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Biochimica et Biophysica Acta 1330 (1997) 127–137

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The use of *cis*-parinaric acid to measure lipid peroxidation in cardiomyocytes during ischemia and reperfusion

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Received 19 March 1997; revised 9 June 1997; accepted 12 June 1997

Abstract

cis-Parinaric acid (PnAc), a fluorescent, polyunsaturated fatty acid, was used to measure lipid peroxidation during simulated ischemia and reperfusion in cultured neonatal rat cardiomyocytes. PnAc was used both as free fatty acid, inserted in the membranes following cultivation of the cells, as well as constituent of the cellular complex lipids by metabolically integrating the fatty acid during growth. In the insertion experiments a pre-incubation with DL-aminocarnitine, an inhibitor of β-oxidation, was necessary to prevent loss of fluorescent signal. Such a pre-incubation resulted in an enrichment of PnAc in the sarcolemma: In pre-treated cells $57 \pm 1.3\%$ of total inserted PnAc is present in the sarcolemma compared to $27 \pm 5.7\%$ in cells containing the integrated probe. Both methods to introduce PnAc into the cells were compared with respect to their sensitivity for an externally applied oxidative stress and thereafter lipid peroxidation during simulated ischemia and reperfusion was assayed. Going from normoxic to ischemic conditions lipid peroxidation did not increase and remained at a low level. When the ischemic cells were subsequently subjected to reperfusion (reintroduction of both oxygen and glucose), large scale lipid peroxidation was obvious. When, on the other hand, oxygen alone was reintroduced (reoxygenation) no increased lipid peroxidation was observed. These observations led to the conclusion that ischemia does not lead to an enhanced lipid peroxidation and that resumption of metabolic activity during reperfusion is necessary to induce lipid peroxidation. © 1997 Elsevier Science B.V.

Keywords: *cis*-Parinaric acid; Lipid peroxidation; Oxidative stress; Phospholipid; Ischemia; Reperfusion

1. Introduction

A growing number of studies provide support for the hypothesis that free radicals contribute to ischemic and reperfusion injury in the myocardium [1–4]. In particular reactive oxygen species such as the superoxide anion ($O_2^{\cdot -}$), which was shown to be generated in the heart during reperfusion following an ischemic period [5–7], may be responsible for extensive damage brought about in DNA, protein and lipids.

Several mechanisms are proposed to explain the formation of oxygen radicals (for review see [4]). During ischemia, the catabolism of cellular ATP results in increased levels of hypoxanthine and xanthine, while xanthine dehydrogenase is converted into xanthine oxidase. Xanthine oxidase mediated

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PII S0005-2736(97)00144-2

(hypo)xanthine degradation is accompanied by $O_2^{\cdot -}$ and H_2O_2 formation, especially when O_2 becomes available during reperfusion. Combined with the release of iron from ferritin, $^{\cdot}OH$ radicals are formed. Another source of oxygen radicals may be related to a fully reduced and possibly damaged mitochondrial respiratory chain. Whereas under normal conditions, only a small amount of the oxygen which is reduced in the mitochondrial respiratory chain is converted into superoxide anion ($O_2^{\cdot -}$), the reintroduction of O_2 and re-establishment of mitochondrial activity in the ischemic cell may lead to an enhanced formation of $O_2^{\cdot -}$ [8]. The superoxide radical is quickly reduced to hydrogen peroxide which will be eliminated by catalase and glutathione peroxidase action but can be converted also, in the presence of iron, to the very reactive hydroxyl radical $^{\cdot}OH$ [9] and may cause damage of several cell components.

Enhanced peroxidative damage may result furthermore from an inhibition of the regular intracellular enzymatic radical scavenging system, mainly consisting of the enzymes superoxide dismutase, catalase and glutathione peroxidase. Such an inhibition, in vivo, was reported by Simmons and Jamall [10] and Coudray et al. [11], although in studies performed with isolated hearts such an inhibition was not observed [12].

Lipid peroxidation during ischemia and subsequent reperfusion has been assayed with a wide variety of methods such as the determination of thiobarbituretic acid reactive components (TBARS) and other breakdown products of lipid peroxides or the measurement of diene conjugation following lipid extraction and purification by HPLC or GLC. In the present study the approach to quantify lipid peroxidation with *cis*-parinaric acid (PnAc) has been applied [13,14]. *cis*-Parinaric acid is a polyunsaturated natural fatty acid with a chain length of 18 C atoms, containing four double bonds in a conjugated form. This double bond system unites several properties convenient to study of lipid peroxidation: It makes the probe very susceptible to lipid peroxidation and it gives the probe its fluorescent properties. The loss of fluorescence upon damage of the double bond system is a direct indicator for the peroxidative stress and damage. This allows us to follow directly the first step in lipid peroxidation: The abstraction of a H atom and double bond rearrangement.

