NAD(P)H, a Primary Target of $^{1}\text{O}_2$ in Mitochondria of Intact Cells*  

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Direct reaction of NAD(P)H with oxidants like singlet oxygen ($^{1}\text{O}_2$) has not yet been demonstrated in biological systems. We therefore chose different rhodamine derivatives (tetramethylrhodamine methyl ester, TMRM; 2',4',5',7'-tetrabromomorhodamine 123 bromide; and rhodamine 123; Rho 123) to selectively generate singlet oxygen within the NAD(P)H-rich mitochondrial matrix of cultured hepatocytes. In a cell-free system, photoactivation of all of these dyes led to the formation of $^{1}\text{O}_2$ which readily oxidized NAD(P)H to NAD(P)$^+$. In hepatocytes loaded with the various dyes only TMRM and Rho 123 proved suited to generating $^{1}\text{O}_2$ within the mitochondrial matrix space. Photoactivation of the intracellular dyes (TMRM for 5–10 s, Rho 123 for 60 s) led to a significant (29.6 ± 8.2 and 30.2 ± 5.2%) and rapid decrease in mitochondrial NAD(P)H fluorescence followed by a slow reincurrence. Prolonged photoactivation (≥15 s) of TMRM-loaded cells resulted in even stronger NAD(P)H oxidation, the rapid onset of mitochondrial permeability transition, and apoptotic cell death. These results demonstrate that NAD(P)H is the primary target for $^{1}\text{O}_2$ in hepatocyte mitochondria. Thus NAD(P)H may operate directly as an intracellular antioxidant, as long as it is regenerated. At cell-injurious concentrations of the oxidant, however, NAD(P)H depletion may be the event that triggers cell death.

Pyridine nucleotides, i.e. NAD(H) and NADP(H), play a central role in metabolism; they are the most important coenzymes acting as hydride (hydrogen anion) donors of various cellular dehydrogenases (e.g. glutathione reductase), functioning as reducing/oxidizing equivalents in essential reactions such as energy supply (aerobic or anaerobic) and photosynthesis, and are required for DNA repair.

The ability of an organism to counteract reactive oxygen species (ROS) or reactive nitrogen species depends on its antioxidative capabilities, which involves destroying of both pro-oxidants (e.g. ROOH, H$_2$O$_2$, ONOOH) and oxidants (e.g. radicals and reactive intermediates like singlet oxygen, $^{1}\text{O}_2$). Whereas pro-oxidants are typically degraded by enzymes (e.g. catalase, glutathione peroxidase, and superoxide dismutase), oxidants are scavenged by relatively small biomolecules (e.g. ascorbic acid, glutathione, and α-tocopherol); these are termed directly operating antioxidants. In this context, NAD(P)H is crucial to maintaining the cellular redox state and/or antioxidative capacity, because of its essential role as a coenzyme in the enzymatic re-reduction of directly operating antioxidants (1, 2). Consequently, NAD(P)H deficiencies are linked with an increased sensitivity to oxidative stress (2, 3).

The capability of NAD(P)H to additionally act as a directly operating antioxidant, i.e. to donate only one electron, was sharply underestimated by various biochemical researchers, a fact that is probably because of the observation that a biochemical standard one-electron oxidant, [Fe(CN)$_6$]$^{3^-}$, oxidizes NADH only very slowly (4). However, we recently demonstrated that, in line with the Marcus theory of electron transfer (1, 5), the reaction constant of Reaction 1

\[ \text{NADH} + \text{Rad}^- \rightarrow \text{NAD}^- + \text{RH} \]  

**REACTION 1**

correlated well with the reduction potential of the oxidizing radical (1). Consequently, putative harmful radicals (ROO', RO', CO$_3^{2-}$) react very fast with NADH ($k = 10^{-6}$–$10^{-8}$ s$^{-1}$). The NAD$^-$ radical thus formed reacts with molecular oxygen near to the diffusion-controlled limit, thereby yielding NAD$^+$ and superoxide, shown in Reaction 2.

\[ \text{NAD}^- + \text{O}_2 \rightarrow \text{NAD}^+ + \text{O}_2^2^- \]  

**REACTION 2**

In chemical systems, O$_2^2$ spontaneously dismutates to H$_2$O$_2$ and $^{1}\text{O}_2$ (6), shown in Reaction 3.

\[ 2\text{O}_2^2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{^{1}\text{O}_2} \]  

**REACTION 3**

In biological systems superoxide dismutase (SOD) catalyzes the dismutation of O$_2^2$ thereby preventing the formation of $^{1}\text{O}_2$, shown in Reaction 4.

\[ 2\text{O}_2^2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + 2\text{O}_2 \]  

**REACTION 4**

The H$_2$O$_2$-consuming enzymes catalase and glutathione peroxidase (GPx) strongly limit the noxious action of H$_2$O$_2$, shown in Reactions 5 and 6.

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{2H}_2\text{O} + \text{^{1}\text{O}_2} \]  

**REACTION 5**

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{2H}_2\text{O} + \text{GSSG} \]  

**REACTION 6**

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§ The abbreviations used are: ROS, reactive oxygen species; $^{1}\text{O}_2$, singlet oxygen; TMRM, tetramethylrhodamine methyl ester; TRB, 2',4',5',7'-tetrabromomorhodamine 123 bromide; Rho, rhodamine; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea; GSSG, glutathione (oxidized form); t-BuOOH, tert-butyl hydroperoxide; HBSS, Hank’s balanced salt solution; MPT, mitochondrial permeability transition; PDT, photodynamic therapy; $k$, rate constant for single electron transfer.

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Given the high concentrations of NADH and NADPH and also the high activity of both superoxide dismutase and glutathione peroxidase in mitochondria, the reduced coenzymes are expected to act as directly operating antioxidants in these organelles (1).

Besides oxidizing radicals, the reactive intermediate $^{1}O_2$ also rapidly reacts with both NADH and NADPH ($k = 4.3 \times 10^7 M^{-1} s^{-1}$ and $8.4 \times 10^7 M^{-1} s^{-1}$) via single electron transfer (7, 8), shown in Reactions 7 and 8.

**NAD(P)H + $^{1}O_2$ → NAD(P)$^+$ + $O_2^+$**

**Reaction 7**

**NAD(P)$^+$ + $O_2$ → NAD(P)$^+$ + $O_2^+$**

**Reaction 8**

In 1976 the thermodynamic capability of NAD(P)H to transfer only one electron to $^{1}O_2$ was estimated by Koppenol (9). Experimental evidence of this reaction in cell-free systems was provided, and consequences of $^{1}O_2$ generation in mitochondria were hypothesized (7, 8, 10, 11) two decades ago. In biological systems, however, direct, i.e. non-enzymatic, oxidation of NAD(P)H by $^{1}O_2$ or by any other oxidant has not yet been demonstrated.

