 0.8 nmol NADH.

Eukaryotic cells contain several enzymes hydrolyzing NAD that are poly-ADP-ribose polymerase, NAD glycohydrolase, and various proteins (29) that mono-ADP ribosylate elongation factor 2, the Gs protein of the adenylate cyclase system and a mitochondrial inner membrane protein. NAD glycohydrolase forms equimolar amounts of free ADP-ribose, which elutes with a re-

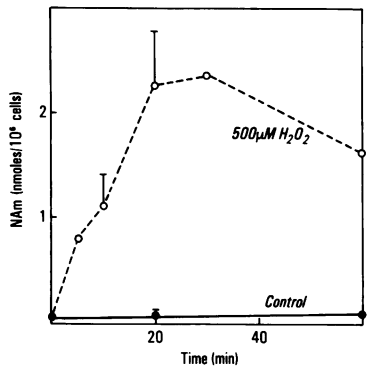

Figure 3. The effect of H_2O_2 on nicotinamide in the extracellular medium. 5×10^6 cells/ml were incubated at 37°C in MGB in the presence (○) or absence (●) of 500 μM H_2O_2. Samples of supernatant were prepared as described in Methods and nucleosides were separated and quantitated by HPLC. Each point represents the mean of two experiments. Error bars show standard deviations for $n = 3$.

tention time of 14.0 min in HPLC nucleotide separation. No more than traces of ADP-ribose were found in HPLC nucleotide separations. Poly-ADP-ribose polymerase forms polymers of ADP ribose which are ester-bound to nuclear proteins. Therefore we looked for changes in activity of poly-ADP-ribose-polymerase after exposure of P388D_1 cells to H_2O_2.

Determination of poly-ADP-ribose polymerase activity. Poly-ADP-ribose-polymerase activity was determined in P388D_1 cells that were incubated with H_2O_2 for various periods of time and then were treated with 0.01% digitonin in order to permeabilize them for ^{3}H-NAD labeled in the adenine moiety. Binding of radiolabel to the TCA precipitable material for 5 min at 37°C was used as a measure of formation of protein-bound poly-ADP-ribose. Basal activity of poly-ADP-ribose polymerase was very high in control P388D_1 cells (22±10 pmol/10^6 cells per min).

Poly-ADP-ribose polymerase activity increased at every time point between 2.5 and 60 min after exposure of P388D_1 cells to 1 mM H_2O_2 (Fig. 4). The activity was doubled from basal levels within 5 min of oxidant exposure, and in the presence of an excess of NAD stayed high for at least 60 min. At this time all

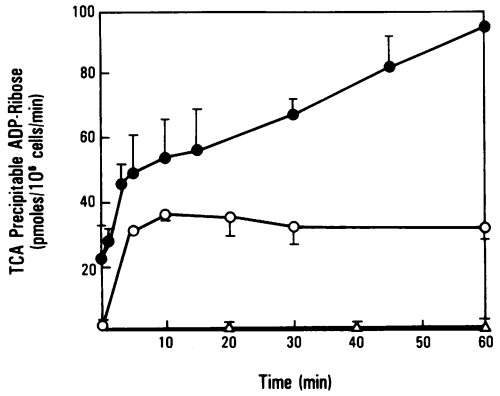

Figure 4. Time-course of poly-ADP-ribose polymerase activity after exposure to H_2O_2. 2×10^6 P388D_1 cells/ml or 5×10^6 lymphocytes/ml were incubated in MGB±H_2O_2 for the periods of time indicated. At these time-points cell samples were centrifuged for 10 s, the supernatant was removed, and the cell pellet was resuspended in 500 μl lysis buffer containing [^{3}H]NAD (see Methods) and incubated at 37°C for 5 min. The reaction was stopped with 200 μl 50% TCA (mean and SD of three experiments). ●, poly-ADP-ribose polymerase activity in P388D_1 cells; ○, poly-ADP-ribose polymerase activity in human peripheral lymphocytes; △, poly-ADP-ribose polymerase activity in P388D_1 cells preincubated with 2.5 mM 3-ABA for 10 min at 37°C.

