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Dependence on the phospholipid polyunsaturated fatty acids of the oxidative injury of isolated cardiomyocytes

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Abstract

We investigated the influence of PUFA in phospholipids (PL) on the functional characteristics of cultured cardiomyocytes (CM) in basal conditions and during free radical (FR) stress provoked either by the xanthine/xanthine oxidase (X/XO) system or by a (9Z, 11E, 13 (S), 15Z)-13-hydroperoxyoctadecatrienoic acid (13-HpOTrE). CM were grown in media containing either $n-3$ (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA) or $n-6$ (arachidonic acid, AA). These two groups of CM displayed different PUFA $n-6/n-3$ ratio in PL. However, their basal electromechanical characteristics were similar. The X/XO system drastically altered CM functions, without difference between the two groups of CM. 13-HpOTrE caused a moderate and reversible depression in action potential parameters, which was dependent upon the PL PUFA, since the $n-3$-enriched CM exhibited an earlier functional depression but faster recovery. Thus, the peroxidative damage of CM depended on a cross relationship between FR species and the PL PUFA composition.

Keywords: *Rat cardiomyocytes, polyunsaturated fatty acids, cell physiology, free radicals, xanthine/xanthine oxidase, 13-hydroperoxyoctadecatrienoic acid*

Introduction

Numerous epidemiological studies have pointed out the key role played by dietary fats in the development of cardiovascular diseases [1]. In particular, the lower incidence of coronary dysfunction in certain populations was correlated with a food rich in fish oil, characterized by their higher content in long chain polyunsaturated fatty acids (PUFA) of the $n-3$ family [2]. In man, the beneficial influence of the $n-3$ PUFA have been mainly explained through their vascular hypolipemiant, antiagregant and hypotensive actions, but the beneficial effects of these fatty acids seems also to involve a direct action on the heart. However, the cellular mechanisms by which dietary PUFA exert their effects on the normal and diseased myocardium are not clearly delineated.

In various animal models, the enrichment of the diet with fish oils significantly reduces the vulnerability of hearts to hypoxia- or ischemia-induced arrhythmias [3–5]. Furthermore, the hearts of rat enriched with $n-3$ PUFA display enhanced oxygen consumption during the reperfusion, whereas the hearts of rats fed with $n-6$ PUFA consume less oxygen and their metabolic function remains low [6]. There were attempts to clarify which long chain $n-3$ PUFA—eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA)—is responsible for beneficial effects of the oil

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of fish in the pathological heart. It seems that $n-3$ PUFA reproduce the effects of fish oils in the heart, although the results remain contradictory. Kinoshita et al. showed that the dog diet enrichment with EPA reduces ischemia-induced arrhythmias by limiting the intracellular calcium overload [7]. Conversely, hearts isolated from rats fed with a DHA-enriched diet showed a significantly increased index of contracture and higher release of lactate dehydrogenase in the coronary effluent [8]. However, McLennan et al. showed that DHA exerts a more pronounced antiarrhythmic effect [9].

Numerous studies stressed the putative role of free radicals (FRs) in the pathogenesis of heart disorders [10]. During reperfusion of ischemic organs indeed, the restoration of oxygen availability seems deleterious because the production of radical species is assumed to be promoted [11,12]. These FR exert their noxious effects through peroxidative damages of membrane and of intracellular components, resulting in significant changes to the cellular metabolism and functions leading ultimately to cell death [13]. In this context, a diet rich in PUFA may favor free-radical mediated lipid peroxidation. However, there are only few studies which dealing with this issue, and they led to discordant results. In women, a 3 months enrichment of the diet with EPA and DHA significantly decreases the plasma content in vitamin E and raised peroxidative reactions [14]. Conversely, a PUFA-based diet does not seem to enhance lipid peroxidation in plasma and urine compared to a diet rich in saturated fats [15]. In animal experimental models, the diet enrichment with oils of vegetable or of marine origin stimulates peroxidative reactions but in turn seems to promote the intracellular anti-oxidant defenses [16,17].

At the cellular scale, the lipid peroxidation seems dependent on the cell fatty acid composition, but the specific influence of each class of PUFA remains ignored [18]. The use of isolated cardiac cell models offers the opportunity of clarifying which type of cells is responsible for results observed in the intact heart and allows a tight control of the growth environment. In this preparation, it has been shown that PUFA content of the cardiomyocyte (CM) phospholipids (PL) reflects the composition in PUFA of the cell culture medium [19,20]. In physiological conditions, the changes in $n-6/n-3$ long chain PUFA ratio in PL had no major influence on CM contraction and on the action potential amplitude and duration parameters [21]. In simulated ischemic conditions, the $n-3$ PUFA-enriched cells displayed an improved recovery during postischemic reoxygenation, in spite of an apparent enhanced vulnerability to substrate-free hypoxia as compared with the $n-6$-rich CM [21,22]. Finally, cultured CM are sensitive to various forms of radical stress [23,24]. However, the influence of the PL PUFA on the severity of the FR attack has not been yet explored in the myocardial cells.

Therefore, the purpose of this study was to evaluate the influence of $n-3$ or $n-6$ PUFA enrichment in PL on the functional characteristics of *in vitro* cultured CM in basal conditions and during FR stress. Since oxidative myocardial injury could be provoked by oxygen radical species or by lipid peroxidation products, PUFA-controlled CM were exposed to the xanthine/xanthine oxidase (X/XO) system, generating hydroxyl radicals and superoxide, and to a hydroxy-peroxidized fatty acid (9Z, 11E, 13(S), 15Z)-13-hydroperoxyoctadecatrienoic acid (13-HpOTrE) [12,25]. In previous works, we have demonstrated that these sources of radical stress exert significant alterations in functional properties and in cell integrity in cultured CM [23,24].

