Direct Measurement of Lipid Peroxidation in Submitochondrial Particles†

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ABSTRACT: The susceptibility of the polyunsaturated fatty acid parinaric acid (*cis*-PnA) to peroxidative damage with concomitant loss of its fluorescent character can be used to detect lipid peroxidation in a direct and sensitive way. The procedure, originally developed to measure peroxidation in lipid vesicles and erythrocyte membranes, has been adapted for the study of submitochondrial particles. Optimal conditions for the concentrations of *cis*-PnA (0.8 mol %), mitochondrial membrane (100 μM membrane phospholipid), and the radical generating system (50 μM NADH and 10 μM:1 mM Fe(III)-ADP) were established. In the absence of peroxidation inducing compounds, a stable fluorescent signal can be detected. Upon addition of NAD(P)H and ADP-Fe(III), lipid peroxidation starts, and the observed fluorescence decrease is a measure of peroxidation. Both NADH and NADPH were able to induce lipid peroxidation in submitochondrial particles in the presence of an iron chelate. The use of NADH resulted in higher rates of peroxidation compared with NADPH at the same concentration. Whereas the rate of NADH-induced lipid peroxidation was maximal at very low NADH concentrations (2.5 μM) and decreased when the concentration became higher, the NADPH-induced lipid peroxidation reaches saturation at 100 μM. NADH-induced lipid peroxidation in submitochondrial particles from different rat tissues (heart, skeletal muscle, and liver) resulted in a clear difference in peroxidation rates. The highest rates were observed in heart submitochondrial particles, while the lowest rates were obtained in submitochondrial particles derived from liver. Skeletal muscle submitochondrial particles showed intermediate rates of lipid peroxidation. The differences in peroxidation rates showed a clear correlation with the activity of NADH:cytochrome *c* oxidoreductase, and a relation between the electron flow through the respiratory chain and the peroxidation rate is demonstrated. All the results obtained with the PnA assay were compared with peroxidation rates measured via oxygen consumption. Both methods gave identical results, demonstrating that the PnA assay is a reliable method to measure lipid peroxidation in submitochondrial particles.

Unsaturated lipids in biological membranes are susceptible to peroxidation by molecular oxygen or reactive oxygen species (free radicals). This lipid peroxidation may cause impairment of membrane functioning, decreased fluidity, inactivation of membrane-bound receptors and enzymes, and increased nonspecific permeability to ions such as Ca^{2+} (Rice-Evans, 1994). Consequently, lipid peroxidation may have impact on biological systems and may lead to disease or severe progression of a disease state (Halliwell, 1992; Halliwell & Gutteridge, 1984) and typical aging characteristics (Sohal & Dubey, 1994; Nohl, 1986). In mitochondria, superoxide radicals, which can be converted to hydrogen peroxide by superoxide dismutase (SOD),[1] are produced as a side product of oxidative phosphorylation (Nohl, 1986; Takeshige et al., 1980; Takeshige & Minakami, 1979). Especially the mitochondrial inner membrane is at risk for lipid peroxidation-induced injuries, because mitochondria utilize oxygen at a high rate and the inner membrane has a large content of polyunsaturated fatty acids, together with peroxidation catalysts (e.g., heme and non-heme iron and copper).

It has been reported that both NADH and NADPH support enzymatically induced lipid peroxidation in submitochondrial particles (SMP) in the presence of an iron chelate (Glinn et al., 1991; Szabados et al., 1987; Takayanagi et al., 1980). Proposed sites of radical production in this system include the NADH dehydrogenase (complex I) and CoQ:cytochrome *c* reductase (complex III) components of the respiratory chain. Also, for succinate dehydrogenase (complex II), indications are found for substrate-induced radical production in the presence of the complex II inhibitor thenoyltrifluoracetone (TTFA) (Eto et al., 1992; Glinn et al., 1991). Glinn et al. (1991) showed that, with succinate as a substrate, an iron chelate is not essential to induce lipid peroxidation.