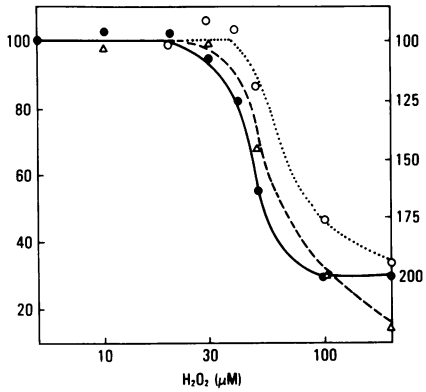

Figure 5. Response of intracellular NAD, ATP, and poly-ADP-ribose polymerase activity to varying doses of H_2O_2. 2×10^6/ml P388D_1 cells in MGB were incubated at 37°C in the presence of various concentrations of H_2O_2. After 20 min the cells were prepared for HPLC (NAD) (●) and ATP (○), or [^{3}H]NAD incorporation (poly-ADP-ribose polymerase activity) (△). Results (mean of 2 experiments) are expressed as percent of values found in cells incubated in the absence of H_2O_2.

but 30 μM H_2O_2 had been consumed by the cells. The dose-response curves for H_2O_2 on NAD levels and poly-ADP-ribose polymerase activity, 20 min after addition of oxidant, are shown in Fig. 5. A minimal concentration of 30–40 μM H_2O_2 was needed to affect either of these two parameters.

Similar results were seen in human peripheral lymphocytes. Within 10 min after exposure to 1 mM H_2O_2 poly-ADP-ribose polymerase activity in these cells increased from 1.8±0.5 pmol/10^6 cells per min to 35.9±1.6 pmol/10^6 cells per min (Fig. 4).

H_2O_2 did not increase the maximal amount of ADP-ribose-protein formation in P388D_1 cells; when the incorporation of radiolabel from [^{3}H]NAD into acid precipitable material was followed for 30 min, and H_2O_2 was added at this time no further increase of radioactivity occurred in the protein pellet. The initial rate of protein-bound ADP-ribose formation was doubled in cells exposed to H_2O_2.

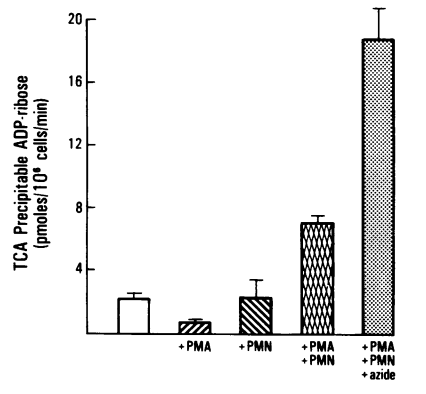

Figure 6. Poly-ADP-ribose polymerase activity in human lymphocytes exposed to PMA stimulated PMN: 5×10^6 peripheral human lymphocytes in 1 ml MGB were incubated for 30 min at 37°C, in the presence or absence of 5×10^6 human PMN, 100 ng PMA, and 5 mM azide as indicated in the figure. At the end of this incubation period, TCA-precipitable ADP-ribose was determined for 5 min at 37°C as described under Methods. Mean and standard deviations for duplicate samples in three experiments.

The formation of TCA precipitable ADP-ribose polymer was reversed in the presence of snake venom phosphodiesterase (Cooper Biomedical, Malvern, PA). Only 13% of the radiolabel in control cells and 8% in cells preincubated with 1 mM H_2O_2 for 30 min was still found in the protein pellet, if cells that had been exposed to [^{3}H]NAD for 5 min were centrifuged, the supernatant removed, and the cells then incubated in 1 ml 50 mM TRIS, 5 mM $MgCl_2$, pH 8.2 containing 250 μg phosphodiesterase for 60 min at 37°C. No decrease in TCA precipitable material was found after DNase or RNase incubation determined as previously described (27).

We examined the target proteins of ADP-ribosylation in order to compare the sizes with those observed under other conditions. Previous studies have suggested that the major acceptor proteins for ADP-ribose are the histones, especially histone Hl with a molecular weight of 22,000 (30), and ADP-ribose polymerase itself with a molecular weight of 110,000 (31). This protein can be so highly ADP-ribosylated that it shows a molecular weight of up to 250,000. In addition, high molecular weight bands with a molecular weight of ~600,000 (32) containing ADP-ribose have been shown on SDS-polyacrylamide gels. We chose a nondenaturing HPLC gel filtration method to separate [^{3}H]ADP-ribosylated proteins. Three major peaks of radioactivity were detected (Fig. 7): a low molecular weight peak which coeluted with histone standards, a broad peak of radioactivity between 100,000 and 200,000 mol wt, which may present ribosylated poly-ADP-ribose polymerase, and a high molecular weight peak, which was associated with DNA as was shown in