Aim of this study was to measure the occurrence of lipid peroxidation during ischemia and subsequent reintroduction of oxygen and glucose in a direct way. Therefore, the original method to measure lipid peroxidation with PnAc, which was based on the addition and insertion of the free fatty acid into vesicles and the erythrocyte membranes, was adapted for use in cultured neonatal cardiomyocytes. Next to this, we were able to metabolically integrate PnAc in the lipids of spontaneous beating neonatal rat heart cells (cardiomyocytes). These two methods of introducing PnAc into the cardiomyocytes were compared, and tested for their sensitivity towards chemically induced oxidative stress, and the distribution of the probe in the cells was determined. Subsequently both methods were applied to the ischemia-reperfusion model system to investigate the initial stages of lipid peroxidation.

The results indicate that lipid peroxidation does not occur during the ischemic period itself. Equally reoxygenation, i.e., the re-introduction of oxygen alone after an ischemic insult, does not result in an increased level of lipid peroxidation. During reintroduction of both oxygen and substrate however large scale lipid peroxidation is obvious.

2. Materials and methods

2.1. Cell culture

Culturing of the cells was done according to a modification of the method of Harary and Farley [15]. Neonatal rats (1–2 days old) were decapitated, their hearts were excised and minced. The mince was incubated in a spinner flask at 37°C with 0.1–0.05% trypsin (Difco, Detroit, MI; in 137 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM glucose and penicillin (100 000 units l^{-1})/streptomycin (100 mg l^{-1})). The incubation fluid was decanted and new medium was added. The incubation medium from the first three incubations (10 min each) was discarded and during the next 8 incubations the mince was almost completely digested. The incubation media were spun (8 min, 430 g) and resuspended in growth medium (Gibco, Paisley, UK, HamF10 supplemented with 10% fetal calf serum, 10% horse serum, 10 μM Arabinose C to inhibit fibroblast growth and $CaCl_2$ (final concentration 1 mM)). The cells were plated in

Falcon 3003 dishes (Falcon plastics, Sumter, SC) for 2–3 h, during which time fibroblasts adhere and myocytes remain freely suspended [16]. Finally, the myocytes were plated on Primaria-treated culture dishes (Falcon 3801, 35 mm in diameter) or on gas dissection discs punched from Falcon 3802 dishes (60 mm in diameter). Within 3 days a confluent monolayer of spontaneously beating cells was formed. Before use the dishes with cells attached were extensively washed in buffer W^+ containing 133 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl and 5 mM glucose, $pH_{37°C}$ 7.4.

2.2. Incubation procedures

To allow insertion of PnAc cells were washed with buffer W^+ and incubated with DL-aminocarnitine (gift of Hülsmann) in buffer W^+ (final concentration 200 μM, 2 h, 37°C), to inhibit β-oxidation [17]. Subsequently PnAc was added from a concentrated stock solution in ethanol to a final concentration of 4 μM. The incubation took place in the dark for 30 min at 37°C. Incubation with PnAc without aminocarnitine pre-incubation was performed in buffer W^+ in the dark for 40 min at 0°C.

Metabolic integration of PnAc in lipids was achieved by adding PnAc, on the day of isolation (day 0) and just before plating, to the growth medium in a final concentration of 30 μM. Cells were used on day 3, when a confluent monolayer of spontaneously beating myocytes was formed.

2.3. Cumene hydroperoxide (CumOOH) and hydrogen peroxide (H_2O_2) induced peroxidation

Cells containing PnAc were incubated in buffer W^+ in the presence of CumOOH (5 mM or 50 μM) or 250 μM H_2O_2 and 25 μM ferrous sulphate. After the chosen time span, EDTA (100 μM) was added to chelate the iron ions.

2.4. Simulation of ischemia, reoxygenation and reperfusion

Simulating ischemia at a cellular level was achieved by using the in vitro model of Vemuri et al. [18] which was modified as described previously [19,20]. Dishes with cultured cardiomyocytes were washed extensively with buffer W^- (133 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl, $pH_{37°C}$ 7.4). Subsequently 1 ml buffer W^- per dish was added. Dishes were then placed in a special ischemia device that consisted of a special Plexiglas constructed chamber which could be tightly closed. Two inlet/outlet tubes allowed an atmosphere of either Argon (Ar: 99.992 vol%), or normal air to be installed. The temperature in the chamber and dishes was kept at 37°C by a continuous stream of heated water, which circulated through the undercompartment of the chamber. This model has been described in detail and has been shown to give rise to morphological and biochemical changes during the anoxic period comparable to in vitro and in vivo preparations that were subjected to ischemia [19]. Dissolved O_2 in the buffer falls to zero within 25 min after the onset of the ischemic conditions [20].