In most cell types, the highest concentrations of reduced nicotinamides are located within the matrix space of mitochondria (12). Taking this into consideration, along with the kinetic data on reactions of different ROS with NAD(P)H in comparison with other biomolecules, $^{1}O_2$ can be expected to be most effective, and most selective, in oxidizing mitochondrial NAD(P)H. We therefore studied the effect of $^{1}O_2$ on the NAD(P)H redox state within the exceptional NAD(P)H-rich mitochondrial matrix space of cultured hepatocytes (12, 13).

To perform these studies, we established a system based on different rhodamine derivatives and on digital fluorescence microscopy to selectively generate $^{1}O_2$ in close proximity to this NAD(P)H pool and to record the effect on mitochondrial NAD(P)H fluorescence with high temporal resolution.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Leibovitz L-15 medium was obtained from Invitrogen; collagenase, collagen (Type I), dexamethasone, and gentamicin were from Serva (Heidelberg, Germany); and KCN and Me$_2$SO were from Merck (Darmstadt, Germany). Bovine serum albumin came from Fluhm Institut (Mannheim, Germany), and the following chemicals were from Sigma: fetal calf serum, superoxide dismutase, NADP-linked isocitric dehydrogenase, 1,3-bis(chloroethyl)-1-nitrosourea (BCNU), $\beta$-hydroxybutyric acid, acetoacetic acid, carbonyl cyanide m-chlorophenylhydrazone, $\beta$-fructose, glutathione (reduced) ethyl ester, dl-isocitric acid, NADH, NADPH, tert-butyl hydroperoxide (t-BuOOH), trifluoperazine, and propidium iodide. Chelex (chelating resin; iminodiacetic acid), 1,3-diphenylisobenzofuran, and 9,10-diphenylanthracene were obtained from Sigma-Aldrich, and digitonin was from Fluka. The fluorescent dyes tetramethylrhodamine methyl ester (TMRM), 2',4',5',7'-tetrabromo- morphodihydrone 123 bromide (TBRB), rhodamine (Rho) 123, and calcine-acetoxymethylester were purchased from Molecular Probes Europe B.V. (Leiden, The Netherlands). Falcon 6-well cell culture plates were obtained from BD Biosciences, and glass coverslips were from Asystent (Sondheim/Rohm, Germany).

**Animals**

Male Wistar rats (200–350 g) were obtained from the Zentrales Tierlaboratorium (Universitätsklinikum, Essen). Animals were kept under standard conditions with free access to food and water. All animals received humane care in compliance with the institutional guidelines.

**Cell Culture**

Hepatocytes were isolated from male Wistar rats as described previously (14). For the fluorescence measurements $1.7 \times 10^2$ cells/cm$^2$ were seeded onto collagen-coated 6.3-cm$^2$ glass coverslips in 6-well cell culture plates. Cells were cultured in L-15 medium supplemented with 5% fetal calf serum, t-glutamate (2.0 mM), glucose (8.3 mM), bovine serum albumin (0.1%), NaHCO$_3$ (14.3 mM), gentamicin (50 mg/L), and dexamethasone (1.0 $\mu$M) at 37°C in a 100% humidified atmosphere of 5% CO$_2/21% O_2/74% N_2$. Two h after seeding, adherent cells were washed three times with Hanks’ balanced salt solution (HBSS, 137.0 mM NaCl/5.4 mM KCl/1.0 mM CaCl$_2$/0.5 mM MgCl$_2$/0.4 mM KH$_2$PO$_4$/0.4 mM MgSO$_4$/0.3 mM Na$_2$HPO$_4$/25.0 mM Hepes, pH 7.4) and supplied with fresh medium as reported previously (15).

**Experiments in a Cell-free System**

**Generation and Detection of $^{1}O_2$—**The $^{1}O_2$ detector molecules, 1,3-diphenylisobenzofuran and 9,10-diphenylanthracene (each 5 $\mu$M; stock solutions 10 mM in Me$_2$SO) were added to HBSS (3.0 ml, 25°C) and transferred into the quartz cuvette of a spectrophotometer (RF-1501; Shimadzu, Kyoto, Japan). After recording the baseline fluorescence of the detector molecules (1,3-diphenylisobenzofuran $\lambda_{nm} = 409$ nm, $\lambda_{em} = 476$ nm; 9,10-diphenylanthracene $\lambda_{nm} = 391$ nm, $\lambda_{em} = 405$ nm) for 5 min at 60-s intervals, TMRM (10 $\mu$M), Rho 123 (10 $\mu$M) or TBRB (10 $\mu$M) were added from concentrated stock solutions (10 mM in Me$_2$SO), and the fluorescence of the $^{1}O_2$ detector molecules was recorded for a further 5 min. Afterward, the samples were transferred into a modified Pentz chamber (diameter, 24 mm) placed on the microscope stage (37°C) of an inverted microscope; a second sample treated the same way up to that point was kept in the dark and served as a control. To photoactivate the different rhodamine derivatives (TMRM $\lambda_{nm} = 535 \pm 17.5$ nm; Rho 123 $\lambda_{nm} = 488 \pm 10$ nm; TBRB $\lambda_{nm} = 535 \pm 17.5$ nm), the 100-watt mercury short arc photo optic lamp (HBO 100; Osram, Göttingen, Germany) of a digital fluorescence microscope (Axioskopt 155 TV; Zeiss, Oberkochen, Germany) equipped with the Attofluor imaging system (Atto Instruments, Rockville, MD) was used. To allow effective irradiation of the whole sample volume, the objective (×63 numerical aperture 1.25 Plan-Neofluar; Zeiss, Göttingen) of the microscope was removed, and the irradiation period was set at 10 min; except for this modification, the same conditions were used to photoactivate the dyes in the cell-free system as those used in experiments with cells (see below). After this treatment, the samples were again transferred to the cuvette of the spectrophotometer, and the fluorescence intensity of the $^{1}O_2$ detector molecules was compared with that of the untreated controls.