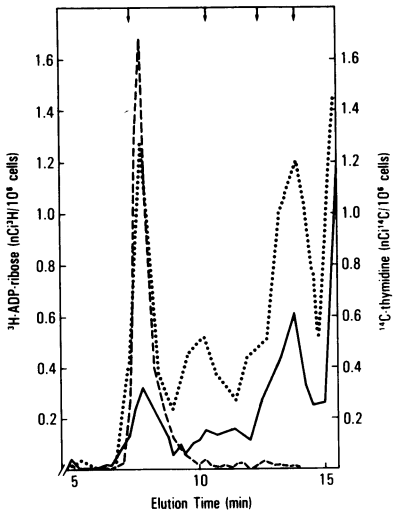

Figure 7. HPLC gel filtration elution of ADP-ribosylated proteins. Cells were labeled with [^{14}C]thymidine (0.1 μCi/ml) overnight and washed three times in MGB. 5 × 10^6 cells were incubated with or without 1 mM H_2O_2 for 30 in at 37°C. After centrifugation for 10 s, the cells were suspended in 500 μl Hepes/digitonin buffer and incubated for 10 min at 37°C in the presence of 10 μCi [^{3}H]NAD. The cells were centrifuged at 10,000 *g* for 4 min at 4°C, the pellets were resuspended in 400 μl HPLC running buffer, sonicated for 30 s, and applied on a TSK 250 HPLC column. Radioactivity in 0.25-min fractions was counted in a liquid scintillation counter. The arrows indicate the elution profile for thyroglobulin (670,000 mol wt), gamma-globulin (158,000 mol wt), ovalbumin (44,000 mol wt), and myoglobin (17,000 mol wt). (——), protein-bound [^{3}H]NAD in control cells; (· · · · ·), protein-bound [^{3}H]NAD in H_2O_2 injured cells; (– – –), [^{14}C]thymidine in control and injured cells.

P388D_1 cells prelabeled with [^{14}C]thymidine. In H_2O_2-injured cells the amount of radioactivity in these three peaks was increased, while the pattern of ADP-ribosylated proteins was not detectably changed, nor was there a change in the amount of coeluting [^{14}C]thymidine radioactivity. When P388D_1 cells were incubated with 2.5 mM 3-aminobenzamide, an inhibitor of poly-ADP-ribose polymerase, for 10 min before the addition of 1 mM H_2O_2, the rate of formation of TCA-precipitable ADP-ribose material dropped to 0.25 pmol/min in the presence or absence of H_2O_2. 3-Aminobenzamide did not decrease the rate of disappearance of H_2O_2 (Schraufstatter, I. U., D. B. Hinshaw, P. A. Hyslop, L. Sklar, and C. G. Cochrane, manuscript submitted for publication). It did prevent the loss of NAD in P388D_1 cells exposed to H_2O_2: cells pretreated with 2.5 mM 3-aminobenzamide and then exposed to 250 μM H_2O_2 for 20 min, contained 1.8±0.1 nmol NAD/10^6 cells. Cells pretreated with 3-ABA but not exposed to H_2O_2 contained 1.4±0.12 nmol NAD/10^6 cells.

To establish whether activation of poly-ADP-ribose polymerase occurs in the proximity of stimulated neutrophils, 5 × 10^6 lymphocytes were coincubated with the same number of PMN in the presence or absence of 100 ng PMA. PMN do not contain poly-ADP-ribose polymerase (33). Neither PMN nor PMA alone activate poly-ADP-ribose polymer formation. PMA-stimulated PMN induced a threefold increase in incorporation of [^{3}H]NAD into TCA-precipitable material in the target lymphocytes (Fig. 6). This increase in activity of poly-ADP-ribose polymerase could be further increased in the presence of azide as an inhibitor of intracellular catalase (Fig. 6).