The control situation (normoxia) was achieved by placing the cells in buffer W^+ at 37°C.

After simulated ischemia, reoxygenation and reperfusion were achieved by adding either 1.5 ml of fresh buffer W^- (no glucose) or 1.5 ml buffer W^+ to each dish, and allowing them to stand at 37°C for the required time.

2.5. ATP measurements

The cellular high-energy phosphate (ATP) was extracted with 50% ethanol for 30 min under gentle shaking (1 ml $dish^{-1}$). An aliquot was dried under a stream of nitrogen and dissolved in 100 μl water. ATP was determined using an luciferine/luciferase assay kit (Sigma). The relative ATP levels were determined by comparing the absolute bioluminescence values of the different samples with those of control cells.

2.6. Measurement of lactate dehydrogenase release

Cell lysis was tested by measurement of lactate dehydrogenase (LDH) release. After control and 'ischemic' incubations the supernatants of the cells were collected and the LDH activity was assayed as described by Van der Schaft et al. [21]. All measurements were performed in duplo, and the relative LDH release was determined by comparing the activities of the different supernatants with the activity of total cell homogenates.

2.7. Lipid extraction

Extraction of the phospholipids was accomplished by placing 2 ml of isopropanol on the 3.5 cm dishes with cells (or 4 ml on a 6 cm dish) [22]. Isopropanol was saturated with nitrogen before use and extractions were performed at room temperature under nitrogen atmosphere in the dark. A standard extraction of 60 min ensured complete extraction. A small amount of plastic was extracted during this procedure, but proved not to interfere with the fluorescence measurements.

2.8. Lipid analysis

Total phospholipid content was determined according to Rouser et al. [23]. After drying the samples under nitrogen, phospholipids were destructed in 70% perchloric acid (> 30 min, 180°C) and inorganic phosphorous content was measured.

2.9. Measurement of fluorescence

All samples were extracted as mentioned above and prepared in 2 ml isopropanol. Fluorescence measurements were carried out at room temperature in a SPF Aminco spectrofluorimeter. The excitation wavelength was 304 nm for free parinaric acid and 324 nm for integrated parinaric acid. The emission was measured at 413 and 420 nm, respectively.

2.10. Isolation of the sarcolemma

The sarcolemma was isolated using the gas-dissection technique previously described by Langer et al. [24] and Post et al. [22]. Briefly, the disc with attached cells was placed at the centre of a mobile platform inside the dissection chamber. The platform, with disc, was then elevated to make a firm contact with the protruding inner horizontal gas outlet. Subsequently, the inlet valve was opened rapidly (< 1 s) to allow the entry of N_2 (gas) at a pressure of 1900–2000 psi. As the N_2 stream bursts radially over the surface of the monolayer, the upper surface of the cells is sheared open, the cellular material is blown out, and the sarcolemma is left in a fenestrated layer and, in some areas, in a wrinkled or rolled form attached to the disks.

2.11. Separation of free fatty acids and phospholipids

Free PnAc was separated from PnAc integrated in phospholipids by means of a silica column. Neutral lipids, e.g., glycerides and fatty acids are washed of the column first with chloroform. Phospholipids are eluted subsequently with chloroform/methanol 1:1 (v/v). Fluorescence in the eluents was checked on a SPF Aminco spectrofluorimeter at excitation wavelength 324 nm and emission wavelength 420 nm.

The percentage of residual free PnAc in the cells in which most of the PnAc was metabolically integrated in the complex lipids was determined after fatty acid extraction according to Dole with small adaptations by Van den Bosch and Aarsman [25]. The extracts were dried under nitrogen, in the dark and redissolved in isopropanol. The level of fluorescence of free fatty acid extracted in this assay was compared to the fluorescence level of a total lipid extract obtained as described above and dissolved in the same solvent.

2.12. Experimental design

After loading the cells with PnAc, cells were subjected to either externally applied oxidative stress or ischemia/reperfusion/reoxygenation. Subsequently lipid extracts were made from the dishes and PnAc fluorescence and P_i contents of the different samples were measured.

3. Results

3.1. Insertion of the probe as free acid

The PnAc insertion assay originally developed by Kuypers et al. [13] and Van den Berg et al. [14] for use in red blood cells and ghosts, was slightly adapted for use in the cardiomyocytes. The optimal concentration of PnAc present in the incubation mixture for 30 min at 37°C (or 40 min at 0°C) appeared to be 4.0 μM. Extraction of PnAc from the incubation buffer showed that 50–70% of added PnAc was indeed inserted in the cells resulting in a final amount of the probe of 4–6 mol% PnAc/phospholipid P_i.