For the detection of lipid peroxidation, many different methods are available and have been used during the last two decades (Gutteridge & Halliwell, 1990). The most commonly used method is detection of thiobarbituric acid-reactive substances (TBARS), which are degradation products of lipid peroxidation. Other methods include measurement of oxygen consumption during the propagation steps of lipid peroxidation as well as measurement of diene

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[1] Abbreviations: BHT = butylated hydroxytoluene, CoQ = coenzyme Q, EDTA = ethylenediaminetetraacetic acid, MDA = malondialdehyde, SOD = superoxide dismutase, TBARS = thiobarbituric acid-reactive substances, TTFA = thenoyltrifluoracetone, SMP = submitochondrial particles, *cis*-PnA = *cis*-parinaric acid.

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conjugation using high performance liquid chromatography (HPLC) and gas liquid chromatography (GLC). Most of these approaches suffer from serious methodological drawbacks. For instance, the method measuring TBARS bears the problem that most of the malondialdehyde (MDA) measured is not formed during the peroxidation but, especially in combination with metal catalysts, is formed during breakdown of lipid peroxides in the acid-heating process. Also, the amount and type of acid added in the TBARS assay may influence the TBA-reactive material formed (Gutteridge & Halliwell, 1990). The measurement of oxygen consumption has the advantage over methods that are based on sample destruction in that it enables continuous registration of lipid peroxidation, but has the disadvantage that it is only indirectly related to lipid peroxidation.

Recently, a method has been developed to measure lipid peroxidation, which uses the fluorescent probe *cis*-parinaric acid (*cis*-PnA) (van den Berg et al., 1988; Kuypers et al., 1987). This method can be a good alternative for some other methods because the *cis*-PnA assay appears to be very sensitive and lipid peroxidation can be followed continuously without destruction of the sample. *cis*-PnA is a polyunsaturated fatty acid, and therefore, data obtained correlate directly with the fate of fatty acids in the membrane.

In the present study, the method using *cis*-PnA is modified to follow lipid peroxidation in SMP. In principle, *cis*-PnA, dissolved in ethanol, is mixed with SMP to incorporate spontaneously into the mitochondrial membrane to yield a stable fluorescent signal. Subsequently, lipid peroxidation is induced by addition of NADH or NADPH in the presence of rotenone and an Fe(III) chelate.

The results obtained with our assay confirm previous reports on peroxidation of lipids in SMP. The observation that our method yields identical results and is also comparable with peroxidation rates detected by measurement of oxygen consumption shows that the PnA assay is a reliable method to measure lipid peroxidation in SMP.

MATERIALS AND METHODS

Chemicals. *cis*-Parinaric acid (*cis*-PnA) was obtained from Molecular Probes (Junction City, OR) and stored in ethanol under nitrogen at −20 °C. NADH, NADPH, ADP, and cytochrome *c* were purchased from Boehringer Mannheim (Mannheim, Germany). Rotenone was obtained from Aldrich (Brussels, Belgium) and TTFA from Sigma (St. Louis, MO). Heparin was obtained from Organon Teknika (Boxtel, The Netherlands) and $FeCl_3 \cdot 6H_2O$ from Merck (Darmstadt, FRG).

Isolation of Mitochondria and Submitochondrial Particles (SMP). All procedures were carried out at 0−4 °C. Bovine heart mitochondria were isolated according to Blair (1967). Rat skeletal muscle mitochondria were isolated from the Musculus gastrocnemius. After removal of fat and connective tissue, the muscle was chopped to pieces with a pair of scissors. The muscle tissue was suspended in SETH buffer (0.25 M sucrose/10 mM Tris-HCl, pH 7.4/2 mM EDTA/50 units/mL heparin) (10% w/v) and homogenized (ten strokes (manual)) using a Teflon glass Potter−Elvehjem homogenizer. The homogenate was centrifuged at $600g_{max}$ for 10 min. The supernatant was again centrifuged at $14\,000g_{max}$ for 20 min (Bookelman, 1978). The final pellet containing the mitochondria was washed twice with 0.25 M sucrose adjusted to pH 7.4 and resuspended in the same buffer. Rat heart mitochondria were isolated according to the same procedure. For the isolation of rat liver mitochondria, the liver of an overnight starved 300 g male Wistar rat was quickly removed and rinsed in 250 mM mannitol/5 mM Hepes, pH 7.4/0.5 mM EGTA/0.1% BSA (further indicated as isolation medium). The liver tissue was chopped with a pair of scissors. Homogenization was preformed by four gentle strokes with a loosely fitting pestle rotating at 650 rpm. After differential centrifugation first at $600g_{max}$ for 5 min and second at $10300g_{max}$ for 10 min, the mitochondria were resuspended in isolation medium, loaded on a Percoll gradient (30% Percoll (v/v) in 225 mM mannitol/25 mM Hepes, pH 7.4/1 mM EGTA/0.1% BSA), and spun for 30 min at $95000g_{max}$. The mitochondrial band was removed and washed twice with 0.25 M sucrose adjusted to pH 7.4. The final pellet was gently resuspended in 0.25 M sucrose, pH 7.4 (Hovius et al., 1990).