Influence of H_2O_2 on DNA and protein synthesis. Poly-(ADP-ribosylation) has been associated with repair of DNA strand breaks (31, 32). We therefore examined the possibility that H_2O_2 treatment induced functional abnormalities of DNA function. Incorporation of [^{14}C]thymidine and [^{3}H]leucine into acid-insoluble material as indicators of DNA and protein synthesis was determined in P388D_1 cells exposed to doses of H_2O_2 between 10 and 1,000 μM. Results are shown in Fig. 8. As noted, a dose-dependent inhibition of both [^{14}C]thymidine and [^{3}H]leucine into the TCA precipitates was observed. The inhibition of [^{3}H]leucine incorporation could not be accounted for by inhibition of amino acid transport across the membrane, since uptake of [^{3}H]aminoisobutyric acid (an amino acid that is not incorporated into protein) was not influenced by H_2O_2 concentrations of 1 mM or less. Higher concentrations of H_2O_2 actually caused a slight increase of uptake of amino acids.

The threshold for inhibition of the incorporation of [^{14}C]thymidine and [^{3}H]leucine was similar to the minimal dose that led to activation of poly-ADP-ribose polymerase and decrease in NAD levels. These data indicate a marked effect of H_2O_2 exposure to the P388D_1 cells on DNA related functions.

Influence of H_2O_2 on DNA SSB. When DNA SSB were determined 5 min after the addition of H_2O_2 by the alkaline unwinding technique, a slight increase in single-stranded DNA was observed with as little as 25 μM H_2O_2. With 100 μM H_2O_2, only 26% of the DNA was still double-stranded under our experimental conditions (Fig. 9). A similar dose response was obtained for peripheral human lymphocytes. The amount of DNA unwinding seen with 100 μM H_2O_2 in lymphocytes was equivalent to that induced in these cells by 500 rad of ^{60}Co irradiation (34).

When catalase was added to P388D_1 cells exposed to 200 μM H_2O_2 at intervals after addition of H_2O_2, and SSB were

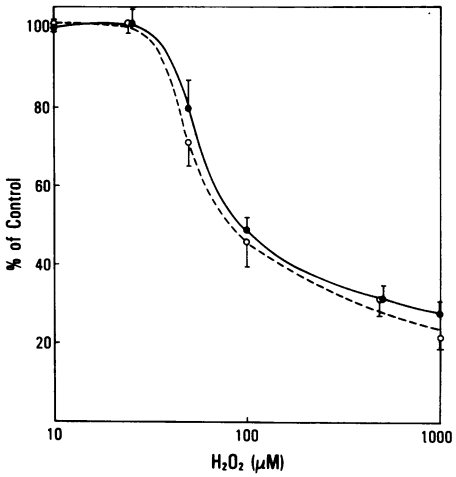

Figure 8. Influence of H_2O_2 on DNA and protein synthesis. 2×10^6 $P388D_1$ cells in full culture medium were incubated for 1 h with concentrations of H_2O_2 between 10 and 1,000 μM in the presence of [^{14}C]thymidine (o) and [^{3}H]leucine (•). The reaction was stopped by the addition of TCA as described in Methods. Results are expressed as percent of incorporation of radiolabel into acid-precipitable material in control cells. Mean and SD of three experiments done in duplicates.

measured in these cells it was observed that an exposure time of 10 s—the shortest time experimentally possible—was sufficient to induce SSB. After 10 s, 65% of the DNA was present as double-stranded DNA; after 30 s, only 37%.

Influence of HMPS activity on cellular NAD levels. Our results indicated that activation of poly-ADP-ribose polymerase played a role in H_2O_2-induced cellular injury, yet it was not the only event induced by oxidant injury.

Activation of the HMPS occurred independently of the activation of poly-ADP-ribose-polymerase. If the HMPS was inactivated (2) by either inhibiting glutathione reductase with 75 μM BCNU (19) or by depleting cellular glutathione to 7% of the initial amount with 0.2 mM BSO overnight (18), NAD levels in cells exposed to H_2O_2 dropped to a similar degree as in cells with an active HMPS. After 10-min incubation with 1 mM H_2O_2 in full medium, NAD levels in cells treated with BCNU were 0.65 nmol/10^6 cells; in BSO treated cells, 0.68 nmol/10^6 cells; and 0.71 nmol/10^6 cells in cells not treated with inhibitors of the glutathione cycle.