**Determination of the Effect of $^{1}O_2$ on NAD(P)H and Seoavenging of $^{1}O_2$ by NADPH—**In other experiments the rhodamine derivatives were photoactivated in the presence of NADPH (20 $\mu$M; stock solution 2.0 mM in HBSS), or the $^{1}O_2$ detector molecules were replaced by NADH or NADPH (20 $\mu$M), and MgCl$_2$ (5.0 mM) was added to the reaction buffer (HBSS, 25°C). NADPH fluorescence intensity was detacted spectrophotometrically $\lambda_{nm} = 340$ nm; $\lambda_{em} = 480$ nm) before and after photoactivation of the rhodamine derivatives (see above). To determine the amount of NADP$^+$ formed, dl-isocitric acid (4.0 mM) and NADP-linked isocitric dehydrogenase (0.21 units/mL) were added to the reaction buffer (HBSS, 37°C) subsequent to the irradiation procedures. The increase in fluorescence (TMRM $\lambda_{nm} = 340$ nm; $\lambda_{em} = 480$ nm) of the irradiated mixture indicating enzymatic re-reduction of NADP$^+$ to NADPH was recorded spectrophotometrically (11). Further experiments were performed in the presence of either superoxide dismutase (100 units/mL) or various HBSS/D$_2$O ratios. Alternatively, experiments were performed with HBSS that had been treated with chelex (15, 16) to minimize the transition metal contamination.

**Experiments with Cultured Hepatocytes**

**Determination of Cellular NAD(P)H Fluorescence and Photoactivation of Intracellular Rhodamines—**Experiments with hepatocytes were started 20–24 h after isolation of the cells. The glass coverslips with adherent cells were transferred to a modified Pentz chamber, and cells were washed twice with warm (37°C) HBSS. Hepatocytes were incubated with TMRM (0.5 $\mu$M), Rho 123 (0.5 or 10.0 $\mu$M), or TBRB (2.0 $\mu$M; stock solutions: 1.0 or 2.0 or 10.0 mM in Me$_2$SO) for 20 min in L-15 cell culture medium (37°C) and then washed three times with HBSS. Afterward, the hepatocytes thus loaded were incubated for another 15 min in dye-free L-15 medium; this incubation period has been found previously to strongly improve the selectivity of the mitochondrial loading with TMRM and Rho 123 (17, 18). The medium was then exchanged, and hepatocytes were covered again with complete L-15 cell culture medium (37°C) to maintain optimal nutrition of the cells during the experiments. The presence of culture medium did not add significant
background to the autofluorescence images at the setting used in this study.

A digital fluorescence microscope was used to measure cellular NAD(P)H fluorescence (see above). Measurements were performed at 37 °C using an excitation filter of 365 ± 12.5 nm and monitoring the emission at 450–490 nm using a bandpass filter. During the measurements cells were flushed with either 5% CO2/21% O2/74% N2 or 5% CO2/85% N2 (in air-tight chambers) to induce hypoxia. Cellular NAD(P)H fluorescence was recorded at 120-s intervals with an excitation period of 0.3 s and the intensity of the mercury lamp attenuated 99% using gray filters to minimize photochemical effects. Single cell fluorescence was determined by confining the regions of interest manually to individual cells. After establishing NAD(P)H baseline fluorescence of 450 nm integral rhodamine derivatives were photostimulated for 1–60 s at the wavelengths cited above, and NAD(P)H fluorescence measurements were continued without delaying the interval for data collection. Rho 123 was excited at either 488 ± 10 nm or 535 ± 17.5 nm as the excitation maximum of this dye has been reported to shift from 507 (19) to 514.5 nm within cells (20, 21).

In some experiments, cultured hepatocytes (in L-15 medium, 37 °C) were preincubated for 1 h with either 300 μM of the glutathione reductase inhibitor BCNU (22, 23) or an ethyl ester of reduced glutathione (4.0 mM) before fluorescence measurements were started (in the presence of these chemicals). All of the further chemicals were added from concentrated stock solutions during NAD(P)H fluorescence measurements at the respective concentrations detailed in the results. None of the chemicals/agents added in this study showed any detectable fluorescence under the conditions applied.

Determination of the Subcellular Distribution of the Different Rhodamine Derivatives—A laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany) equipped with both argon and helium/neon lasers was used to study the subcellular distribution of the different rhodamine derivatives and their effect on mitochondrial integrity after photoactivation. Subcellular distribution of TMRM (λexc. = 543 nm; λem. ≥ 560 nm), Rho 123 (λexc. = 488 nm; λem. ≥ 505 nm), and of TRB (λexc. = 543 nm; λem. ≥ 505 nm) was determined from the subcellular fluorescence of the probes at the respective wavelengths. The objective lens was a numerical aperture 1.4 oil-immersion Plan-Apochromat. The scanning parameters were as follows. The pinhole was set at 130 μm, producing confocal optical slices of less than 1.0 μm in thickness. Confocal images (scanning time 3.9 s, zoom factor 0.7 to 2.5) were collected at different intervals and with different parameters. The power of the helium/neon laser was set at 10%, and that of the argon laser was set at 0.1% to minimize photochemical damage.

Similar to the experiments based on digital fluorescence microscopy, after establishing the baseline fluorescence (5–10 min), the rhodamine derivatives were photoactivated for 5–60 s using the 100-watt mercury short arc photo optic lamp of the LSM 510 system. In some experiments, hepatocellular autofluorescence was excited at 488 nm with the power of 5 mW at 10%, collecting fluorescence emission through a 565-nm long pass filter. Image processing and evaluation were performed using the “physiology evaluation” software of the LSM 510 imaging system.

Recording of the Mitochondrial Membrane Potential and Detection of Onset of Mitochondrial Permeability Transition—Mitochondria were identified, and their functional integrity was confirmed by membrane potential-dependent staining with TMRM, using either digital fluorescence microscopy or laser scanning microscopy. Hepatocytes were incubated with TMRM (0.5 μM) as described above. When digital fluorescence microscopy was used, intracellular TMRM fluorescence (λexc. = 535 ± 17.5 nm; λem. ≥ 580 nm) was recorded at 120-s intervals with the intensity of the mercury lamp attenuated 40% using gray filters to minimize photochemical effects; using laser scanning microscopy, mitochondrial TMRM fluorescence (λexc. = 543 nm; λem. ≥ 560 nm) was scanned at different intervals as given above. In some experiments hepatocytes were incubated simultaneously with TMRM (0.5 μM) and Rho 123 (0.5 μM). In experiments with double-stained mitochondria, red fluorescence was attenuated 65% for the logging of fluorescence of Rho 123 (λexc. = 488 nm; λem. = 505–530 nm) were optically isolated in successive scans.