To assess the stability of the probe, cells were labelled with PnAc at 0°C as mentioned above and incubated at 37°C in buffer W^+ for various time spans. It can be clearly seen (Fig. 1) that in this case

PnAc fluorescence disappears within a few minutes. Obviously the probe was degraded rapidly after insertion and increase of the temperature. Since it is known that fatty acid metabolism is a major energy source of heart muscle cells we considered the possibility that β-oxidation might be the cause of this quick degradation. Therefore we pre-incubated the cells with DL-aminocarnitine (AC), a competitive inhibitor of carnitine-acyltransferase. By inhibiting the acyl-CoA transport into mitochondria, inhibition of β-oxidation is achieved.

When cells were pre-treated with AC and subsequently incubated at 37°C for extended time periods the PnAc fluorescence signal remained constant. Several inhibitor concentrations and incubation periods were tested and it appeared that a pre-incubation of 2 h with 200 μM DL-aminocarnitine proved to be sufficient to obtain a stable fluorescence signal for at least 2 additional h of incubation which was sufficient to carry out the ischemia and reperfusion experiments (Fig. 1). Therefore, in all PnAc insertion experiments, cells were pre-treated with AC at those conditions.

3.2. Incorporation of the probe in cellular lipids

To be able to compare peroxidation of free fatty acids with peroxidation occurring in the acyl chains

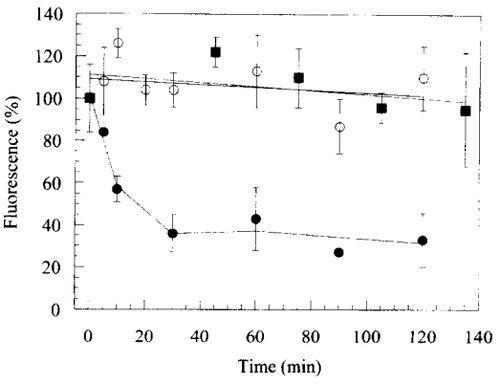

Fig. 1. Probe stability after PnAc insertion or integration into lipids. PnAc was added as mentioned in Section 2. Fluorescence was measured after incubations for various time spans in buffer W^+ and plotted as percentage of the fluorescence level at $t = 0$. (○: insertion experiments, −AC; ●: insertion experiments, +AC; ■: integrated PnAc). Each experiment was repeated at least three times, with three samples per time point each. Shown are standard deviations of means (SEM).

of the phospholipids we developed a method to metabolically integrate PnAc in the complex lipids of cardiomyocytes. It appeared to be sufficient to add PnAc to the growth medium for 3 consecutive days. To determine the optimal PnAc concentration for these integration experiments, a range of concentrations was tested (10–100 μM). At a final concentration in the growth medium of 30 μM PnAc the integration was optimal. At lower concentrations the incorporation was not sufficient to obtain a suitable fluorescent signal and at higher concentrations (> 50 μM) cell viability was affected. Concentrations above 70 μM proved to be lethal. Pre-incubation with AC was not necessary in these integration experiments to obtain a stable fluorescence signal upon labelling (Fig. 1).

To assure that integrated PnAc was indeed covalently coupled to the lipids, we used a silica column to separate in the total lipid extract free fatty acids from phospholipids. Part of the fluorescence signal was present in the free fatty acid containing fraction, however most of the signal (roughly 50–90%) was recovered in the phospholipid fraction. To get a more accurate determination of this distribution an extraction of free fatty acids alone was performed. A comparison between the fluorescence levels in this extract with that of a total lipid extract demonstrated that 75% of the total signal resulted from PnAc incorporated in complex lipids. The fraction of integrated PnAc could be further enlarged by adding fresh, PnAc free growth medium to the cells a few hours before use. This treatment extracted free PnAc from the cells and in this way 80 to 90% of PnAc present in the cell is integrated in its lipids. Since this last approach ensured the highest amount of metabolically integrated PnAc, we used this method for all subsequent integration experiments.

3.3. Characterization of accumulated PnAc

To validate both methods of probe insertion in cardiomyocytes, the distribution of the PnAc in the cell was tested, as well as the sensitivity of the probes to oxidative stress.

To test sensitivity of inserted and integrated PnAc to oxidative stress, cells were incubated for various time spans with hydrogen peroxide or cumene hydroperoxide. Fig. 2A shows the degradation of inserted,

free PnAc and Fig. 2B shows degradation of integrated PnAc. In both cases PnAc is readily degraded in the presence of these peroxides.