To determine if inhibition of the ADP-ribose polymerase affected the glutathione cycle in cells exposed to oxidant, $P388D_1$ cells were pretreated with 3-aminobenzamide before exposure to H_2O_2. BCNU or BSO had no effect on cells without exposure to H_2O_2. The HMPS activity was not influenced by inhibition of poly-ADP-ribose polymerase activity with 2.5 mM 3-aminobenzamide (Fig. 10). Exposure of $P388D_1$ cells to 2.5 mM H_2O_2 induced an initial 10-fold increase in HMPS activity in the presence as well as in the absence of 3-aminobenzamide.

Discussion

The exposure of $P388D_1$ cells to H_2O_2 induced a fall in NAD and ATP levels starting within 1 min after its addition, which reached a maximum after ~30 min. The evidence suggested that the fall in intracellular NAD resulted from increased catabolic rate. The rate of formation of catabolic products nicotinamide and poly-ADP-ribose was increased at a time when NAD levels were falling. These data suggested that exposure to H_2O_2 leads to stimulation of the enzyme poly-ADP-ribose-polymerase. The threshold dose of H_2O_2 needed to induce a fall of NAD was identical to the minimal dose of H_2O_2 necessary to activate poly-ADP-ribose-polymerase.

Inhibition of poly-ADP-ribose polymerase with 2.5 mM 3-aminobenzamide or 2.5 mM theophylline prevented the fall in NAD induced by H_2O_2. This underscores the role of poly-ADP-ribose polymerase and increased catabolism of NAD as the cause of its depletion. In addition, in studies to be reported separately (Schraufstatter, I. U., D. B. Hinshaw, P. A. Hyslop, L. Sklar, and C. G. Cochrane, manuscript submitted for publication), inhibition of this enzyme was found to prevent the sustained fall in ATP, the increase in intracellular Ca^{++}, and lysis of $P388D_1$ cells.

The fall in ATP followed closely the decrease in NAD observed either as a time-course or a dose-response relationship. Whenever intracellular NAD fell by ~50% (0.7 mM), ATP levels started to decrease as well (Fig. 5). Similarly, when NAD levels increased again (with low concentrations of H_2O_2 of 250 μM or

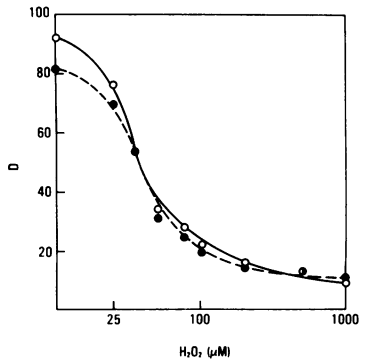

Figure 9. DNA single-strand breaks as determined by alkaline unwinding technique. $P388D_1$ cells (•) or human peripheral lymphocytes (o) (2×10^6/ml MGB) were incubated with different doses of H_2O_2 for 5 min at 37°C, H_2O_2 was removed by centrifugation, the cell pellet was resuspended in 450 μl ice-cold meso-inositol buffer, and 200-μl samples were lysed in alkaline medium. Ethidium bromide fluorescence was determined (excitation, 520 nm; emission, 590 nm) after a 45-min incubation at 15°C. D represents percent double-stranded DNA at the end of this incubation period as described in Methods. Mean of three experiments determined in duplicate.

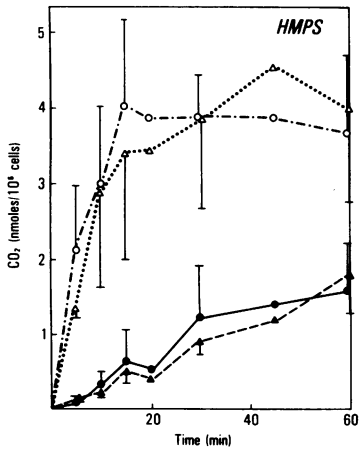

Figure 10. Influence of inhibition of poly-ADP-ribose polymerase on HMPS activity in the presence and absence of H_2O_2. 4×10^6 $P388D_1$ cells in 2 ml MGB were incubated with 2 μCi C_6-[^{14}C]glucose or 0.5 μCi C_1-[^{14}C]glucose (2). For inhibition of poly-ADP-ribose polymerase, cells were preincubated with 2.5 mM 3-aminobenzamide. 2.5 mM H_2O_2 was added at the same time as the radiolabel. The reaction was stopped at the time-points indicated by the addition of 200 μl 50% trichloroacetic acid and CO_2 was trapped on cotton swabs soaked in NaOH. HMPS activity was calculated by subtracting $^{14}CO_2$ formed from C_6-labeled glucose from $^{14}CO_2$ formed from C_1-labeled glucose.

lower), ATP concentrations started to recover as well. Note that the threshold concentration of H_2O_2 that would lead to cell lysis within 3–6 h was also in the 250–500-μM range. Permanent energy depletion thus was incompatible with cell survival. The glycolytic pathway that generates 75% of the ATP in P388D$_1$ cells (1) is inhibited in the presence of H_2O_2.