The onset of mitochondrial permeability transition (MPT) was detected according to the procedure described in Ref 24, with slight modifications. Briefly, cells were loaded simultaneously with calcine-AM (1.0 μM) and TMRM (0.5 μM) and scanned above for the loading with TMRM alone and then washed three times with HBSS and covered again with L-15 cell culture medium (for 15 min) that contained propidium iodide (5 μg/ml but not TMRM (100 nm) as originally reported (24). This incubation period and the following experiments were performed in the absence of any TMRM within the supernatant to make sure that the probe was located exclusively within the mitochondrial matrix of the cells (see above). Using laser scanning microscopy, red fluorescence of TMRM (λexc. = 543 nm; λem. ≥ 585 nm) and green fluorescence of calcine (λexc. = 488 nm; λem. = 505–530 nm) were recorded in successive scans. Loss in mitochondrial TMRM fluorescence and redistribution of cytosolic calcine fluorescence (into the mitochondrial matrix) were considered as qualitative measures of a decrease in mitochondrial membrane potential and an increased permeability of the inner mitochondrial membrane, respectively, known to indicate the onset of MPT as high conductance permeability transition pores are opened (24–27).

Cell Viability—The uptake of the vital dye propidium iodide (5 μg/ml) was directly determined either during or at the end of the experimental procedures to detect loss of cell viability. The red fluorescence of propidium iodide excited at 543 nm was collected through a 560-nm long pass filter when laser scanning microscopy was used; using digital fluorescence microscopy, propidium iodide was detected at λexc. = 535 ± 17.5 nm and λem. ≥ 590 nm.

Statistics—All experiments with hepatocytes were repeated at least three times using cells from different animals, and experiments in a cell-free system were repeated at least twice. Cellular microfluorographs and traces shown in the figures are representative of all the corresponding experiments performed. The results are expressed as means ± S.D. or S.E.

RESULTS

Oxidation of NAD(P)H by 1O2 in a Cell-free System—Before starting with the cellular measurements, we studied in a cell-free system whether photoactivation of the different rhodamine derivatives (TMRM, Rho 123, and TRB) intended to be used for intramitochondrial generation of 1O2 did in fact generate sufficient 1O2. Additionally, we tested whether NAD(P)H, when reacting with this ROS, underwent significant oxidation to enzymatically active NAD(P)− as reported previously (8, 10, 11).

When the known (20, 21, 28) 1O2 generators TRB and Rho 123 (10 μM) were photoactivated, the fluorescence of both 1O2 detector molecules, 1,3-diphenylisobenzofuran (5 μM) and 9,10-diphenylanthracene (5 μM), was markedly quenched (data not shown). Very surprisingly, TMRM, for which 1O2 generation has not yet been quantified, was even more effective than Rho 123, presumably because of the small O2 quantum yield of the latter (20, 28). Using TMRM, the fluorescence of 1,3-diphenylisobenzofuran was quenched more strongly (54.5 ± 3.0%) than that of 9,10-diphenylanthracene (14.1 ± 1.0%), in line with their rate constants for single electron transfer to 1O2 (20, 28). When the known 1O2 generators, no quenching of the detector molecules became apparent. To confirm the conclusion that TMRM is highly effective in generating 1O2, the fluorescence quenching of 1,3-diphenylisobenzofuran and 9,10-diphenylanthracene was performed in the presence of D2O, which is known to increase the lifetime and thus the steady state level of 1O2 (30, 31). In line with our view, the fluorescence quenching of the 1O2 detector molecules was enhanced 2–5-fold in the presence of D2O (data not shown). In summary, the data presented here clearly demonstrated that photoactivation of all rhodamines resulted in the generation of 1O2.

When the 1O2 detector molecules were replaced by NADPH (20 μM), its fluorescence significantly decreased after photoactivation of the selected rhodamines (each 10 μM; see Table 1). Similar to the experiments performed with 1,3-diphenylisobenzofuran and 9,10-diphenylanthracene, NADPH fluorescence decreased more strongly (50–80%) in the presence of D2O (data not shown). Again, the strongest decrease in fluorescence was observed with TRB as a 1O2 generator. The decrease in NADPH fluorescence was found to be independent of the pres-
ence of either superoxide dismutase or contaminant transition metal ions (Table I). The latter possibility was excluded by treating the reaction solution with chelax. Thus, the fluorescence of NADPH was neither affected by O$_2$, which may arise during 1O$_2$ generation (see Reactions 7 and 8), nor by OH, resulting from transition metal-dependent Fenton reactions. In the absence of the 1O$_2$ generators the NAD(P)H fluorescence hardly decreased (2%/h) via autoxidation (data not shown).

To verify that NADPH was actually oxidized to its enzymatically active non-fluorescent form (NADP$^+$), we tested whether reduction was possible, using the procedure described by Bodaness (11), with slight modifications. When di-isocitric acid and NADP$^+$-linked isocitric dehydrogenase were added to the incubation buffer after photoactivation of the selected rhodamine derivatives, NADPH fluorescence was largely restored within minutes (Table I). These results strongly indicated that NAD(P)H was oxidized by 1O$_2$ via Reactions 7 and 8 as suggested by Peters and Rodgers (7, 8).

If NAD(P)H were a primary target of 1O$_2$, this would prevent, or partially prevent, the oxidation of other molecules targeted by 1O$_2$. In fact, when 1O$_2$ was generated by photoactivation of TMRM, NADPH (20.0 μM), but not NADP$^+$, significantly (22.4 ± 3.0%) and almost completely (98.6 ± 0.5%) diminished the decrease in fluorescence of both 1,3-diphenylisobenzofuran and 9,10-diphenylanthracene (5.0 μM), respectively. In summary, in the cell-free system, 1O$_2$ as generated by photoactivation of Rho 123, TMRM, or TBRB, respectively, mainly oxidized NAD(P)H to enzymatically active NADP$^+$.

**Oxidation of NAD(P)/H by 1O$_2$ in Mitochondria of Hepatocytes**—When primary cultured hepatocytes were loaded with the rhodamine derivatives, the intracellular fluorescence of TMRM ($\lambda_{\text{exc}}$ = 543 nm, $\lambda_{\text{em}}$ = 560 nm; see below) and Rho 123 ($\lambda_{\text{exc}}$ = 488 nm, $\lambda_{\text{em}}$ = 505 nm), detected using laser scanning microscopy, was detectable entirely within intact mitochondria, whereas TBRB ($\lambda_{\text{exc}}$ = 543 nm, $\lambda_{\text{em}}$ = 560 nm), which was hardly taken up by the cells even at higher concentrations (2.0 μM), was mainly located within the lysosomes/endosomes and the cytosol of the cells (data not shown). Under these conditions, none of the rhodamine derivatives affected either cell viability (as detected by propidium iodide uptake) or mitochondrial functionality as assessed by recording the mitochondrial membrane potential.