SMP from bovine heart, rat skeletal muscle, and rat heart mitochondria were prepared by sonication according to Lee (1979) except that the EDTA was omitted from the isolation buffer. SMP from rat liver mitochondria were prepared by swell−shrink and sonication according to Hovius et al. (1990). The inner membrane fraction was used as SMP in the lipid peroxidation assay.

Measurement of Lipid Peroxidation in SMP. Lipid peroxidation in SMP, in the presence of 0.5 μM rotenone, was induced by addition of 50 μM NADH and 10 μM ADP-Fe(III). Prior to addition, the Fe(III) ions were complexed with ADP by addition of 1 volume of 4 mM $FeCl_3 \cdot 6H_2O$ to 1 volume of 400 mM ADP and adjustment to pH 7.0−7.5 with 1 M NaOH. The final concentrations in the reaction were 10 μM Fe(III) and 1 mM ADP, respectively.

Lipid peroxidation was measured by the PnA method described by Kuypers et al. (1987) and was adjusted for SMP. Measurements were performed in a 1 mL thermostated cuvette at 25 °C, and the fluorescent signal was followed continuously with a SLM Aminco SPF-500 spectrofluorometer using an excitation wavelength of 324 nm, slidwidth 1 nm, and an emission wavelength of 413 nm, slidwidth 20 nm. The fluorescent probe *cis*-PnA dissolved in ethanol (0.2 mM) was added to a cuvette containing a SMP membrane suspension. Final concentrations were as follows: *cis*-PnA, 0.8 μM, and SMP, equivalent to 100 μM membrane phospholipid (approximately 200 μg of SMP protein), in 125 mM KCl/25 mM Tris-HCl, pH 7.4. The rate of peroxidation was followed for 15 min. The peroxidation rate was determined as described in the Results.

As during the propagation steps oxygen is consumed, lipid peroxidation was also followed by measuring the rate of oxygen consumption by polarographic methods in a chamber of 170 μL fitted with a Clark type microelectrode (YSI Inc., Yellow Springs, OH).

Enzyme Assay for Complex I and III of the Respiratory Chain. To determine the combined activities of complex I and III of the respiratory chain, the NADH:cyt *c* oxidoreductase assay was used (Hatefi et al., 1961; Boffoli et al., 1994). The reaction medium contained 200 μM NADH, 2 mM KCN, 2.5 μM TTFA, and 5 mM $MgCl_2$ in 25 mM K_2HPO_4 buffer, pH 7.4 (set with 25 mM KH_2PO_4), with 0.25% BSA. The sample was added to the reaction medium and freeze/thawed three times in liquid nitrogen. After thawing, 50 μM cytochrome *c* (final concentration) was added, and the

