Oxidative Damage of the Membrane Lipids after Electroporation

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Abstract. Electric field pulses used for cell manipulation can cause irreversible cell damage. The mechanisms of the processes leading to such cell damage are very complicated. Our work demonstrated that exponential electric pulses with intensity of 2–7.5 kV/cm and duration of 5.2 ms were able to initiate peroxidation of fatty acid emulsions, liposomal membranes, red blood and Ehrlich ascite tumor cells. Electric pulses-induced peroxidation of erythrocyte membranes was followed by hemolysis. The electric treatment caused damage of E. coli membrane lipids which was accompanied by decreased cell survival. All these effects depended on field intensity. A relatively good correlation between pulse-induced peroxidation of erythrocyte membranes and hemolysis was observed. These results suggest that free radical mediated processes as lipid peroxidation and/or lipid degradation or fragmentation may be possible causes for electric pulses-induced irreversible cell damage.

Key words: Lipid peroxidation — Electroporation — Membrane lipids

Introduction

Short electric field pulses of high intensity have been used for cell manipulation (electrotransformation, somatic cell electrofusion and other cell engineering techniques) (Zimmermann 1983; Mathew and Tsong 1989). Depending on field intensity and pulse duration reversible or irreversible breakdown of cell membranes takes place (Zimmermann 1983). If the intensity and/or the duration of the pulse exceed a critical value the reversible electrical breakdown of the cell membrane goes over into irreversible one with hard consequences for the cell survival (Zim-
The exact mechanism of this adverse side effect is not clear yet. Recently, it has been demonstrated that exposure of plant protoplasts to electric pulses is associated with ethane release (Biedmeier et al. 1990) which seems to be an indication of lipid peroxidation (Pitkanen et al. 1989).

Peroxidation of lipids is a powerful destructive process (Slatei 1984). It may be initiated by different factors one of which seems to be electric field with high intensity. In this context, the aim of the present work was to verify the possibility for initiation of lipid peroxidation in artificial membranes and in cells exposed to varying field intensities.

**Abbreviations:** TBARS thioarbituric acid reactive substances MDA malondialdehyde TBA thioarbituric acid HO hydroxyl radical, UV ultra-violet SOD superoxide dismutase E1 2,5,7,8-tetramethyl-6-chromanol

**Materials and Methods**

Phospholipids were isolated from egg yolk as described by Clark and Switzer (1977). Liposomes (2 mg phospholipid/ml glass distilled water) were prepared by the method of Batsz and Korn (1973). Linoleic acid 1-o-phosphatidylincholine type A-E from egg yolk, 1,2-dilinoleoyl-sn-glycero-phosphocholine, 1,2-dioleoyl-sn-glycero-phosphocholine, and 1,2-dipalmitoyl-sn-glycero-phosphocholine were obtained from Sigma. 2,5,7,8-tetramethyl-6-chromanol (F1) synthesized in the laboratory of prof. E. Kagan was a generous gift from prof. V. Kagan.

Ehrlich ascite tumor cells from mice peritoneal exudate E. coli strain LE 392 in logarithmic phase and rat erythrocytes were used. All cell preparations were three times washed and resuspended in 290 mmol sucrose or in isotonic glycerol to a final concentration of 1.5 x 10^6 cells/ml for E. coli and Ehrlich ascite tumor cells or to 50% by hematocrit for red blood cells. The conductivity of all samples was less than 10^-2 S m^-1.

High voltage source in combination with capacitor (26 μF) was used for generation of exponentially decaying electric pulses. The time constant of the exponential pulse is usually in the order of milliseconds (Anderson and Evans 1988). Thyrister was utilized as switching element to discharge the capacitor into the electroporation chamber. The chamber had two stainless steel electrodes. The interelectrode distance was 0.15 mm. The circuit included 20 Ω resistor in series and 200 Ω resistor in parallel with the sample which determined a theoretical time-constant of 5.2 ms.