At 5 mM CumOOH the probe is degraded rapidly, but this concentration was lethal for the cells after about 60 min. At 50 μM CumOOH a slower peroxidative process was observed, and cells survived the applied time span of peroxidative stress. In experiments with cells containing free PnAc, degradation with 50 μM CumOOH was about as effective as with 250 μM H_2O_2. In the case of lipid incorporated PnAc 50 μM CumOOH is less effective than 250 μM H_2O_2. Inserted, free PnAc seems to be more sensitive to peroxidative stress than PnAc integrated in lipids.

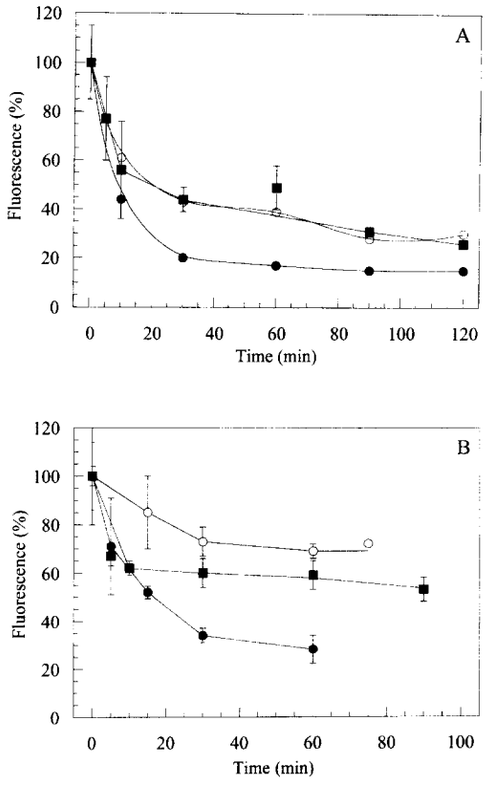

Fig. 2. PnAc degradation due to peroxide induced oxidative stress. Cells were incubated with buffer W containing either 250 μM H_2O_2 and 10 μM ferrous sulphate (■), or 5 mM (●) or 50 μM CumOOH (○), as also described in Section 2. (A) insertion experiments, (B) results from metabolic integration experiments. Fluorescence values are depicted as percentage of the fluorescence value at $t = 0$. Values are obtained from three separate experiments with three samples per time point per experiment. Shown is the standard deviation of mean, $n = 3$.

Secondly the fraction of PnAc present in the sarcolemma of cardiomyocytes was determined. We used the gas dissection technique, developed by Langer et al. [24] to isolate the plasma membrane of our cells and compared the fluorescence in the sarcolemmal lipid extract with that in lipid extracts of whole cells. The myocardial plasma membranes were shown to be devoid of sarcoplasmic reticulum markers and were highly enriched in the Na^+/K^+-ATPase sarcolemmal marker. Sarcolemmal recovery was 40–46% [22].

Based on the observation that 38% of total lipid–phosphorous of the cell is present in the plasma membrane [22], the percentage of total PnAc present in the plasma membrane was calculated. It was found that in aminocarnitine treated cells about 57 ± 1.3% ($n = 3$) of total PnAc is present in the plasma membrane. In contrast, labelling without AC pre-treatment resulted in a random distribution of the probe, with 36 ± 0.5% ($n = 3$) of total PnAc present in the sarcolemma. Metabolically integrated PnAc appears to be enriched in intracellular membrane fractions, because only 27 ± 5.7% ($n = 3$) of the total PnAc is present in the sarcolemma.

3.4. Lipid peroxidation in cardiomyocytes

Applying protocols as described previously [19,20,26] myocardial cells were studied under normoxic conditions; under conditions simulating ischemia and, following this ischemic period, under conditions simulating reperfusion with O_2 both in the absence (reoxygenation) and presence (reperfusion) of glucose. Three parameters were tested to evaluate the conditions of the cells. Loss of cell integrity was assessed by measuring the release of lactate dehydrogenase and the degree of ischemia was determined by measuring intracellular ATP contents. Furthermore, cell morphology was checked by means of light microscopy. The results shown in Table 1, are comparable to data published before [19,20,26]. Cells remain intact during 60 min of ischemia and the low ATP values indicated a severe simulated ischemia. Recovery is limited in case reoxygenation is performed but extensive upon the addition of both oxygen and glucose for energy production. Under these conditions also, the resumption of spontaneous contractile activity of the cells was observed.

Table 1
LDH release and ATP contents of cardiomyocytes after ischemia and reoxygenation/reperfusion

Incubation time (min)	LDH release (%)	ATP content (%)
0 I	3.2 ± 1.5	100
60 I	4.3 ± 1.3	28 ± 8
120 I	20.6 ± 4.5	18 ± 6
60 I + 60 O	n.d.	35 ± 12
60 I + 60 R	n.d.	76 ± 14

Incubations were performed as mentioned in Section 2, ATP of control (no ischemia) was set at 100%. Values are mean ± SEM, $n = 3$.
I: ischemia, O: reoxygenation, R: reperfusion, n.d.: not determined.