Materials and methods

Culture of rat cardiomyocytes

Primary rat CM cultures were prepared from 2- to 4-day old Wistar rats, as previously described [26]. After 6 proteolytic treatments (0.1% trypsin; Difco, Detroit, MI), the proportion of CM was increased by two successive pre-platings (30 and 120 min, respectively). The myocyte-enriched suspension was seeded in 60 mm Petri dishes (Falcon Primaria; Becton Dickinson, Oxnard, CA) at a density of 2×10^6 cells/dish. The standard culture medium was composed of Ham's F10 medium supplemented with 10% fetal calf serum, 10% human serum and antibiotics (streptomycin 150 mg/ml and penicillin 200 IU/ml; Seromed, Berlin, Germany). From this standard culture medium, two fatty acid-enriched media were prepared: a $n-6$ rich medium supplemented with arachidonic acid (AA, C20:4$n-6$; 0.1 mM), and a $n-3$ medium supplemented with EPA (C20:5$n-3$; 0.05 mM) and DHA (C22:6$n-3$; 0.05 mM), as previously detailed [19]. In all media, pH was adjusted to 7.4. Sterilization was performed by ultrafiltration (0.22 μm; Millipore, Bedford, MA). Just before use, the calcium activity was standardized at 1.2 mM by addition of a sterile solution of $CaCl_2$, $2H_2O$ (27 mg/ml; Merck, Darmstadt, Germany). These cells were used after a 5-day incubation period at 37°C in a humidified atmosphere containing 5% CO_2.

Fatty acid analysis

The phospholipid fatty acids were analyzed as previously described [27]. The cell lipids were extracted according to Folch et al. [28] and the PL were separated from the non-phosphorus lipids in silica cartridges [29]. Then, the PL fatty acids were analyzed by gas chromatography on a Carbowax 20M

capillary column after transmethylation with BF3-methanol.

Functional characteristics

Forty-five minutes before the experiments, the incubation media were replaced by a Puck's F balanced salt solution and covered with a paraffin oil layer. The experiments were conducted at 36°C in static bath conditions. The dish was placed in an experimental chamber fixed on the stage of an inverted phase contrast microscope (Diavert; Leica-Leitz, Wetzlar, Germany). The dish was continuously flushed with air (600 ml/min). Under these conditions, mean values of pH, PO_2 and PCO_2 of the experimental medium were 7.4 ± 0.1, 100 ± 6.3, 15 ± 2.1 mm Hg, respectively, and remained unchanged during the course of experiments. Intracellular voltages were recorded using conventional KCl-filled, glass–fiber micropipettes (Clark Electromedical Instruments, Reading, UK). The microelectrode mean impedance was 52 MΩ. Intracellular voltages were measured using a high-impedance microelectrode amplifier (VF 180 Biologic, Grenoble, France). The voltage and contraction signals were displayed on a storage oscilloscope (DSO 1604 Gould, Ilford, UK), stored on magnetic tapes (DTR400 Biologic) and transcribed on paper chart with an analogic polygraph (WR3320 Graphtec, Yokohama, Japan). Addition of FR compounds in the bath was performed with a microsyringe (Hamilton, Bonaduz, Switzerland) fixed on a micromanipulator (Leica-Leitz). A quick agitation of the dish allowed a homogenous diffusion of added compounds in the experimental medium. Physiological parameters were recorded over one hour after FR addition.

The following physiological parameters were measured: maximal diastolic potential (MDP), action potential overshoot (OS), action potential amplitude (AP), maximal rate of initial depolarization (V_{max}), action potential duration at 80% duration (APD80), action potential rate (APR), excitation–contraction delay (tC20; time for 20% cell shortening, starting from the action potential upstroke), contraction duration at 80% relaxation (CD80).

Oxygen free radical generation

FR species were generated by the X/XO reaction according the procedure previously published [23]. Xanthine oxidase (XO; Boehringer Mannheim, Mannheim, Germany) was dialyzed overnight against phosphate buffer (pH 7.4) at 4°C. The enzyme activity was adjusted to 0.5 IU/ml with the phosphate buffer by a colorimetric test. Xanthine (X; Sigma, Saint Quentin-Fallavier, France) was prepared in 0.004 N NaOH. Xanthine oxidase and xanthine were added to the extracellular fluid as 100 µl aliquots to obtain the final concentration (0.01 IU/ml and 0.1 mM, respectively). At this concentration, the X/XO mixture mainly produces superoxide and hydroxyl radicals as detected by electron paramagnetic spectroscopy and is able to induce significant functional and cell integrity defects [23,24].

Peroxidized fatty acid: 13-hydroperoxyoctadecatrienoic acid

(9Z, 11E, 13 (S), 15Z)-13-HpOTrE was a kind gift of Servier Research Institute (Suresnes, France). This peroxidized fatty acid was produced by incubation of α-linolenic acid (C18:3$n-3$) with soybean lipoxygenase as previously described [25]. The lipoxygenase metabolites of unsaturated fatty acids were purified by isocratic normal-phase HPLC. Each molecule was identified and quantified by comparison of its retention time with those of references and by analysis of UV spectra, using 2,3000 cm^{-1} as molar extinction coefficient at 235 nm. The purified 13-HpOTrE was stored at −80°C at the stock concentration of 8.64 mM in methanol. Just before experiments and after evaporation, a solution of 13-HpOTrE (1 mM) was prepared in iced Na_2CO_3 (100 mM) (Merck). This solution was added to the experimental medium as 15 µl aliquots to achieve a final concentration of 3×10^{-6} M of 13-HpOTrE, at which functional damage and enzyme loss have been previously described [24,25].