The extent of lipid peroxidation was estimated using 100 μl samples for measurement of thioarbituric acid reactive substances (TBARS) (Fodor and Marx 1988) and conjugated dienes (Buerg and Auster 1978). To avoid artificial oxidation during the extraction of lipids all solvents were saturated with nitrogen. Results are expressed as nmol of malondialdehyde (MDA) per ml suspension and nmol of conjugated dienes per ml suspension. Absorption coefficients of 1.56 x 10^5 mol^-1 at 532 nm (Fodor and Marx 1988) and 2.8 x 10^4 mol^-1 at 233 nm (Lichtenberg et al. 1988) were used to estimate the amount of MDA and conjugated dienes, respectively.

Peroxidation of linoleic acid was estimated by measuring the absorbance of the emulsion at 233 nm before and immediately after the exposure to electric pulses.
For the measurement of hemolysis erythrocytes were washed two times to remove hemoglobin released at the moment of electro-exposure and were resuspended to the same hematocrit in isotonic saline. After 120 min of incubation and centrifugation of the samples the absorbance of the clear supernatant was measured at 525 nm after suitable dilution. 100% hemolysis was obtained by replacing saline with distilled water containing 0.1% Triton X 100. In part of the samples O2 concentration was decreased and they were electro-treated under nitrogen stream. These samples were washed with nitrogen-saturated saline and were resuspended in the same medium. The water-soluble radical scavenger uric acid was added to the suspending medium immediately before the electro-exposure. 2,5,7,8-Tetramethyl-6-chromanol (E3) was introduced by means of 2,3-dipalmitoyl-sn-glycerophosphocholine liposomes as described by Niki et al. (1988). After the electro-exposure the samples were washed and resuspended as described above.

E. coli cell suspension in saline was oxidized either by incubation with Fenton's reagent (10 μmol/ml H2O2 + 5 μmol/ml FeSO4) or by UV irradiation. UV irradiation was performed for 20 mm using an ultra-violet lamp BLM-12 at a distance of 25 cm. Aliquots were taken and lipids were extracted with chloroform/methanol 2:1 (v/v). The chloroform layer was washed two times with distilled water and was evaporated under nitrogen. The residue was dissolved in ethanol. UV spectrum of ethanol solution was recorded on Shimadzu UV-VIS Recording Spectrophotometer UV-200.

Results are mean ± S.E.M. All experiments were performed at least in triplicate independent preparations.

**Results and Discussion**

Fig. 1 presents the level of conjugated dienes in linoleic acid emulsion as a function of the number of the electric pulses. As it is seen, linear relationship exists between

![Figure 1](image_url)

**Figure 1.** Relationship between the number of electric pulses and conjugated dienes in linoleic acid emulsion. Linoleic acid (100 nmol/ml) in glass distilled water was exposed to electric pulses of 5 kV/cm (△). In part of the samples pO2 was decreased by bubbling with nitrogen (○).
pulses number and conjugated dienes. The decrease of $pO_2$ in the samples by bubbling with nitrogen decreases conjugated dienes level. These results indicated that conjugated dienes were formed practically at the moment of the exposure and that oxygen was involved in this process.

Figure 2. UV absorption spectra of phosphatidylcholine with varying double bonds number. Phospholipid liposomes prepared from 1,2-dipalmitoyl-sn-glycerophosphocholine, 1,2-dioleoyl-sn-glycerophosphocholine, 1,2-dilinoleoyl-sn-glycerophosphocholine or egg-yolk phosphatidylcholine were exposed to single electric pulse (10 kW/cm). Immediately after the treatment lipids were extracted and UV spectra in ethanol were recorded. Controls were not electo-treated. 1 - control; 2 - electo-treated.

To be sure that the increase of absorbance is due to conjugated dienes, i.e. to lipid peroxidation, liposomes prepared from phosphatidylcholine containing different number of double bonds were subjected to electric pulses (Fig. 2). It was
found that peaks characteristic for conjugated dienes and possibly to ketone dienes (Recknagel and Glende 1984) appeared only in the preparations containing two or more double bonds (1,2-dilinoleoyl-sn-glycerophosphocholine and egg yolk phosphatidylycholine). The electric treatment of liposomes prepared from 1,2-dipalmitoyl-sn-glycerophosphocholine (without double bonds) or 1,2-dioleoyl-sn-glycerophosphocholine (with one double bond) did not cause increase of the absorption in the 230–300 nm region.