Hepatocellular autooxidation as excited at $\lambda_{\text{exc}}$ = 365 ± 12.5 nm and detected at $\lambda_{\text{em}}$ = 450–490 nm using digital fluorescence microscopy has been considered to be almost exclusively represented by the fluorescence of NADP(H) (32, 33) and was found to be well co-localized with TMRM and Rho 123 here (data not shown). These results are in line with previous studies where reduced pyridine nucleotides, as well as TMRM and Rho 123, were found to be almost exclusively located within the mitochondria of cultured rat hepatocytes (17, 18, 24–26). As the intramitochondrial concentration of NADH has been reported to be significantly smaller than that of NADPH in hepatocytes (12), we considered the dominant fluorophore under investigation here to be NADPH. The assumption that the hepatocellular autooxidation at these settings was largely represented by mitochondrial NAD(P)H and not by other cellular fluorophores was further supported by the observation that the addition of KCN (5 μM), an inhibitor of the respiratory chain, markedly (14.5 ± 2.5%) and rapidly increased cellular auto fluorescence, which, on the other hand, was decreased by 37.5 ± 5.3% when oxidative phosphorylation was uncoupled from respiration with carbonyl cyanide m-chlorophenylhydrazone (10 μM; data not shown). These changes in cellular auto fluorescence exhibited the same tendencies as were observed in other studies with cultured hepatocytes (26).

When hepatocytes loaded with 0.5 μM TMRM or Rho 123 were continuously irradiated (TMRM $\lambda_{\text{exc}}$ = 535 ± 17.5 nm; Rho 123 $\lambda_{\text{exc}}$ = 488 ± 10 or 535 ± 17.5 nm) for 60 s using the 100-watt mercury short arc photo optic lamp of the inverted microscope, a rapid decrease in NADP(H) fluorescence depending on the time of photoactivation was observed in TMRM-loaded cells, whereas only a slight decrease in NADP(H) fluorescence was evident in cells loaded with Rho 123 (Fig 1A). However, when the cells were loaded with 10.0 μM Rho 123, i.e. with a concentration as previously used in studies of photodynamic therapy with different types of tumor cells and animal models (20, 21, 34–36), photoactivation of intramitochondrial Rho 123 provided essentially the same effect on cellular NADP(H) fluorescence as TMRM. In cells loaded with TBRB (2.0 μM), no decrease in fluorescence showed through even after prolonged (60-s) photoactivation ($\lambda_{\text{exc}}$ = 535 ± 17.5 nm) of the dye. This result is in apparent contrast to the strong oxidation of NADP(H) after photoactivation of TBRB in the cell-free system (Table I), but it is a good reflection of the fact that TBRB is not co-localized with the mitochondrial NADP(H) pool. In line with the stronger oxidation of NADP(H) after photoactivation of TMRM in the cell-free system, TMRM also decreased NADP(H) fluorescence in mitochondria noticeably more strongly than Rho 123 under comparable conditions (Fig 1A). In controls, the intensity of mitochondrial NADP(H) fluorescence was not affected by mitochondrial loading with either TMRM or Rho 123, and in hepatocytes, which were not loaded with the dyes, no decrease in NADP(H) fluorescence was observed after photoactivation. In contrast to the experiments performed in the cell-free system, the decrease in cellular NADP(H) fluorescence was not intensified when intramitochondrial TMRM was excited in the presence of D$_2$O-enriched

![Table I](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Rhodamine derivative</th>
<th>Additions/treatments</th>
<th>NADPH oxidized</th>
<th>Recovery of NADPH fluorescence</th>
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<td>% of control</td>
<td>%</td>
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<td>Rhodamine 123</td>
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<td>11.2 ± 1.4</td>
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<td>TMRM</td>
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<td>TMRM</td>
<td>Superoxide dismutase (100 units/ml)</td>
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Mitochondrial NAD(P)H and $^{1}$O$_2$

L-15 medium (data not shown). As photoactivation of TMRM most effectively decreased mitochondrial NAD(P)H fluorescence intensity (29.6 ± 8.2% after 10 s of irradiation; Rho 123: 30.2 ± 5.2% after 60 s of irradiation), most of the following experiments with hepatocytes were performed using TMRM.

As known from studies of photodynamic therapy (PDT), $^{1}$O$_2$ can lead to a marked oxidation of proteins and membrane lipids, resulting in leakage of small biomolecules from the damaged cells/cellular compartments. In line with this, photoactivation of TMRM has been reported to result in generation of free radicals (37, 38) leading to a gradual and reversible decline in membrane potential of isolated individual rat heart mitochondria because of repetitive opening and closing of the mitochondrial transition pore (37). Therefore, to exclude the possibility that the observed decrease in NAD(P)H fluorescence was a result of mitochondrial NAD(P)H leakage, we studied the capability of rhodamines of inducing MPT. When intramitochondrial TMRM or Rho 123 (after loading with 10 μM), mitochondrial NAD(P)H fluorescence (λ$_{ex}$ = 365 ± 12.5 nm; λ$_{em}$ = 450–490 nm) (A), as well as mitochondrial TMRM and nuclear propidium iodide fluorescence (λ$_{ex}$ = 535 ± 17.5 nm; λ$_{em}$ ≥ 590 nm) (B), were recorded at 120-s intervals using digital fluorescence microscopy and are given in arbitrary units (a.u.). After establishing the baseline fluorescence (10-min), intramitochondrial TMRM was photoactivated (λ$_{ex}$ = 535 ± 17.5 nm) for 20 s (arrow), and fluorescence measurements were continued. The mitochondrial TMRM fluorescence was used as a measure for the mitochondrial membrane potential; the uptake of the vital dye propidium iodide was determined to detect loss of cell viability. Mitochondrial NAD(P)H fluorescence (A) is shown for 33 single cells as not evident from the figure. Each trace shown in B is the average of 15–30 cells. Data are representative of at least three experiments using hepatocytes from different animals; compare with Figs. 1B, 3, and 4.