Statistics

Each protocol was performed in 6 cultures from 6 different preparations. In each dish, 3 measurements were performed in 3 defined areas of the cell monolayer during the pre-treatment control period and every ten minutes over one hour after drug addition. The results were expressed as mean ± s.e.m. The data were submitted to a two-way analysis of variance including a random factor (culture dish) and a fixed factor (treatment). The differences were considered as significant for $p < 0.05$.

Results

Fatty acid composition of cell phospholipids

Figure 1 shows the summarized fatty acid content of CM PL. The two groups of CM displayed similar phospholipid contents in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and PUFA (Figure 1(A)). Conversely, important changes were found in the PUFA fractions (Figure 1(B)). The PL of the $n-3$ CM were characterized by a high content in both EPA (5.5%) and DPA (docosapentaenoic acid, C22:5$n-3$; 4.3%) and in DHA (8.3%). Those of the $n-6$ CM were enriched in AA (28.1 vs 12.5% in

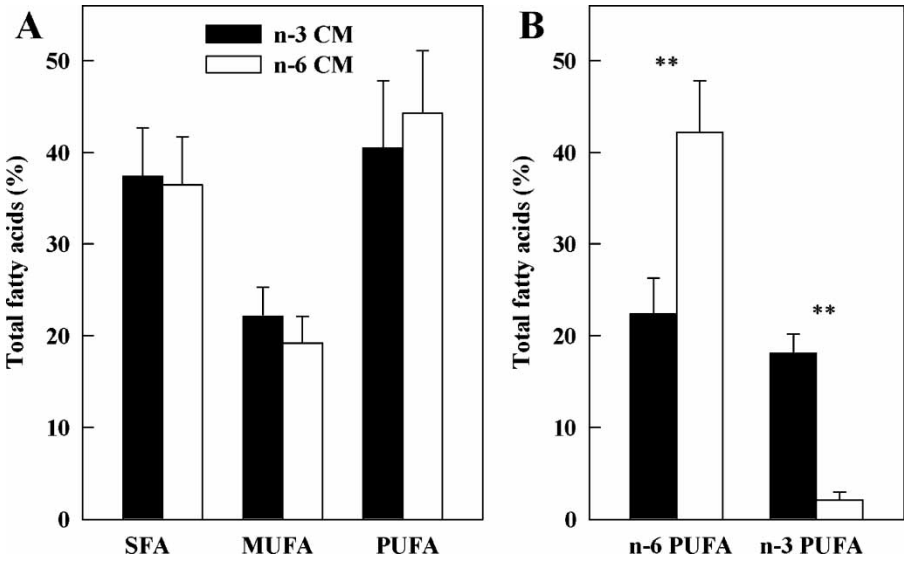

Figure 1. Fatty acid content of membrane phospholipids of rat cardiomyocytes (CM) incubated in the $n-3$ and $n-6$ fatty acid-controlled media. (A) Total phospholipid contents in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA); (B) $n-3$ and $n-6$ PUFA composition of phospholipids. Values are means ± s.e.m. ($n = 2 \times 3$), **: $p < 0.01$. Total phospholipid fatty acids were 128 and 119 μg/mg proteins in $n-3$ and $n-6$ CM, respectively.

$n-3$ CM) and in docosatetraenoic acid (C22:4$n-6$; 8.2%). Therefore, $n-6/n-3$ ratio in the PL fell down to 1.2 in the $n-3$ CM and rose up to 20.1 in the $n-6$ CM.

Nevertheless, in spite of this unbalance in PL $n-6/n-3$ ratio, the two groups of CM displayed similar electrical and contractile characteristics in basal conditions (Figures 2–7, CTRL), apart from a moderately slower initial upstroke of action potentials in $n-6$ CM (Figures 3(B) and 6(B)). These observations are consistent with our previously published data [21].