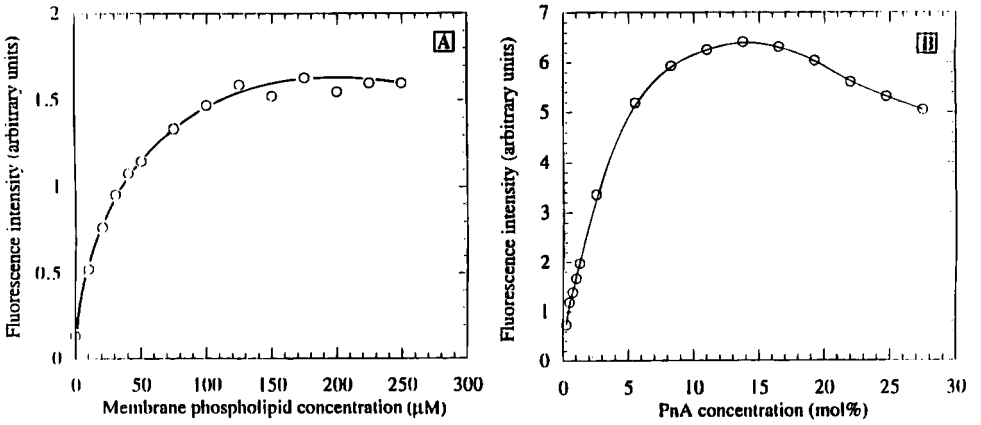

FIGURE 1: Fluorescence intensity of *cis*-PnA in bovine heart SMP membranes as a function of concentration. (A) 2 mL of buffer (125 mM KCl/25 mM Tris-HCl, pH 7.4) containing 0.8 μM *cis*-PnA and various concentrations of SMP membrane phospholipid and (B) 2 mL of buffer containing 100 μM SMP membrane phospholipid and various concentrations of *cis*-PnA.

increase in reduced cytochrome *c* was measured for 5 min at 550 nm at room temperature.

Determination of Phospholipid and Protein Content of the Membranes. The phospholipids of the mitochondria and the SMP were extracted according to Bligh and Dyer (1959), and phospholipid phosphate was analyzed according to Rouser et al. (1970). Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

RESULTS

Dependence of PnA Fluorescence on Phospholipid Concentration. As the *cis*-PnA assay is based on the susceptibility of the fluorescence properties of *cis*-PnA to peroxidation, optimal concentrations of *cis*-PnA and its ratio to phospholipid content were determined. In Figure 1A is shown that at 0.8 μM *cis*-PnA maximal fluorescence was reached at a SMP content equivalent to 100 μM phospholipid phosphate. When the content of SMP was held at 100 μM, the intensity of fluorescence increased linearly with the concentration PnA up to 2.5 mol %, reaching saturation at 5 mol % followed by a decrease in fluorescence. The divergence of a linear relation above 2.5 mol % is due to self-quenching of the probe in the phospholipid membranes (Figure 1B). These results are similar to those reported by van den Berg et al. (1988) for erythrocytes and by Dinis et al. (1993) and Laranjinha et al. (1992) for sarcoplasmic reticulum and low density lipoproteins, respectively.

In all subsequent experiments, concentrations of 0.8 μM (mol %) of *cis*-PnA and 100 μM SMP membrane phospholipid phosphate were used. Under these conditions, the fluorescence intensity of the PnA probe is a linear function of its concentration in the membranes.

NADH-Induced Lipid Peroxidation in SMP. Lipid peroxidation in SMP was induced via NADH in the presence of an iron(III) chelate (ADP-Fe(III)) according to previous reports (Glinn et al., 1991; Takeshige et al., 1980; Takayanagi et al., 1980). This made it possible to compare our results obtained with the *cis*-PnA assay.

The recorder tracing of an experiment in which lipid peroxidation was followed in bovine heart SMP, using the *cis*-PnA method in the presence of rotenone, is shown in Figure 2. Upon addition of *cis*-PnA, a rapid increase in fluorescence intensity is seen which is due to incorporation of the probe in the SMP membranes. After 3 min the