FIG. 1. Effect of short term photoactivation of different rhodamine derivatives on mitochondrial NAD(P)H fluorescence of cultured hepatocytes. Cells were cultured on glass coverslips and loaded with Rho 123 (0.5 or 10.0 μM), TMRM (0.5 μM), or TBRB (2.0 μM) for 20 min in L-15 cell culture medium (37 °C). Hepatocytes were then washed three times with HBSS and incubated for another 15 min in dye-free L-15 medium. Cellular NAD(P)H fluorescence was recorded at 120-s intervals using digital fluorescence microscopy (λ$_{ex}$ = 365 ± 12.5 nm; λ$_{em}$ = 450–490 nm) and is given in arbitrary units (a.u.). After establishing the baseline fluorescence (10 min), the rhodamine derivatives were photoactivated (TMRM λ$_{ex}$ = 535 ± 17.5 nm; Rho 123 λ$_{ex}$ = 488 ± 10 nm; TBRB λ$_{ex}$ = 535 ± 17.5 nm; open arrows) for the periods indicated, and NAD(P)H fluorescence measurements were continued. Rho 123 was additionally irradiated at λ$_{ex}$ = 535 ± 17.5 nm (filled arrow). Each trace shown in A is the average of 15–25 cells. Data are representative of at least three experiments using hepatocytes from different animals. In B the effect of TMRM irradiation (10-s) on mitochondrial NAD(P)H fluorescence is shown for 28 single cells.

FIG. 2. Effect of prolonged photoactivation of mitochondrial TMRM on mitochondrial NAD(P)H fluorescence, mitochondrial membrane potential, and viability of cultured hepatocytes. Hepatocytes were cultured on glass coverslips and loaded with TMRM (0.5 μM) for 20 min in L-15 cell culture medium (37 °C). Cells were then washed three times with HBSS and incubated for another 15 min in dye-free L-15 medium that contained propidium iodide (5 μg/ml). Cellular NAD(P)H fluorescence (λ$_{ex}$ = 365 ± 12.5 nm; λ$_{em}$ = 450–490 nm) (A), as well as mitochondrial TMRM and nuclear propidium iodide fluorescence (λ$_{ex}$ = 535 ± 17.5 nm; λ$_{em}$ ≥ 590 nm) (B), were recorded at 120-s intervals using digital fluorescence microscopy and are given in arbitrary units (a.u.). After establishing the baseline fluorescence (10-min), intramitochondrial TMRM was photoactivated (λ$_{ex}$ = 535 ± 17.5 nm) for 20 s (arrow), and fluorescence measurements were continued. The mitochondrial TMRM fluorescence was used as a measure for the mitochondrial membrane potential; the uptake of the vital dye propidium iodide was determined to detect loss of cell viability. Mitochondrial NAD(P)H fluorescence (A) is shown for 33 single cells as not evident from the figure. Each trace shown in B is the average of 15–30 cells. Data are representative of at least three experiments using hepatocytes from different animals; compare with Figs. 1B, 3, and 4.
Mitochondrial NAD(P)H and $^{1}{O}_2$

TMRM (0.5 μM), no cytotoxic effects became apparent in experiments with Rho 123 (0.5 μM) and calcein-AM (1.0 μM) for 20 min in L-15 cell culture medium (37°C). Cells were then washed three times with HBSS and incubated for another 15 min in L-15 medium that contained propidium iodide (5 μg/ml). The intracellular fluorescence of TMRM and propidium iodide ($\lambda_{\text{exc}} = 543\text{ nm}; \lambda_{\text{em}} = 585\text{ nm}$) (B and D), as well as of calcein ($\lambda_{\text{exc}} = 488\text{ nm}; \lambda_{\text{em}} = 505-530\text{ nm}$) (A and C) was imaged using laser scanning microscopy. After establishing the baseline fluorescence, images were collected before (A and B) and 4 min after (C and D) photoactivation of TMRM for 10 s. The mitochondrial TMRM fluorescence was used as a measure for the mitochondrial membrane potential and cytosolic calcein to assess the permeability of the inner mitochondrial membrane. The uptake of the vital dye propidium iodide was determined to detect loss of cell viability. Note that mitochondria continued to exclude calcein after photoactivation, and TMRM fluorescence was only slightly decreased (C and D). Bar indicates 10 μm; compare with Figs. 1B, 2, and 4.

A major protective effect of NADPH is associated with its ability to inhibit MPT (data not shown) (26). The inability of trifluoperazine/fructose to inhibit the initial decrease in NAD(P)H fluorescence further suggests that ROS other than $^{1}{O}_2$, large amounts of which may be generated during the onset of MPT leading to NAD(P)H oxidation (26, 39), were not responsible for the decrease in NAD(P)H fluorescence observed here. In summary, the decrease in mitochondrial NAD(P)H fluorescence upon short term photoactivation of intramitochondrial TMRM and Rho 123 did not result from mitochondrial damage but from NAD(P)H oxidation. In contrast to the short term photoactivation, prolonged (~15 s) photoactivation of TMRM caused a rapid decrease in membrane potential, onset of MPT within minutes, and subsequently apoptotic cell death (Fig. 4).
Mitochondrial NAD(P)H and \(^1\text{O}_2\)

**Fig. 5.** Effect of BCNU on the photoactivation-induced decrease in mitochondrial NAD(P)H fluorescence of cultured hepatocytes. Hepatocytes were cultured on glass coverslips and incubated for 1 h in L-15 cell culture medium (37 °C) with or without the glutathione reductase inhibitor BCNU (300 μM; Control). Cells were then loaded with TMRM (0.5 μM), and cellular NAD(P)H fluorescence was recorded in the presence of BCNU at 120-s intervals as described in the legend for Fig. 1. After establishing the baseline fluorescence (10-min), either intramitochondrial TMRM was photoactivated (λ_{exc} = 535 ± 17.5 nm) for 10 s (arrows) or t-BuOOH (100 μM), known to lead to NAD(P)H oxidation via the glutathione peroxidase/reductase system, was added to the supernatant, and fluorescence measurements were continued. Note that BCNU completely inhibited the effect of t-BuOOH but not that of TMRM photoactivation on NAD(P)H fluorescence. Each trace shown is the average of 20–30 cells and is representative of at least three experiments using hepatocytes from different animals.