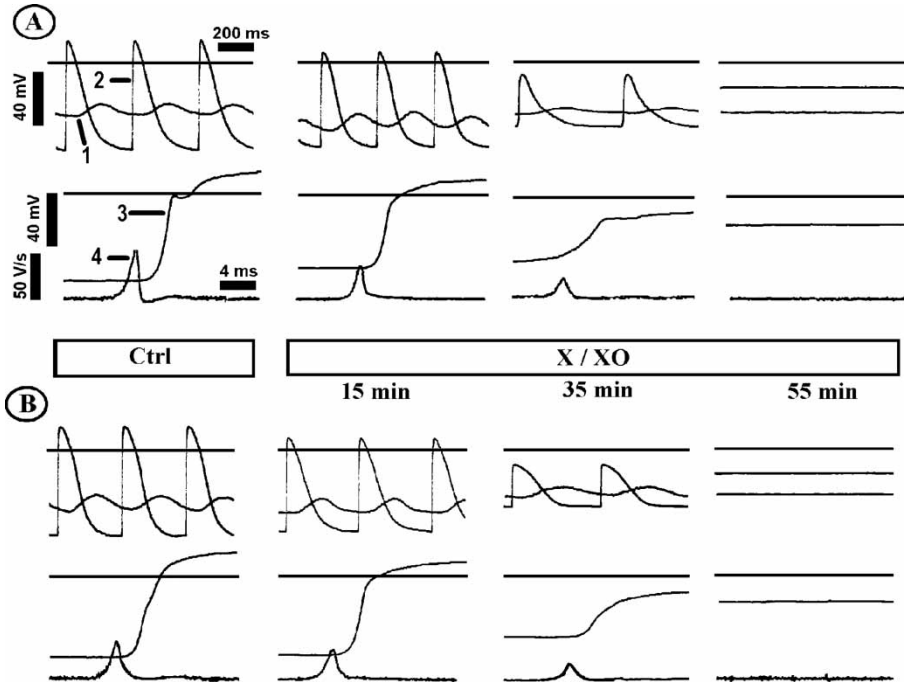

Figure 2. Representative cellular contractions (1), action potentials at slow (2; upper traces) and fast (3; lower traces) sweep speeds, and first derivative (4) of the voltage changes (peak = V_{max}) recorded from cultured rat cardiomyocytes incubated in media enriched with $n-3$ PUFA (A) and in $n-6$ PUFA (B) in basal conditions (CTRL) and submitted to oxidative stress by xanthine/xanthine oxidase (X/XO; 0.1 mM/0.01 IU/ml, respectively). The horizontal baseline indicates the zero potential level. Calibration for contraction (1) is arbitrary. In each row, unretouched trace records from the same culture dish.

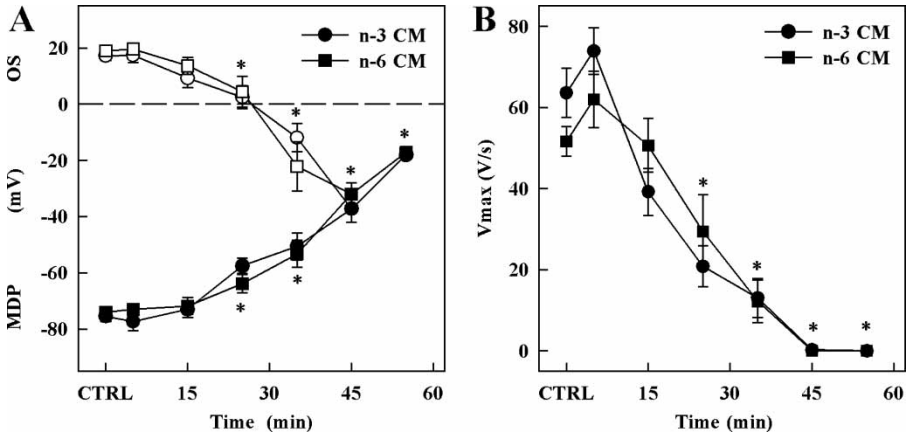

Figure 3. Influence of the addition of the xanthine/xanthine oxidase mixture (0.1 mM/0.01 IU/ml, respectively) on the electrophysiological parameters measured from rat cardiomyocytes (CM) incubated in media enriched with $n-3$ PUFA (circles) and in $n-6$ PUFA (squares). (A) MDP, Maximal diastolic potential (dark symbols); OS, overshoot (open symbols); B: V_{max}, maximal rate of depolarization. Data are means ± s.e.m. ($n = 6$). Differences vs Ctrl: ⋆ $p < 0.05$.

Xanthine/xanthine oxidase peroxidizing system

Figure 2 shows representative recordings of the effects of X/XO system on the electromechanical function of CM enriched with $n-3$ or $n-6$ PUFA. The corresponding measured functional parameters are given in Figures 3 and 4.

In both cell groups, the exposure to X/XO mixture caused a progressive and drastic decrease in the