An increase in nicotinamide concentrations in the extracellular medium paralleled the depletion of NAD intracellularly. Since NADH is not a substrate for poly-ADP-ribose polymerase (15), one must assume that the NADH was lost from the cell as a result of oxidation and then conversion into nicotinamide and poly-ADP-ribose. Moreover, when poly-ADP-ribose polymerase was inhibited with 3-ABA, we observed that there was an increase in NAD of ~0.8 nmol/10^6 cells during the first 10 min in the presence of 250 μM H_2O_2 (Schraufstatter, I. U., D. B. Hinshaw, P. A. Hyslop, L. Sklar, and C. G. Cochrane, manuscript submitted for publication), which was accompanied by a similar fall in NADH levels (unpublished results).

The loss of NAD after exposure to H_2O_2 was higher than the amount of protein-bound ADP-ribose recovered and preceded the measured activation of poly-ADP-ribose polymerase. With a concentration of 1 mM H_2O_2 350 pmol poly-ADP-ribose bound to protein were formed during the first 10 min of injury; 850 pmol NAD were lost under the same conditions. The reasons for the apparent discrepancy may lie in the fact that protein-bound ADP-ribose can be further metabolized by poly-ADP-ribose-glycohydrolase, which hydrolyzes the ribose-ribose bond, by phosphodiesterase, which hydrolyzes the pyrophosphate bond, and by ADP-ribosyl-histone splitting enzyme (35, 36). ADP-ribose itself is further metabolized into AMP and ribose phosphate (37) (Fig. 11), which then can be used for ATP formation. The half-life of polymeric ADP-ribose has been measured by adding 3-aminobenzamide, an inhibitor of poly-ADP-ribose-polymerase, to Ehrlich ascites tumor cells, treated with alkylating agents (37), and following the disappearance of poly-ADP-ribose. The half-life under those conditions was <1 min. The half-life of disappearance in our cells was ~5 min for H_2O_2-injured cells and ~13 min for control cells. An increase in catabolism in poly-ADP-ribose polymer in the presence of H_2O_2 might explain the lag in time between the measured increase of poly-ADP-ribose polymerase activity and the loss of NAD during the first 5 min. Similar discrepancies between loss of NAD and recovery of protein-bound ADP-ribose, determined by various methods, have been described for several other situations (37–39) that are associated with activation of poly-ADP-ribose polymerase.

The basal level of incorporation of [^{3}H]NAD into acid precipitable material was very high in P388D$_1$ cells, which made it difficult to measure small increases of poly-ADP-ribose polymerase activity. Human peripheral lymphocytes contain only ~10% of the basal activity, but show the same absolute increase in ADP-ribose polymer formation as P388D$_1$ after the addition of H_2O_2 (Fig. 4). For this purpose lymphocytes were chosen as the target cells for oxidants produced by stimulated PMN. The total amount of H_2O_2 produced by 5×10^6 PMN stimulated with 100 ng PMA during 30 min averaged 50 nmol/ml. Activation of poly-ADP-ribose polymerase in the target cells lymphocytes was observed under those conditions.

Poly-ADP-ribose polymerase activity is dependent on the presence of DNA (35, 36, 40), and causes an NAD-dependent cross-linking of modified oligonucleosomes (41, 42). A physical association of DNA with poly-ADP-ribose was suggested in the present data in which ADP-ribose and DNA eluted together in HPLC.

Activation of poly-ADP-ribose polymerase has been described for various situations that are associated with DNA strand breaks: (*a*) following irradiation (43–45), (*b*) in the presence of alkylating agents (43–49), (*c*) or deoxyadenosine (50), and (*d*) in permeabilized cells treated with DNAse (51). DNAse treatment induced activation of poly-ADP-ribose polymerase under the experimental conditions used here. Thus, the question arose whether DNA was the primary target of H_2O_2 induced injury, secondarily leading to activation of poly-ADP-ribose polymerase.