To confirm that the mitochondrial NAD(P)H was oxidized by \(^1\text{O}_2\) and not directly by the photoactivated rhodamine derivatives, we studied the influence of the environmental pO\(_2\) on the decrease in intramitochondrial NAD(P)H oxidation. When the cells were flushed with 95% N\(_2\)/5% CO\(_2\) for 20 min, hypoxia, as indicated by a slight increase in NAD(P)H fluorescence, completely prevented the decrease in NAD(P)H fluorescence after photoactivation of TMRM (Fig. 6). This strongly suggested that NAD(P)H was oxidized under normoxia by (most likely) \(^1\text{O}_2\), the main ROS generated in photochemical processes, rather than by products (radicals, radical ions) of the photochemically activated process or any photochemical activation of TMRM itself in a type-1 photoreaction. As expected, hypoxic cells were found to resist photoactivation of TMRM. In contrast to normoxic conditions no loss in cell viability was observed even after prolonged photoactivation (data not shown). Rather than completely preventing NAD(P)H oxidation, hypoxia only partly prevented the decrease in intramitochondrial TMRM fluorescence (Fig. 6) (see above); it follows that this probably resulted from both uncoupled, i.e. \(^1\text{O}_2\)-independent, and coupled photobleaching of the dye. The fact that the decrease in TMRM fluorescence showed a relatively weak dependence on the environmental pO\(_2\) further suggested that the indicator molecule itself did not react with \(^1\text{O}_2\) very well, which possibly explains its high oxidizing effect on NAD(P)H.

**DISCUSSION**

NAD(P)H, the Primary and Restorable Target of \(^1\text{O}_2\) in Mitochondria of Viable Cells—During the past 25 years, a good deal of thermodynamically and experimentally based data...
have been reported concerning the rapid reaction of $^1\text{O}_2$ with NAD(P)H (see Reactions 7 and 8). However, the resulting consequences for intracellular conditions have hardly been considered. The rate constants for single electron transfer ($k_e$) from NADH or NADPH to $^1\text{O}_2$ are significantly higher ($4.3 \times 10^6$ and $8.4 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) than the $k_e$ values for the well known directly operating antioxidants ascorbate ($3.8 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$), glutathione ($2.4 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$), and a-tocopherol ($5.0 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$) (8). When the respective intramitochondrial concentrations of these biomolecules are taken into consideration for hepatocytes (NADH: 4.0 mM, NADPH: 6.0 mM, glutathione: 10.0 mM (43); ascorbate: 0.1–0.5 mM (44); a-tocopherol: 0.05–0.228 nmol/mg protein (45, 46); $\sim 10–450 \mu$M)), NADH can be expected, and NADPH even more so, to be the primary targets of $^1\text{O}_2$ within the mitochondrial matrix of this cell; the concentrations given were calculated in part from the mitochondrial content of each compound, assuming that about $7.2 \times 10^6$ rat liver mitochondria contain 1 mg of protein, and the volume of a single mitochondrion is 0.71 $\mu$m$^3$ (12).

In line with this assumption, selective generation of moderate amounts of $^1\text{O}_2$ within the mitochondrial matrix space of cultured hepatocytes by local photoactivation of TMRM and Rho 123 led to a rapid oxidation of mitochondrial NAD(P)H followed by obviously enzymatic re-reduction of NAD(P)H (Fig. 1, A and B). Prolonged photoactivation of TMRM further increased NAD(P)H oxidation and resulted in a rapid decrease in mitochondrial membrane potential (37, 38), the onset of MPT, loss of mitochondrial NAD(P)H, and finally apoptotic cell death (see Figs. 2 and 4). In controls, NAD(P)H oxidation in the cell-free system was found to be independent of $^1\text{O}_2$ and contaminating heavy metal ions (Table I); this is in line with previous studies that reported that $^1\text{O}_2$ quenching other than contaminating heavy metal ions (Table I); this is in line with these considerations, after $^1\text{O}_2$ generation the cells were unable to fully restore their NAD(P)H levels (Fig. 1), most likely because the very high concentration of NAD(P)H and the low pO$_2$ present within this compartment enhances the yield of (NAD(P))$_2$.

The role of NAD(P)H as a Directly Operating Antioxidant—In the cell-free system NADPH significantly diminished the reaction of $^1\text{O}_2$ with both 1,3-diphenylisobenzofuran and 9,10-di-phenylanthracene. This is in line with a previous study where NADPH protected NADP-linked isocitric dehydrogenase against photochemically generated $^1\text{O}_2$, thus allowing the enzyme to regenerate the NADPH responsible for its own protection (11). The results obtained from experiments in cell-free systems clearly indicate that NAD(P)H has the potential to directly protect targets against attack by $^1\text{O}_2$ and would therefore be expected to play a role as a directly operating antioxidant in living cells, as well.

It also follows that the ability of a mitochondrion to resist $^1\text{O}_2$ ought to depend on its NAD(P)H concentration, as well as on its capability to re-reduce oxidized nicotinamides. However, it is very problematic to experimentally manipulate the well regulated mitochondrial NAD(P)H levels in living cells without affecting the basic cell metabolism, metabolic compartmentation, or cell viability. Because of this experimental limitation it is not possible to differentiate unequivocally between the indirect and the direct antioxidative and protective effect of reduced pyridine nucleotides. For instance, in preliminary studies, it was only possible to slightly (5.2–7.8%) increase the mitochondrial NAD(P)H concentration using $\beta$-hydroxybutyric acid (10 mM), whereas hardly any decrease in NAD(P)H fluorescence was detectable when acetoacetic acid (10 mM) was added to the supernatant. Consequently, none of these substrates provided either significant protection of cultured hepatocytes or diminished mitochondrial integrity/cell viability when $^1\text{O}_2$ was generated during photoactivation of intracellular TMRM. Despite the limitation that any protective effect offered by mitochondrial NAD(P)H against $^1\text{O}_2$ is very difficult to demonstrate experimentally, the fact that mitochondrial NAD(P)H is the primary and restorable target of $^1\text{O}_2$ (see above) leaves almost no doubt that NAD(P)H acts as a directly operating antioxidant in this compartment. As a directly operating antioxidant, NAD(P)H is likely to act collectively and on a concerted basis with the cellular enzymes superoxide dismutase, catalase, and glutathione peroxidase, which can degrade the O$_2^-$ generated during the $^1\text{O}_2$-mediated NAD(P)H oxidation (see Reactions 7 and 8).