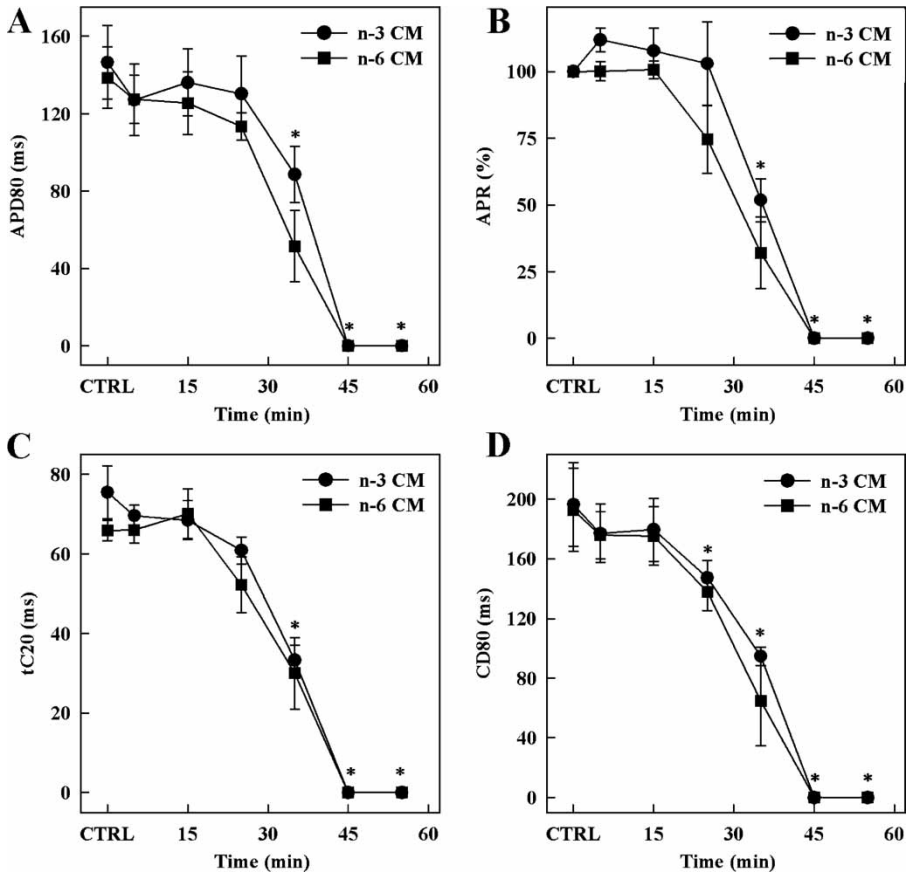

Figure 4. Influence of the addition of the xanthine/xanthine oxidase mixture (0.1 mM/0.01 IU/ml, respectively) on the time and contractile parameters measured from rat cardiomyocytes (CM) incubated in media enriched with $n-3$ PUFA (circles) and in $n-6$ PUFA (squares). (A) APD80, Action potential duration at 80% duration; (B) APR, action potential rate (change, %); (C) tC20, excitation–contraction delay (time for 20% cell shortening, starting from action potential upstroke); (D) CD80, contraction duration at 80% relaxation. Data are means ± s.e.m. ($n = 6$). Differences vs Ctrl: ⋆ $p < 0.05$.

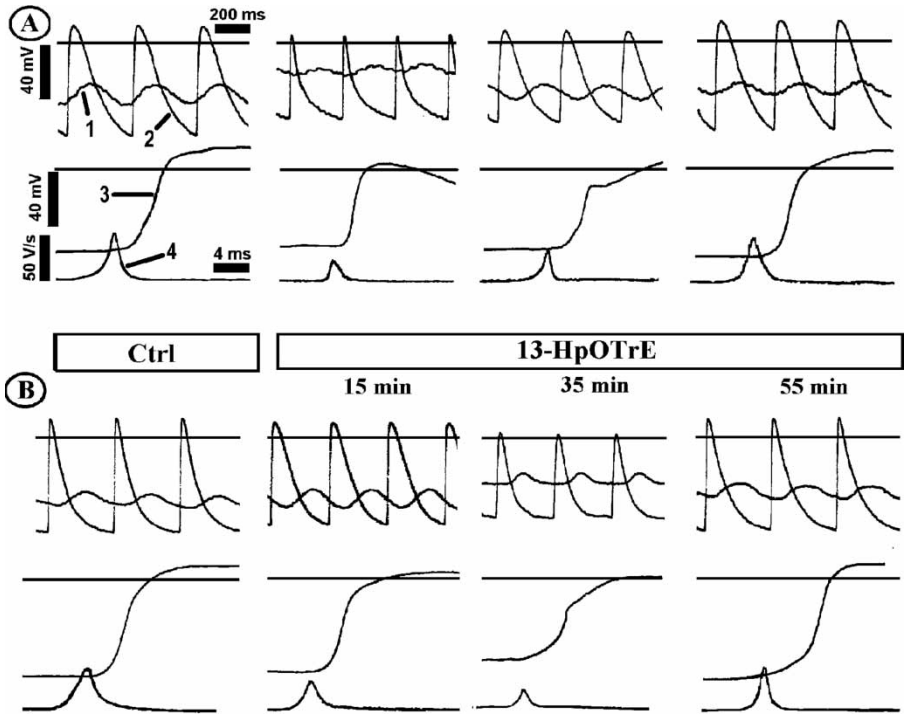

Figure 5. Representative cellular contractions (1), action potentials at slow (2; upper traces) and fast (3; lower traces) sweep speeds, and first derivative (4) of the voltage changes (peak = V_{max}) recorded from cultured rat cardiomyocytes (CM) incubated in media enriched with $n-3$ PUFA (A) and in $n-6$ PUFA (B) in basal conditions (CTRL) and submitted to oxidative stress by (9 Z, 11 E, 13 (S), 15 Z)-13-hydroperoxyoctadecatrienoic acid (13-HpOTrE; 3×10^{-6} M). The horizontal baseline indicates the zero potential level. Calibration for contraction (1) is arbitrary. In each row, unretouched trace records from the same culture dish.

amplitude and duration parameters of the action potential (AP) in the two groups of PUFA-enriched CM (Figures 3 and 4). The MDP was reduced by about 30% after 35 min of X/XO-induced oxidative stress (Figure 3(A), MDP). At the same time, AP OS displayed a similar decrease (Figure 3(A), OS), whereas the upstroke velocity (V_{max}) fell to about only 20% of the value measured in CM before adding the oxidative mixture (Figure 3(B)). At the same step, AP duration at 80% repolarization (APD80) was shortened by about 45% (Figure 4(A)) and the spontaneous APR was only 60% of the initial control values (Figure 4(A)).

The parallel changes in CM contraction parameters are shown in Figure 4(C),(D). Within 35 min of X/XO treatment, the average excitation–contraction delay (tC20) was measured at 33.3 ± 3.7 ms in the $n-3$ CM and at 29.9 ± 8.9 ms in $n-6$ CM (Figure 4(C)).