Fig. 3 shows the degradation of PnAc during ischemia, both of inserted PnAc and metabolically integrated PnAc. For free PnAc, as well as for metabolically integrated PnAc, a small decrease in fluorescence is visible. In the time span used, about 15% of the PnAc was lost, a value comparable to probe degradation during normoxia. Therefore, we conclude that during ischemia no increase in lipid peroxidation occurs.

After 60 min of simulated ischemia, followed by 0–120 min reoxygenation, the PnAc fluorescence signal decreased little (Fig. 4). As the observed decline is comparable to the decline occurring during ischemia and the control normoxic situation, it can be concluded that also during reoxygenation lipid peroxidation is at a low level and not increased in comparison with normoxic and ischemic conditions.

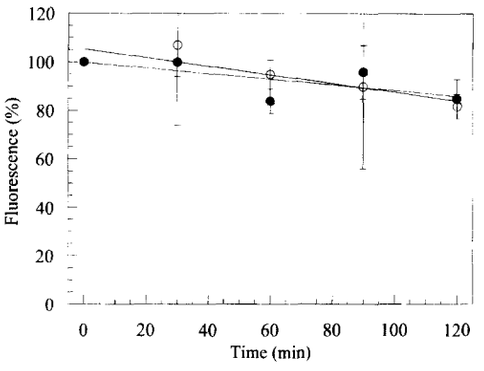

Fig. 3. PnAc degradation during ischemia. Cells were subjected to various time spans of ischemia as described in Section 2. Lipids were extracted and fluorescence was measured. All values are depicted as percentage of fluorescence at $t = 0$. ●: insertion experiments; ○: integration experiments. $n = 3$, shown are SEM.

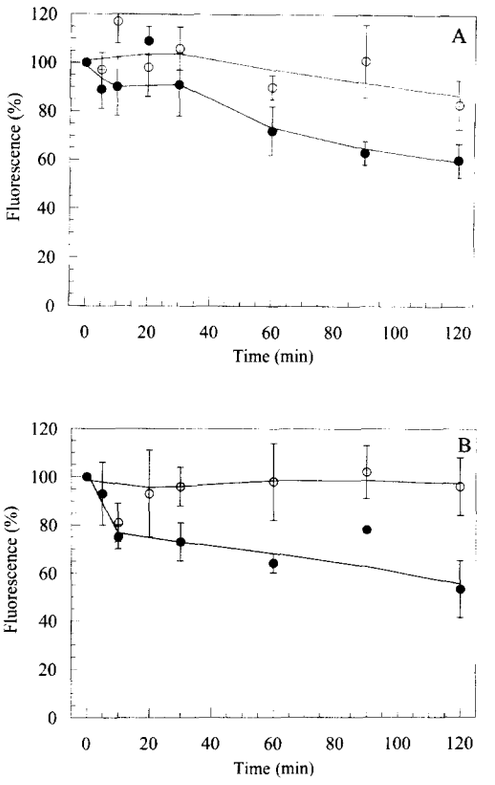

Fig. 4. Degradation of PnAc during reoxygenation and reperfusion. Depicted are relative fluorescence values ($F_{t=0} = 100\%$). Cells were first subjected to ischemia for 60 min as described before and subsequently placed in fresh buffer W^- for reoxygenation (○) or buffer W^+ for reperfusion (●). (A) Insertion experiments, (B) integration experiments. $n = 3$, shown are SEM.

This was the case for both inserted (Fig. 4A) and integrated PnAc (Fig. 4B).

When on the other hand recovery from ischemia was obtained by the addition of both oxygen and glucose, i.e., reperfusion, increased degradation of PnAc was obvious. Again the results for inserted PnAc (Fig. 4A) and integrated PnAc (Fig. 4B) were comparable. Apparently reflow of oxygen alone is not sufficient to induce large scale peroxidative damage; availability and probably the consumption of substrates is necessary.

4. Discussion

Damage to cellular structure and impairment of biological functions during several degenerative processes, and in a steadily increasing number of diseased states, has been ascribed to the production of

free radicals and subsequent peroxidation of membrane lipids. Methods to investigate this lipid peroxidation are available, be it that most are time consuming and/or indirect, e.g., measuring breakdown products of lipid peroxides, but not the damage to lipids itself (for review see [27])

A more direct way to detect lipid peroxidation was offered by the use of parinaric acid as a peroxidation sensitive membrane probe [13]. *cis*-Parinaric acid is a highly fluorescent, polyunsaturated fatty acid and destruction of any part of its conjugated double bond system, for example due to an attack by reactive oxygen species, causes the loss of its fluorescence. In this way, its fluorescent properties and peroxidation susceptibility are united and the disappearance of PnAc fluorescence is direct evidence for lipid peroxidation. The method has been successfully applied to investigate lipid peroxidation in erythrocytes and ghosts [14], sarcoplasmic reticulum [28], low density lipoprotein particles [29], and submitochondrial particles [30]. Hedley and Chow [31] used PnAc in flow cytometric measurements of lipid peroxidation.