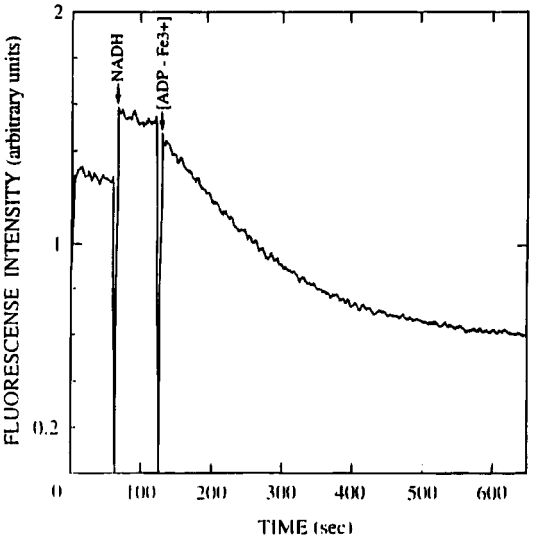

FIGURE 2: Fluorescence intensity signal followed in time during a lipid peroxidation experiment in bovine heart SMP. Assay conditions: buffer 125 mM KCl/25 mM Tris-HCl, pH 7.4, 100 μM SMP membrane phospholipid, 0.8 μM *cis*-PnA, 0.5 μM rotenone, 50 μM NADH, and 1 mM:10 μM ADP-Fe(III).

fluorescence signal stabilized and remained nearly constant in the absence of peroxidation inducing compounds. The small time-dependent decrease in fluorescence is due to bleaching of the probe. Addition of NADH resulted in an increased fluorescence intensity signal because *cis*-PnA and NADH have overlapping excitation and emission spectra. Immediately after addition of ADP-Fe(III), lipid peroxidation starts, as visualized by a decrease of the fluorescence signal due to breakdown of *cis*-PnA. The remaining fluorescence at the end of the reaction is almost completely due to NADH.

In Table 1 different conditions of NADH-induced lipid peroxidation (in the presence of rotenone) measured with the *cis*-PnA method are summarized. The rate of fluorescence decrease depends on the initial *cis*-PnA concentration and follows first-order kinetics. For quantification, the reaction rate constant, defined as percentage of the initial fluorescence intensity (% min^{-1}), is expressed per milligram of SMP protein (see legend to Table 1). The peroxidation rates were corrected for the fluorescence decrease measured in the absence of *cis*-PnA due to NADH oxidation (see legend, Table 1). This fluorescence decrease is independent of the NADH concentration and is linearly dependent on the

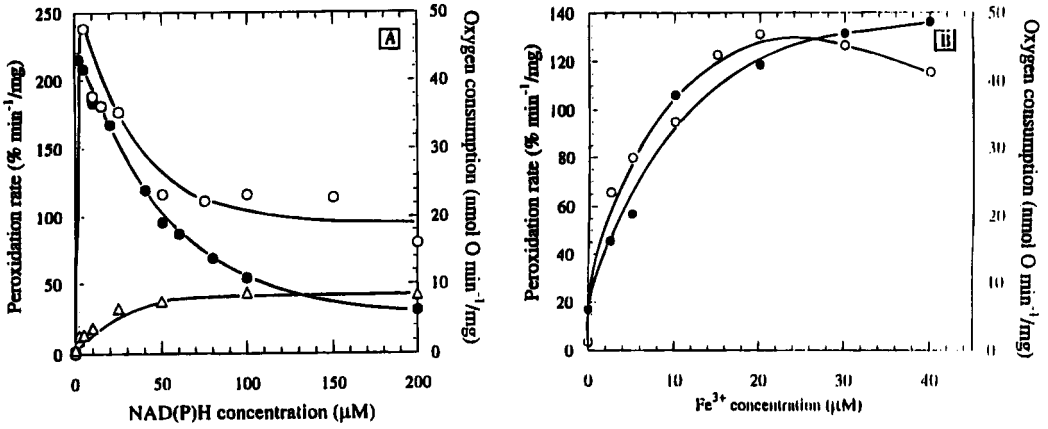

FIGURE 3: Peroxidation rates measured with the *cis*-PnA assay and measurement of the oxygen consumption in SMP from bovine heart at different concentrations of NADH (● (PnA) and ○ (O_2)) or NADPH (△ (PnA)) (A) and [ADP-Fe(III)] (● (O_2) and ○ (PnA)) (B). Assay conditions: buffer 125 mM KCl/25 mM Tris-HCl, pH 7.4, 100 μM SMP, membrane phospholipid, 0.8 μM PnA, 0.5 μM rotenone, and in (A) 1 mM:10 μM ADP-Fe(III) and in (B) 50 μM NADH.