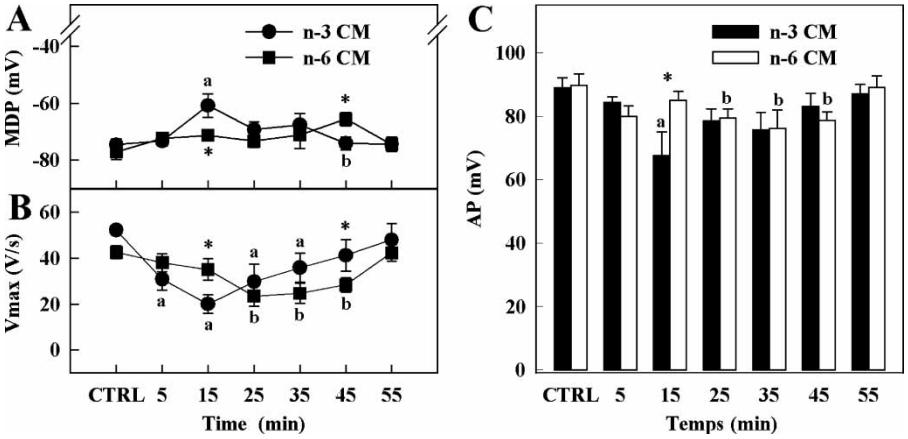

Figure 6. Influence of the addition of (9Z, 11E, 13(S), 15Z)-13-hydroperoxyoctadecatrienoic acid (13-HpOTrE; 3×10^{-6} M) on the electrophysiological parameters measured from rat cardiomyocytes (CM) incubated in media enriched with $n-3$ PUFA (circles and filled bars) and in $n-6$ PUFA (squares and empty bars). (A) MDP, Maximal diastolic potential; (B) V_{max}, maximal rate of depolarization; (C) AP, action potential amplitude. Data are means ± s.e.m. ($n = 6$). *: Differences vs Ctrl; a, b; differences between media at the same time of oxidative treatment; $p < 0.05$.

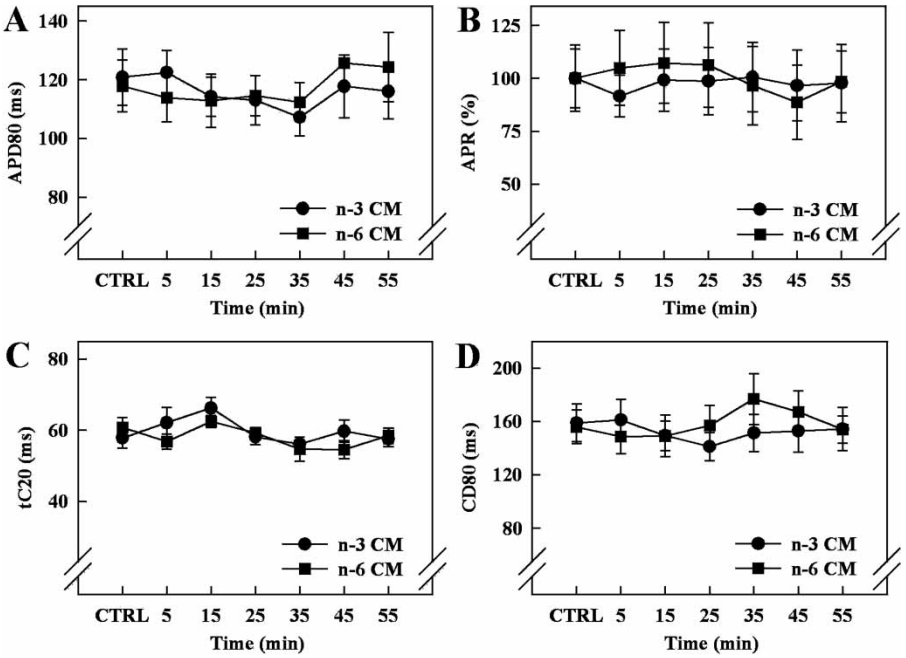

Figure 7. Influence of the addition of (9Z, 11E, 13(S), 15Z)-13-hydroperoxyocta-decatrienoic acid (13-HpOTrE; 3×10^{-6} M) on the time and contractile parameters measured from rat cardiomyocytes (CM) incubated in media enriched with $n-3$ PUFA (circles) and in $n-6$ PUFA (squares). (A) APD80, action potential duration at 80% duration; (B) APR, action potential rate (change, %); (C) tC20, excitation–contraction delay (time for 20% cell shortening, starting from action potential upstroke); (D) CD80, contraction duration at 80% relaxation). Data are means ± s.e.m. ($n = 6$). *: Differences vs Ctrl; a, b; differences between media at the same time of oxidative treatment; $p < 0.05$.

At this time, the contraction duration at 80% of relaxation (CD80) represented only 48 and 38% of their initial values in $n-3$ and $n-6$ CM, respectively (Figure 4(D)).

After 45 min of X/XO treatment, we did not record any spontaneous electrical activity in the $n-3$ and the $n-6$ CM (Figures 2–4(A),(B)) and MDP kept on reducing down to −18 mV at the end of the protocol (55 min; Figure 3(A), MDP). At the same time also, the contractile activity was similarly abolished in the two groups of PUFA-enriched CM (Figures 2, 4(c),(d)).

Whatever the time of exposure to the FR generating system, these data did not show any significant differences in the changes in electrical and contractile parameters between $n-3$ and $n-6$ CM.