In this study, we applied the methodology to investigate the initial stages of lipid peroxidation in cultured neonatal cardiomyocytes during ischemia, reoxygenation and reperfusion, i.e., reoxygenation in the presence of glucose necessary to resume energy production. Two probe conditions were applied: The insertion as free acid and the incorporation of PnAc in membrane lipids.

The original method for PnAc insertion, as developed by Kuypers et al. [13], had to be adapted for use in cardiomyocytes with respect to the optimal concentration and optimal incubation time. During these experiments it appeared that PnAc was quickly degraded, which is understandable because fatty acids are a major energy source of heart cells. This metabolic degradation could be eliminated and a stable fluorescence level was reached by pre-incubation of the cells with an inhibitor of β-oxidation, DL-aminocarnitine (AC). At the chosen conditions up to 60% of the PnAc present in the growth medium or the incubation buffer was inserted. Additional experimentation showed that under these conditions the probe is not equally distributed over the plasma and intracellular membranes. Whereas 38% of total phospholipid is present in the plasma membrane up to 57% of the total inserted PnAc can be found here, an effect which has to be ascribed to the fact that fatty acid transport to the mitochondrial inner membrane is blocked by the aminocarnitine, resulting in a cytosolic accumulation of free fatty acids (FFA) [32]. This might subsequently lead to a reduced FFA flux across the sarcolemma into the cytoplasm and accumulation of PnAc in the sarcolemma.

PnAc could also be integrated in the phospholipids of the cell. Experiments with other fatty acids, for example arachidonic acid, showed that addition of this fatty acid to the growth medium resulted in metabolic integration in the lipids [33]. Recently Ritov et al. [34] reported that they were also able to integrate PnAc metabolically in phospholipids of eukaryotic cells. They used four different rapidly dividing secondary cell lines and showed that PnAc bound to human serum albumin (1.8 mM PnAc) was integrated in the phospholipids of these cells within 2 h. Because of the occurrence of cell divisions, lipid turnover in these cells is much higher than in primary cell lines, like the terminally differentiated, non-dividing cardiomyocytes, and shorter incubation times will therefore be sufficient in those cells. Not only incubation times but also the concentration of added PnAc may be of importance as cytotoxicity of PnAc in undifferentiated, malignant cells, was reported by [35], even at low concentrations (5 μM) and after 24–48 h incubation time. Comparable differentiated cells were resistant to concentrations of PnAc up to 30 μM. The difference in sensitivity was thought to be due to differences in uptake rates, which were 3–4 times higher in malignant, undifferentiated cells. It turned out that incorporation of PnAc in the cardiomyocytes could be obtained by the presence of PnAc in the growth medium for 3 consecutive days at an initial concentration of 30 μM. Under these conditions, a substantial part of the PnAc is taken up by the cell. Analysis of the fate of this PnAc showed that not all of the fatty acid is incorporated in complex lipids: a substantial part remains present as free acid. Incubating the cells however with PnAc-free growth medium, removed most of this free acid. From the incorporated PnAc 27% appeared to be present in the plasma membrane lipids.

When cardiomyocytes, loaded with the fluorescent probe, were incubated with the peroxidation inducing molecules cumene hydroperoxide (CumOOH) or hydrogen peroxide (H_2O_2) a rapid loss of fluorescence

was observed. CumOOH appeared to be more effective than H_2O_2, which is probably caused by the fact that CumOOH is a more hydrophobic peroxide than H_2O_2. It is inserted in the plasma and intracellular membranes immediately and forms radicals in the direct vicinity of the fatty acids.

Free parinaric acid has a higher sensitivity for peroxidative stress than parinaric acid integrated in complex lipids. Comparable results were obtained before in model systems in which the susceptibility of free PnAc and PnPC (1-palmitoyl-2-parinaroyl-phosphatidylcholine) to applied oxidative stress were compared [13]. The explanation of this phenomenon could be that the free fatty acid may be more mobile than the parinaroyl-containing lipids, covering a bigger membrane surface in a given time span, thereby increasing its changes to encounter a free radical species.

Taken together we conclude that parinaric acid (free or lipid incorporated) can be used to provide the membranes of cardiomyocytes with a convenient probe to investigate early events in lipid peroxidation during ischemia and reperfusion. PnAc fluorescence is stable for at least 2 h under normoxic conditions, the probe is sensitive to peroxidative stress and the distribution of the probe in the cell is known.