Table 1: Lipid Peroxidation in Bovine Heart SMP with and without NADH, ADP, and Fe(III) Using the PnA Assay for the Detection[a]

additions	peroxidation rate (% min^{-1} mg^{-1})[b]
−NADH	2.63
+Fe(III)	3.68
+ADP	2.35
+ADP + Fe(III)	2.38
+ADP-Fe(III)	2.93
+NADH	3.06
+Fe(III)	4.08
+ADP	2.49
+ADP + Fe(III)	3.46
+ADP-Fe(III)	115.60
+ADP-Fe(III) + BHT	2.48
+ADP-Fe(III) + excess EDTA	2.38

[a] Assay conditions: To 1 mL of 125 mM KCl/25 mM Tris-HCl, pH 7.4, were added depending on the additions stated (− absent and + present): 50 μM NADH, 10 μM Fe(III), 1 mM ADP, 10 μM:1 mM ADP-Fe(III), 0.01% BHT, 0.5 mM EDTA, and 0.5 μM rotenone. [b] Peroxidation rate calculated according to the following equation:

$$\frac{\Delta F_i - \Delta F_{NADH}}{F_i} \times [\text{protein}] \times 100\%$$

in which ΔF_i = initial fluorescence decrease expressed in arbitrary units/min, ΔF_{NADH} = fluorescence decrease as a result of NADH oxidation expressed in arbitrary units/min, F_i = initial fluorescence intensity expressed in arbitrary units, and [protein] = protein concentration expressed in mg/mL.

ADP-Fe(III) concentration between 0 and 40 μM (not shown).

In the absence of NADH, the rate of lipid peroxidation was low and could not be enhanced by the addition of Fe(III), ADP, or ADP-Fe(III). In the presence of NADH, lipid peroxidation could only be induced by the ADP-Fe(III) chelate. The process of lipid peroxidation was completely abolished in the presence of the radical scavenger butylated hydroxytoluene (BHT) or at high concentrations of EDTA. At low EDTA concentrations, an EDTA-Fe(III) (1:1) complex is formed, which could also induce lipid peroxidation, albeit at lower rates compared to ADP-Fe(III) (not shown).

Influence of NAD(P)H and ADP-Fe(III). The dependence of the rate of lipid peroxidation on the concentration of NADH and NADPH is shown in Figure 3A. In varying the concentration of NADH, a biphasic relationship was observed. The rate of lipid peroxidation increased with increasing concentration of NADH up to 2.5 μM, whereas at higher NADH concentrations lipid peroxidation was prevented. A similar biphasic curve for NADH-induced lipid peroxidation in SMP was reported by Glinn et al. (1991), Takeshige et al. (1980), and Takeshige and Minakami (1979).

Table 2: Lipid Peroxidation Rates and Enzyme Activities of SMP of Different Tissues

sample	peroxidation rate (% min^{-1} mg^{-1})	oxygen consumption (nmol of O min^{-1} mg^{-1})	NADH:cyt *c* oxidoreductase (mU mg^{-1})
rat liver SMP	37	44	71
rat skeletal muscle SMP	61	79	135
rat heart SMP	83	161	241

It has been reported that NADPH could also induce lipid peroxidation in SMP. In Figure 3A the results are shown of the dependence of the peroxidation rate on NADPH. Up to 100 μM, this dependence on NADPH shows saturation kinetics. These data confirm earlier studies (Glinn et al., 1991; Takeshige et al., 1980; Takeshige & Minakami, 1979).

The dependence of the rate of *cis*-PnA breakdown on the concentration of ADP-Fe(III) showed a characteristic saturation pattern (Figure 3B). Increasing concentrations of the ADP-Fe(III) chelate up to 15−20 μM resulted in a faster degradation of the PnA fluorescence corresponding with a higher rate of lipid peroxidation. The same kinetic characteristics were observed when the rate of oxygen consumption was used as a measure of lipid peroxidation.

For reproducibility and accuracy of the measurements, concentrations of 50 μM NADH and 10 μM ADP-Fe(III) were chosen in the following experiments.