Peroxidized fatty acid: 13-Hydroperoxyoctadecatrienoic acid

The influence of 13-HpOTrE (3×10^{-6} M) was investigated in the two groups of CM incubated in media enriched in EPA and DHA ($n-3$ CM) or in AA ($n-6$ CM), respectively. Figure 5(A),(B) shows representative recordings of the action potentials and the contractions of the $n-3$ and $n-6$ CM exposed to 13-HpOTrE during 1 h, and the corresponding measured parameters are given in Figures 6 and 7.

The exposure to the peroxidized fatty acid 13-HpOTrE induced a partial transitory decrease in the action potential amplitude (AP) in the two groups of PUFA-controlled CM (Figures 5(a),(b) and 6(c)). In the $n-3$ CM, this reduction in action potential amplitude was observed after 15 min in the presence of 13-HpOTrE, whereas this diminution occurred from 25 to 45 min of treatment in $n-6$ CM (Figures 5(a),(b) and 6(c)). As a consequence, the 13-HpOTrE-induced changes in action potential amplitude in the two groups of PUFA-controlled CM appeared significantly different at 15 min of treatment, but turned out to be similar in the other protocol phases (Figure 6(C)). These changes in action potential amplitude partly resulted from the reduction in MDP, which occurred earlier in $n-3$ CM than in $n-6$ CM (Figure 6(A)). Because of this distinct time response in the CM membrane potential, the respective changes in MDP in the $n-3$ and $n-6$ CM were significantly different after 15 min (Figure 6(A)). Conversely, MDP recovery in $n-3$ CM was hastened (Figure 6(A), 45 min), as compared with $n-6$ CM, whose MDP restoration was observed later.

The addition of 13-HpOTrE provoked also a significant reduction in the maximal upstroke velocity (V_{max}) in the two groups of CM (Figure 6(B)). This slowing down in the initial action potential depolarization was observed from 5 to 35 min in $n-3$ CM and was again delayed in $n-6$ CM (from 25 to 45 min). As in the case of MDP (Figure 6(A)), the decreases in V_{max} observed in the presence of

13-HpOTrE in $n-3$ and in $n-6$ CM, respectively, were significantly different after 15 min and 45 min of treatment. Given that cultured CM in our conditions obey the voltage dependence of the upstroke velocity, [30] it seems reasonable to assume that the 13-HpOTrE-induced decrease in V_{max} resulted from the diminution in MDP. At the end of the protocols, however, there was no more difference in action potential amplitude, in MDP and in V_{max} in the two groups of PUFA-controlled CM (Figures 5, 6, 55 min).

The effects of 13-HpOTrE addition on the time and contractile parameters are shown in Figure 7(A)–(D). The action potential duration at 80% repolarization (APD80) displayed in the two group of PUFA-controlled CM a slight, non-significant decrease from 15 to 35 min after 13-HpOTrE addition, and this parameter reverted back to values close to control ones at the end of the protocol (Figure 7(A)). On the other hand, the APR remained practically unchanged throughout the protocol (Figure 7(B)). Similarly, the excitation–contraction delay (TC20, measured between the action potentials upstroke and 20% shortening) and the CD80 did not significantly vary in the presence of 13-HpOTrE (Figure 7(C),(D), respectively). A fortiori, these parameters did not indicate any difference in the tolerance of $n-3$ and $n-6$ CM toward 13-HpOTrE.

Discussion

The goal of this study was to determine whether the PUFA lipid profile might influence the extent of oxidative damage to cardiac muscle cells subjected to different FRs attacks. Indeed, there is an evident but theoretical relation between the chemical nature of unsaturated fatty acids and their sensitivity to peroxidation. PUFA are particularly susceptible to autoxidation since they have chemically labile, bisallylic hydrogens, and accordingly the oxidizability of PUFA is dependent on the number of bisallylic positions present in the molecules [31]. PUFA represents thus a preferential site for the initiation of peroxidative processes [32]. However, in spite of this widely invoked theoretical link, this question received little experimental support and the cross relationship between the PUFA lipid content of cells and the nature of the FR stress remains largely elusive.

To examine this, two groups of CM differing by their content in $n-3$ and in $n-6$ phospholipid PUFA of their PL were obtained by incubating the cultured CM in two different PUFA-enriched media [19,22]. As previously described, the basal electromechanical characteristics of $n-3$ and of $n-6$ CM were similar, apart from a moderate decrease in the initial depolarization phase (V_{max}) in $n-6$ CM [21]. These two groups of PUFA-controlled CM were subjected to the X/XO peroxidizing system and to a peroxidized fatty acid (13-HpOTrE). These source of oxidative stress were selected in order to simulate in vitro the initial phase and the propagation phase of the FR attack, respectively [23–25].

As in the case of CM grown in the standard culture medium, our results show that X/XO mixture provoked a progressive and irreversible fall in the electric and mechanical activities of CM enriched in long chain PUFA [23,24]. The main result was that the $n-6/n-3$ PUFA ratio did not affect the extent and the kinetics of X/XO-induced CM dysfunctions, since there was no significant difference in the electromechanical parameters between the two groups of CM in every steps of the protocols. Nevertheless, our previous data demonstrated an effect of X/XO on the membrane contents in long chain fatty acids such as arachidonic, eicosapentaenoic and DHAs [24]. This insensitivity of the X/XO action towards the PUFA profile of the CM can receive two explanations. First, the X/XO generating mixture may exert an oxidative attack of the cellular surface too strong to reveal a difference among the different PUFA-enriched CM. Moreover, our preceding biochemical data showed that the X/XO caused a rather moderate breakdown of the long chain PUFA compared to the profound and irreversible CM injury, suggesting that the cellular proteins may be the main target of X/XO-generated free-radicals [24,33,34].