During ischemia itself we observed no increased lipid peroxidation. After 2 h of simulated ischemia about 15% of initial fluorescence level was lost (Fig. 3) but this decrease was comparable to the decrease under normoxic conditions (Fig. 1). The observed absence of lipid peroxidation might indicate that also radical generation is diminished during simulated ischemia in our system. Data presented by others are contradictory and seem to depend on the sensitivity and suitability of the applied method or on the type of material used for these studies (cultured cells vs. perfused hearts). Zweier et al. [5] used electron paramagnetic resonance spectroscopy (EPR) to show that reactive oxygen centred free radicals are generated in hearts during the ischemic period itself and that a burst of oxygen radical generation occurred within moments of reperfusion. On the other hand, Lesnefsky et al. [36], using electron spin resonance spectroscopy (ESR) did not detect radical formation during the ischemic insult itself, but detected a sudden increase in free radicals during reperfusion. Also enhanced chemiluminescence was used to measure radical formation directly [7] and it was shown that radical formation decreased during ischemia but markedly increased during the first 3–5 min of reperfusion. Furthermore, the level of radical production during reperfusion varied with the duration of the preceding ischemic period [37,38]. In agreement with these data is our observation that during reperfusion a significant increase in lipid peroxidation occurred, and not during the ischemic period.

To exclude the possibility that the decrease in PnAc signal intensity during reperfusion is due to β-oxidation instead of lipid peroxidation, we inhibited β-oxidation using DL-aminocarnitine (AC) in the insertion experiments. Pre-incubation with AC and its presence during PnAc insertion is sufficient to inhibit β-oxidation for the whole time course of the ischemic and reperfusion episodes (Fig. 1). Prolonged incubation with AC did not result in a further stabilization of the PnA fluorescence signal. During experiments in which the PnAc had been metabolically integrated into phospholipid molecules, the fatty acid is not available for β-oxidation. Furthermore, the same decrease of PnA intensity was observed during reperfusion in integration experiments compared to the insertion experiments (Fig. 4). These observations strongly indicate that the loss of PnAc fluorescence during reperfusion indeed should be ascribed to lipid peroxidation, rather than to its degradation in the β-oxidation pathway.

Increased lipid peroxidation during reperfusion following simulated ischemia was reported by several groups. Blasig et al. [39] showed that during reperfusion 4-hydroxynonenal (HNE) levels increased. HNE is a specific product of lipid peroxidation and it is formed after interactions with free radicals only. During reperfusion also an increase of conjugated dienes and the liberation of thiobarbituretic acid reactive substances (TBARS) such as malondialdehyde (MDA) have been observed [36–38]. Our data furthermore show that during reoxygenation, i.e., the reintroduction of oxygen alone, lipid peroxidation does not occur. The combined supplementation of both oxygen and glucose, which can be used to supply energy to the system, are required to lead to an increase in the lipid peroxidation. The latter observation suggests that mitochondrial activity is involved in the generation of radicals and the induction of peroxidative damage. Such an involvement of

mitochondrial activity has been put forward indeed recently, when Piantadosi and Zhang showed that formation of radicals in rat brain after ischemia takes place in the mitochondria [40].

Several mitochondrial enzymes are affected during ischemia. Borutaite et al. [41] showed that the main effects of ischemia on mitochondrial respiration are an inhibition of the respiratory chain, some inhibition of the phosphorylation system and an increase of the proton leak. Under normal conditions 1% or 2% of the oxygen reduced in the respiratory chain is converted into reactive oxygen species via, i.e., the reduction of O_2 by a single electron to form O_2^-. Superoxide anion rapidly dismutates to H_2O_2 in the mitochondria either spontaneously or enzymatically via manganese superoxide dismutase. The rate of O_2^- production by mitochondria increases as the mitochondrial $[Ca^{2+}]$ increases and/or the pH decreases. Furthermore, the rate of H_2O_2 production is a function of the oxidation–reduction state of the electron carriers; as the carriers become reduced, the rate of H_2O_2 production increases [8]. Since during ischemia a lowering of the pH, a mitochondrial Ca^{2+} overload, as well as a full reduction of the electron transport system occurs, this might set the stage for the production of ROS upon restoration of O_2 and substrate supply. This then subsequently might lead to the observed lipid peroxidation as determined in the present study.

Acknowledgements

The authors wish to thank Dr. W.C. Hülsmann for his kind gift of DL-aminocarnitine. This research was carried out under the auspices of The Netherlands Foundation for Chemical Research (SON) and with financial support from The Netherlands organization for Scientific Research (NWO). The research of J.A. Post has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).

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