Correlation between Enzyme Activity of the Respiratory Chain and Lipid Peroxidation. Because lipid peroxidation in SMP is induced via complex I, a possible correlation between the electron flow through the respiratory chain and the rate of lipid peroxidation was investigated by comparing this process in SMP prepared from mitochondria isolated from different rat tissues. The results of the studies using SMP from rat heart, skeletal muscle, and liver are summarized in Table 2.

The rates of lipid peroxidation obtained with the PnA assay show a remarkable difference between the SMP prepared

from mitochondria isolated from different rat tissues, with decreasing peroxidation rates from heart muscle (83% min^{-1} (mg of protein)$^{-1}$) to skeletal muscle (61% min^{-1} (mg of protein)$^{-1}$) and finally to liver (37% min^{-1} (mg of protein)$^{-1}$). The results from oxygen consumption rates (in the presence of rotenone) showed the same order of decrease. Finally, the flux of electrons through the respiratory chain, measured as the activity of NADH:cyt *c* oxidoreductase activity, again showed the same correlation.

DISCUSSION

Lipid peroxidation is increasingly recognized as an important determinant in the impairment of biological functions and in the development of disease state. During the last decade a number of methods have been developed for the detection of reactive oxygen species or to monitor lipid peroxidation. However, most of these methods either require sophisticated techniques such as electron spin resonance, are discontinuous and require sample destruction, suffer from serious methodological drawbacks, or are indirectly related to the process of lipid peroxidation (for review, see Gutteridge & Halliwell, 1990).

The method developed by Kuypers et al. (1987) is based on the use of a polyunsaturated fatty acid, *cis*-PnA, which after incorporation into biological membranes is highly fluorescent. The fluorescent properties of *cis*-PnA are destroyed when the conjugated allylic bonds are attacked by reactive oxygen species. Thus, the disappearance of *cis*-PnA fluorescence is directly related to lipid peroxidation and can be followed continuously. The method has been successfully applied in the study of lipid peroxidation in erythrocyte membranes (van den Berg et al., 1988), sarcoplasmic reticulum (Dinis et al., 1993), and low density lipoprotein particles (Laranjinha et al., 1992).

To study the possible relationship between NAD(P)H-induced lipid peroxidation in mitochondrial membranes and the pathobiochemistry of inherited or acquired mitochondrial respiratory chain disorders, the PnA assay was adopted for SMP. NAD(P)H-induced lipid peroxidation in rotenone-inhibited SMP assayed by either polarographic or MDA-based methods has already been reported (Glinn et al., 1991; Szabados et al., 1987; Tretter et al., 1987; Takayanagi et al., 1980).

For the adaptation of the PnA fluorescence method, optimal ratios between [*cis*-PnA] and the amount of SMP phospholipid phosphate were determined. Maximal fluorescence of *cis*-PnA (0.8 μM) was obtained at a membrane phospholipid concentration of 100 μM (Figure 1A) while a linear relationship between *cis*-PnA fluorescence and [*cis*-PnA] was observed between 0 and 2.5 mol % *cis*-PnA (Figure 1B). Using experimental conditions of 0.8 mol % *cis*-PnA and 100 μM SMP phospholipid phosphate, NADH-induced *cis*-PnA breakdown (and thus lipid peroxidation) in rotenone-inhibited SMP in the presence of ADP-Fe(III) could be followed by the decrease in fluorescence (Figure 2).

As *cis*-PnA and NADH both have fluorescent properties and have overlapping emission spectra induced by the excitation at 324 nm, quantification of the decrease in fluorescence due to *cis*-PnA breakdown is complex. The total fluorescence yield when both *cis*-PnA and NADH are present is the result of *cis*-PnA fluorescence, NADH fluorescence, and quenching of *cis*-PnA fluorescence due to absorption by and energy transfer to NADH. The residual fluorescence after the complete peroxidation of *cis*-PnA was shown to be due to the NADH still present in the system. Furthermore, apart from *cis*-PnA peroxidation, NADH is also oxidized by SMP, although this rate is very low due to the presence of rotenone (typically, 1.7 μM NADH oxidized min^{-1} at 100 μM SMP phospholipid phosphate, not shown). To correct the initial rate of total fluorescence decrease for the decrease of fluorescence due to NADH oxidation, duplicate experiments were performed in the absence of *cis*-PnA to determine the rate of NADH-dependent fluorescence decrease; subsequently, this rate was subtracted from the initial rate observed when both *cis*-PnA and NADH were present. In this way, errors due to quenching of *cis*-PnA fluorescence by NADH are minimized, but the importance of performing experiments under standardized conditions (when NADH oxidation is low) is stressed.