The presence of DNA SSB seen with concentrations of H_2O_2 as low as 25 μM agrees with this hypothesis, especially since DNA SSB occurred within seconds after the addition of oxidant. Threshold doses of H_2O_2 needed to induce poly-ADP-ribose polymerase activation are approximately the same as those inducing DNA SSB. DNA synthesis measured during the first hour after exposure to varying doses of H_2O_2 showed a similar dose-response as that observed for SSB. There was a linear relationship between $Q_d = (100 \times (\log D_{control} - \log D\ H_2O_2))$ (34) determined for SSB and the percent inhibition of DNA synthesis for the range of concentrations of H_2O_2. DNA damage induced by various agents blocks semiconservative DNA synthesis, but the mechanisms involved are not well understood in eukaryotic cells (52).

Cell lysis in P388D$_1$ cells during a period of 6 h was observed with doses of H_2O_2 of ~250 μM (at a cell concentration of 2×10^6/ml) and higher (2). This dose corresponds to the concentration of H_2O_2 needed to induce an irreversible loss of NAD and ATP. Considerably lower doses of H_2O_2 (threshold, ~30 μM) were sufficient to transiently deplete cells of their high energy phosphates, to inhibit DNA and protein synthesis, and to induce DNA SSB. DNA alterations occurring in this low dose range of H_2O_2 might be passed on into further cell generations, unless efficient DNA repair is taking place. Recent reports have shown that concentrations of H_2O_2 that can be produced by stimulated neutrophils can induce sister chromatid exchanges in target cells (52–54) and even lead to malignant growth (55).

Activation of the glutathione redox cycle (2)—an important cellular oxidant defense mechanism (4, 56)—measured by an increase in HMPS activity, occurred independently of the activation of poly-ADP-ribose polymerase. Inhibition of the poly-ADP-ribose polymerase with 3-ABA was without effect on oxidant stimulation of the glutathione cycle. The inverse experiment, inhibition of the HMPS activity, had no influence on poly-ADP-ribose polymerase activity and on cellular NAD levels, and only a minor effect on cellular ATP levels (2).

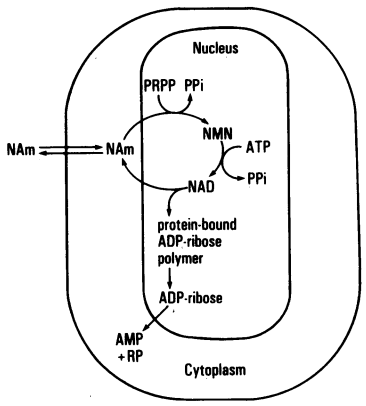

Figure 11. Synthetic and catabolic steps of poly-ADP-ribose metabolism. *NAm*, nicotinamide; *NMN*, nicotinamide mononucleotide; *PRPP*, phosphoribose pyrophosphate; *PPi*, pyrophosphate; *RP*, ribose phosphate.

In summary, we have shown that early in cell injury H_2O_2 can induce depletion of NAD followed by a fall in ATP due to activation of poly-ADP-ribose polymerase activation. Note, however, that this represents only one of several possible mechanisms of cell killing by oxidants. Cell lysis in cells that have a lower activity of poly-ADP-ribose polymerase (57–59) and HMPS activity, might be mediated by other mechanisms such as (*a*) enzyme inhibition due to oxidation and amino acids such as tryptophane, tyrosine, and the sulfhydryl containing amino acids or (*b*) following lipid peroxidation (60) or (*c*) be the result of irreparable DNA damage such as double strand breaks.

The observation that the oxidant, H_2O_2, or an intracellular metabolite of it, induces rapid onset of DNA strand breaks at low concentrations focuses attention on the relationship of inflammation, mutagenesis and malignant transformation. This relationship has received clear experimental support recently (53, 55, 61).

Acknowledgments

We acknowledge the excellent secretarial help from Monica Bartlett.

This work was supported in part by funds from U. S. Public Health Service (HL23584, AI17354, and HL16411), and also by a fellowship from The Puritan Bennett Foundation to Dr. Schraufstatter, a fellowship from the American Lung Association to Dr. Hinshaw, and a fellowship from the American Heart Association to Dr. Hyslop.

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