It was also found (Fig. 3) that in phospholipid liposome suspension containing ADP-Fe$^{2+}$ (concentration 10 nmol/ml) the initiated by single 7.5 kV/cm pulse lipid peroxidation proceeded significantly faster. This effect strongly depended on field intensity (Fig. 4). As it is seen, the rate of MDA generation increases progressively with the increase of field strength. In the interval between 1 and 5 kV/cm similar relationship was observed also in samples which did not contain ADP-Fe$^{2+}$ (Fig. 4). One possible explanation of this observation is that significant amount of iron is liberated from the electrodes during the pulse. Using the method of Buettner (1988) we found, however, that our preparations contained not more than 0.8 μmol/l transition metal(s) and the liberation of iron from the electrodes was negligible (less than 0.1 μmol/l).

To shed some light on the reactive species involved in the initiation of lipid peroxidation the effect of $^{1}$O$_{2}$ and HO$^{'}$ scavengers at varying concentrations was tested. In Table 1 only scavengers which do not interfere with lipids UV spectrum are listed.
Figure 4. Relationship between field intensity and TBARS generation in phospholipid liposomes. Phospholipid liposomes with (○) or without (△) addition of ADP Fe³⁺ were exposed to varying field intensities. After the exposure the samples were incubated 60 mm at 37°C and TBARS was estimated.

Table 1. Effect of HO⁻ and ¹⁸O₂ scavengers on electric pulses induced conjugated dienes generation. 1,2-dilinoleoyl-sn-glycerophosphocholine liposomes (2 mg phospholipid/ml) were exposed to single electric pulse with intensity 10 kV/cm. Scavengers were added before the electro exposure to a final concentration of 100 mmol/l. Controls contained the same ingredients but were not exposed to electric pulses. Immediately after the treatment, the lipids of the samples and the respective controls were extracted with N₂-saturated chloroform/methanol (2:1 v/v) and then UV spectra in ethanol were recorded. Conjugated dienes were calculated by the differences in absorbance at 233 nm.

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Conjugated dienes (nmol/ml)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>NaN₃</td>
<td>110.5 ± 2.8</td>
<td>81</td>
</tr>
<tr>
<td>Formate</td>
<td>20.3 ± 1.4</td>
<td>90</td>
</tr>
<tr>
<td>Benzoate</td>
<td>10.7 ± 1.1</td>
<td>73</td>
</tr>
<tr>
<td>DMSO</td>
<td>29.8 ± 0.9</td>
<td>97</td>
</tr>
<tr>
<td>Mannitol</td>
<td>31.1 ± 0.6</td>
<td>93</td>
</tr>
</tbody>
</table>

At concentration 100 mmol/l all HO⁻ scavenger inhibited conjugated dienes generation. Similar effect was observed with the singlet oxygen scavenger NaN₃. At lower concentration (10 mmol/l) the inhibitory effect of the scavengers was negligi-
able (data not shown). Almost complete inhibition was observed by the lipid-soluble radical scavenger 2,6-ditert-butyl-p-cresol at much lower concentration-0.1 mmol/l (data not shown). We also found that catalase (1100 U/ml) and SOD (33 U/ml) exerted significant inhibition. This effect seems to be not due to their enzymatic functions. Their inactivation by heating did not abolish the inhibition. The inhibition by hydroxyl radical- and singlet oxygen-scavengers may be taken as an evidence for HO and/or $^{1}\text{O}_2$ involvement in electric pulse-initiated lipid peroxidation. However, results based on the use of free radical scavengers should be interpreted with caution. As a rule they have low specificity (Packer et al. 1981; Niki 1987) and can interact with a number of activated species. On the other hand only radicals or activated species formed in the close proximity to the membranes or even in the lipid phase will be able to initiate peroxidation. Because of the heterogeneity of the system the effective concentration of the scavenger at the radical formation site may be low (Chevion 1988). This possibly explains the fact that the water-soluble scavengers are effective inhibitors only at high concentrations.