This relation between the FR stress and the lipid profile has been already approached in other experimental models and through different means, although the literature remains rather poor on this subject. Some studies suggested that PUFA enrichment promotes lipid peroxidation. Indeed, a fish oil-rich diet was reported to induce a decrease in the liver content in vitamin E and to increase the plasmatic and urine markers of the peroxidation in human and rat [14,35]. Moreover, the incorporation of $n-3$ PUFA stimulates the FR production from human tumoral cells and favors the lipid peroxidation in human platelets [36,37]. Lamers et al. [38] showed that ischemia-reperfused hearts from PUFA-fed pigs release less MDA than hearts from saturated fats-fed animals. Conversely, PUFA enrichment may influence cell protection against FRs. Increased content in $n-6$ PUFA in rat hearts raises mitochondrial MnSOD activity [16]. In addition, Fisher et al. [39] showed in human that a decreased $n-3/n-6$ PUFA ratio is linked with a significant reduction in the low-density lipoproteins (LDL) oxidation by macrophages. These data support the notion that a relation may exist between the phospholipid PUFA and the sensitivity towards FR stress of the whole heart, the liver and various cell types but until now there is no clear comparison between the respective influence of $n-3$ and $n-6$ PUFA. Our work relied on an effective comparison between PUFA $n-3$ and $n-6$. The data from the present study confirmed that

X/XO-generated FRs caused drastic functional injury to isolated CM but, because of the severe oxidative attack and the comparatively low fatty acid peroxidation, [24] the PUFA configuration did not seem to have a significant influence in these specific experimental conditions.

Oxygen radicals react with PUFA in the cell membrane to form lipid hydroperoxides and these chain reaction products may also exert damaging effects [32,40,41]. In order to simulated in a more "physiological" manner this subsequent step of oxidative attack, the $n-3$ and $n-6$ enriched CM were exposed to (9Z, 11E, 13(S), 15(-Z))-13-HpOTrE derived from α-linolenic acid [25]. The presence in the bath of 13-HpOTrE caused moderate and reversible electrophysiological changes in the two groups of CM, as previously described in different experimental models [24,41]. These effects are due to the lipid hydroperoxide itself, since the reduced derivative was ineffective [24,25]. Consistently, lipid hydroperoxides provoke enzyme loss, membrane alterations and disturb the activities of various enzymes in vascular cells [42–45]. The reversibility of the lipid hydroperoxide noxious effects is considered to be due to the progressive decay in its membraneous content [46,47]. Conversely, 13-HpOTrE had no effect on CM contractility, whereas in guinea-pig papillary muscle lipid hydroperoxides induced transient rise in diastolic and contraction tension, [41] which cannot be measured in our cellular preparation.

In regard to the respective PUFA enrichment of the cultured CM, our data indicated that the reduction in the electrical parameters is more premature in CM enriched in $n-3$ fatty acids than in $n-6$ CM. Conversely, $n-3$ CM displayed an earlier recovery of the maximal diastolic depolarization (MDP).

This result could be temptatively explained by the higher number of double bounds in the $n-3$ PUFA than in $n-6$ PUFA. This greater degree of unsaturation may affect the rate of peroxidation of $n-3$ PUFA, causing earlier peroxidative functional damage in EPA and DHA enriched CM. Of relevance was the fact that the $n-3/n-6$ PUFA ratio exerts similar influence in conditions of hydroperoxide attack and in those of simulated ischemia-reperfusion, i.e. faster functional defect and recovery are seen in the n3-rich CM [21].

There is no comparative data from the literature concerning the influence of the nature of the phospholipid PUFA on the cardiocellular effects of lipid hydroperoxides. In a different approach however, Illiou et al. [25] investigated the effects of hydroperoxides derived from either $n-3$ PUFA (linolenic acid) or $n-6$ PUFA (AA), respectively, on guinea pig papillary muscle. The authors showed that these two different lipid peroxidation products caused disturbances in electromechanical activity similar in amplitude and kinetics. Therefore, the differences noted in the present study in the sensitivity of $n-3$ and of $n-6$ CM towards 13-HpOTrE should rely on the specific vulnerability to oxidative damage of PUFA incorporated in the membrane and did not seem dependent upon differences in PUFA derivatives resulting from the lipid peroxidative cascade initiated by 13-HpOTrE.

To summarize, the present study demonstrated for the first time the $n-3/n-6$ PUFA ratio of PL modulates the functional injury caused to isolated CM by a C18 lipid hydroperoxide, a protocol designed as a closer approach of an *in situ* FR stress. In these conditions indeed, CM enriched with $n-3$ PUFA displayed both slightly but significantly hastened alteration and restoration of their functional properties, as compared with the $n-6$ CM. How to reconcile these observations with the widely accepted cardioprotective influence of fish oil fatty acids? Taking into consideration our previous investigations, it can be assumed that an earlier arrest may drive the stressed cells into a protective, hibernation state, [48] improving the capability of the cells to recover upon stress alleviation [21,22]. Conversely, the present study demonstrated that this PUFA-dependent modulation of cellular peroxidative damage could not be revealed in a case of lethal, direct attack by oxygen FRs produced from the X/XO system, indicating the key importance of the experimental conditions intended to mimic *in vitro* the FR injury.

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