The present results of NADH-induced lipid peroxidation monitored with the *cis*-PnA assay were compared with data obtained by polarographic measurements of oxygen consumption. Both methods yield similar results when applied under the same experimental conditions (Figure 3, Table 2). The data presented are also, in general, in accordance with literature (Glinn et al., 1991; Takayanagi et al., 1980). Furthermore, the reliability of the *cis*-PnA assay has been established by comparison with several other methods available to detect lipid peroxidation (Kuypers et al., 1987; van den Berg et al., 1988). The only finding which is not in agreement with previous reports is the fact that succinate does not protect the mitochondrial membrane toward lipid peroxidation when peroxidation was followed with the *cis*-PnA assay. Succinate has been reported to protect mitochondrial membrane lipid peroxidation (Szabados et al., 1987; Tretter et al., 1987; Takayanagi et al., 1980), probably by keeping ubiquinol (CoQ) in the reduced form (Beyer, 1992; Takayanagi et al., 1980). At present, no explanation can be offered for this discrepancy.

Table 1 shows that, only in the presence of an iron chelate such as ADP-Fe(III), NADH-induced lipid peroxidation in rotenone-inhibited SMP occurs at an appreciable rate. Furthermore, this process is correlated with the activity of NADH:cyt *c* oxidoreductase (Table 2). Clearly, electron flow through the respiratory chain is a prerequisite for the initiation of lipid peroxidation, but the mechanism by which free radicals and thus reactive oxygen species are produced in this system is not completely clear. The following processes in lipid peroxidation can be considered (Minotti & Aust, 1992):

$$I^{\bullet} + LH \rightarrow IH + L^{\bullet} \quad \text{(initiation)} \qquad (1)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (O_2 \text{ addition}) \qquad (2)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad \text{(propagation)} \qquad (3)$$

The chain is promoted by some initiator ($I^{\bullet}$) which catalyzes the generation of a lipid alkyl radical ($L^{\bullet}$). This species, in turn, rapidly binds oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$), which eventually converts to LOOH via hydrogen abstraction from neighboring allylic bonds. Both iron and partially reduced species of dioxygen have been implicated in the formation of initiators, although the mechanisms of their involvement are still unclear. One possible mechanism

is the superoxide anion (O_2^-) driven Fenton reaction:

$$O_2^- + Fe(III) \rightarrow O_2 + Fe(II) \quad (4)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^\bullet \quad (6)$$

where lipid peroxidation is initiated by the most reactive oxidant, the hydroxyl radical ($OH^\bullet$). Alternatively, the formation of a superoxide anion, a hydroxy peroxide, or some other reducing system promotes the reduction of Fe(III) until the appropriate Fe(II)/Fe(III) ratio is reached, which leads to the generation of lipid peroxidation initiating species (reaction 7 followed by reaction 6) (Minotti & Aust, 1992).

$$Fe(II) + O_2 + 2H^+ \rightarrow Fe(III) + H_2O_2 \quad (7)$$

Thus, the electron flow through complex I in rotenone-inhibited SMP may produce the superoxide anion radical to initiate reactions 4–6 directly (Turrens & Boveris, 1980; Takeshige & Minakami, 1979) or may be involved in the reduction of Fe(III), a catalytic activity assigned to NADH dehydrogenase (Takeshige et al., 1980), to initiate reactions 7 and 6.

In conclusion, we have developed a sensitive, continuous, reliable fluorescence assay to follow lipid peroxidation in SMP. This gives the possibility to show the biochemical mechanisms by which electron flow through the respective complexes of the respiratory chain produces reactive oxygen species.

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