Mitochondrial NAD(P)H Depletion as a Decisive Trigger of Apoptotic Cell Death—Besides being a major site of intracellular generation of reactive oxygen species (O$_2^-$, H$_2$O$_2$) (50), mitochondria are also very vulnerable to attacks by these species (50, 51). Accordingly, mitochondrial photosensitizers have been reported to induce apoptosis very rapidly (42, 49, 52, 53). However, the initial events leading to the several apoptotic cascades and pathways involved in cell death are often unknown (54) and have almost invariably been attributed to oxidation of proteins and membrane lipids, leading to disruption of the inner mitochondrial membrane (28, 38, 42, 49, 52, 55–58). In this context, it appears doubtful whether, among the photochemical drugs applicable for PDT in clinical trials, real mitochondrial photosensitizers (like TMRM and Rho 123) actually exist. Irradiation of cultured hepatocytes loaded with the photochemical drugs photofrin, Al(III) phthalocyanine chloride tetrarsulfonyl acid (AlPcS4), meso-tetra(4-sulfonatophenyl) porphine dihydrochloride (TPPS4), or 5-aminolevulinic acid-(5-ALA-) generated protoporphyrin IX, in no case led to a direct oxidation of mitochondrial NAD(P)H, most likely because the required co-localization was not given. The observed decreases in NAD(P)H fluorescence always resulted from loss in cel
viability/mitochondrial integrity (most likely mediated by extramitochondrial $^{1}\text{O}_2$ generation) and were thus in contrast to the effects of TMRM and Rho 123 described here.

In the present study, TMRM was found to rapidly induce onset of MPT in cultured rat hepatocytes followed by apoptotic cell death in less than 30 min when the photoactivation periods of the probe were prolonged (see Figs. 2 and 4). Given the rapidity of apoptotic cell death observed here and in other studies (42, 49, 52, 53), it seems rather unlikely that any intermediate steps of biosynthesis and signal transduction pathways were required (49).

In this context, the finding that NAD(P)H is a primary target of $^{1}\text{O}_2$ in living cells is likely to be of major importance for the general understanding of the photochemotherapeutic potential of photosensitizing molecules. When small amounts of $^{1}\text{O}_2$ are generated, NAD(P)H should act as a directly operating antioxidant thereby terminating the attacking $^{1}\text{O}_2$ molecules (see above). However, when the amount of $^{1}\text{O}_2$ generated exceeds the capacity of this antioxidative system, excess oxidation of NAD(P)H probably actually becomes a trigger for cell damage (see Figs. 2 and 4). Large amounts of the pro-oxidant $^{1}\text{O}_2$ may, for example, promote formation of hydroxyl radicals, are generated within the mitochondrial matrix space (see Reactions 7 and 8), and under these conditions of oxidative stress both the direct antioxidative function of NAD(P)H and the whole antioxidative network will be impaired, the latter because of the central role of NADPH as an indirectly operating antioxidant in the regeneration of others that operate directly (1, 2). Thus, NAD(P)H-dependent enzymatic reductions will be abruptly terminated. Consequently, mitochondrial energy status/ATP levels and membrane potential will be disturbed by deprivation of electrons from metabolic processes (7, 8, 10, 11). Such an imbalance in mitochondrial energy and redox status is known to be involved in modulating the mitochondrial permeability transition pore, thus promoting further ROS generation and onset of MPT, a well known trigger of apoptosis (26, 39, 59). This presumption is supported by the fact that after photoactivation of low concentrations of Rho 123 or TBRB, which had no significant effect on NAD(P)H redox state, almost no toxic effects became apparent within 60 min. The lack of a NAD(P)H oxidizing effect of both dyes under these conditions is likely to reflect either the low $^{1}\text{O}_2$ quantum yield of Rho 123 (20, 28) or the weak cellular uptake and predominantly cytosolic/lysosomal localization of TBRB. Although the photochemical effects observed here will inevitably differ with cell type, for instance high phototoxicity of Rho 123 and especially TBRB has been convincingly demonstrated for MGH-U1 bladder carcinoma cells (20, 21), the data presented here should have implications for phototherapy. The effective generation of $^{1}\text{O}_2$ in close proximity to the main pool of cellular NAD(P)H by using a photosensitizer with a high $^{1}\text{O}_2$ quantum yield should improve the photochemotherapeutic potential of cancer treatment and diminish side effects because of the effective destruction of the antioxidative network of the targeted cells.

In this context, recording of NAD(P)H fluorescence intensity was found to be a reasonable dosimetric measure of cell damage induced by TMRM here and during PDT (33). Especially mitochondrial photosensitisers should be advantageous for PDT as they (i) are effective inducers of apoptosis, which, in contrast to necrosis is normally not accompanied with an inflammatory response, and (ii) do not lead to sublethal nuclear DNA damages and thus genome aberrations, a risk accompanied with the application of nuclear photosensitizers.

TMRM is a potentiometric fluorescent probe that is widely used for several tasks in cell biology and physiology. It serves as a marker for identifying mitochondria (17) and for recording their membrane potential (37, 38, 60), for example, and is used in an assay for detecting onset of MPT (24–27). In the light of the present results, however, one should keep in mind that TMRM, even when excited only for a short period, most likely affects cellular NAD(P)H homeostasis and consequently weakens the antioxidative capacity of the cells. This will be of relevance especially when TMRM, combined with the cytosolic marker calcein, is used to study the MPT-inducing potential of ROS and reactive nitrogen species (24–27).

**CONCLUSIONS**

The results presented, obtained from experiments both in the cell-free system and in primary cultured rat hepatocytes, strongly suggest that NAD(P)H is the primary and enzymatically restorable target of $^{1}\text{O}_2$ within mitochondria of viable cells. It follows that mitochondrial NAD(P)H is likely to act as a directly operating antioxidant and thus provides protection when $^{1}\text{O}_2$ is generated within this organelle. However, when the amount of $^{1}\text{O}_2$ generated exceeds the capacity of the NAD(P)H-regenerating systems, one-electron oxidation of NAD(P)H by $^{1}\text{O}_2$ might even be an as-yet unnoticed pathogenetic event responsible for effects (including photodynamic ones) like inhibition of respiration and electron transport, disruption of the mitochondrial electrochemical gradient, oxidation of NAD(P)H-dependent compounds in mitochondria, onset of MPT, and finally apoptosis.

Having regard to the susceptibility of NAD(P)H to one-electron oxidations when reacting with oxygen-centered species (1) and to the ubiquitous distribution of NAD(P)H within the cell, it is most likely that both roles of NAD(P)H, i.e. as a directly operating antioxidant and as a decisive trigger of cell injury, are also of relevance in connection with other ROS. One of the most likely candidates is the carbonate radical (CO$_3^{•−}$), donated from peroxynitrite.

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