It appears likely that electric pulses are able to initiate peroxidation and accelerate the iron-catalyzed peroxidation in model lipid systems. This, however, does not mean that electric pulses will be able to initiate peroxidation in cells. Cell membranes are protected against peroxidation, and oxygen radicals and other activated species are rapidly scavenged by specific enzyme systems. To test the possibility for initiation of lipid peroxidation in cells we used suspensions of erythrocytes, Ehrlich ascite tumor cells and E. coli. These three types of cells differ considerably with respect to their susceptibility towards peroxidation. Cancer cells are highly protected against peroxidation (Chevion 1988, Tretter et al. 1989) and E. coli membranes do not contain polyunsaturated fatty acids (Trauble and Overath 1973). In contrast erythrocyte membrane are rich in polyunsaturated fatty acids and might be expected to be more susceptible to peroxidation (Niki et al. 1988). As seen in Fig. 5 exposure of erythrocytes to pulses with increasing intensity elevated the MDA content. As far as this is an indication of lipid peroxidation it might be expected that membrane disruption supervene. Indeed, hemolysis was observed if erythrocytes were resuspended in isotonic saline and allowed to stand. The extent of hemolysis depended on the field strength and correlated with MDA level (Fig. 5). Calculations based on MDA concentration indicated that a small part of all membrane lipids was oxidized by electric pulses. A question arises whether such an extent of peroxidation may lead to hemolysis. To answer this question, in separate experiments we incubated erythrocytes with varying concentrations of KO$_2$. It was found that KO$_2$ caused hemolysis and increased MDA level (Table 2). As seen in the Table, MDA level corresponding to significant hemolysis is at the same order of magnitude as in suspensions exposed to electric pulses. The low level of TBARS observed at the highest KO$_2$ concentration seems very likely to be due to superoxide interaction with MDA (Bonnes-Taurel et al. 1992) or to formation
**Figure 5.** Peroxidation and hemolysis of erythrocytes exposed to electric pulses. 50% erythrocyte suspension was exposed to varying field intensities. Aliquots were taken for TBARS (△) and hemolysis (○) measurement. For the measurement of hemolysis erythrocytes were washed and resuspended in isotonic saline. After 120 min incubation at 37°C the samples were centrifuged and absorbance of clear supernatant was measured at 525 nm.

**Table 2.** KO₂-induced lipid peroxidation and hemolysis. 50% erythrocyte suspension in isotonic saline was incubated in presence of KO₂ 100 μl aliquots were taken for TBARS measurement. Hemolysis was estimated by measuring the absorbance at 525 nm of clear supernatant after centrifugation of the samples.

<table>
<thead>
<tr>
<th>KO₂, μmol/ml erythrocytes suspension</th>
<th>MDA, nmol/ml</th>
<th>Hemolysis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.8 ± 0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>1.6 ± 0.3</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>2.5 ± 0.3</td>
<td>5.2</td>
</tr>
<tr>
<td>10</td>
<td>6.6 ± 0.4</td>
<td>59.0</td>
</tr>
<tr>
<td>20</td>
<td>1.2 ± 0.1</td>
<td>86.0</td>
</tr>
</tbody>
</table>

of excess of H₂O₂ which degrade MDA (Kostka and Kwan 1989). Thus, it appears that peroxidation induced by electric pulses may be the reason for hemolysis. It should be kept in mind however, that calculations based on TBARS are not correct. TBARS does not reflect directly the amount of oxidized lipids (Bonnes-Taurel et
al. 1992) and the formation of the red pigment depends on various conditions (Kosugi et al. 1989; Janero and Bughardt 1989). Additional support of the proposal that electric pulses-induced hemolysis is due to lipid peroxidation was given by the experiments in which the effect of free radical scavengers was tested (Table 3). It was found that uric acid at concentration 100 mmol/l and E₁ at concentration 0.3 mmol/l strongly suppressed pulses-induced hemolysis. Significant decrease of the hemolysis was observed also in the samples where the concentration of oxygen was low.

Table 3. Effect of radical scavengers on electric pulses-induced hemolysis. 50% erythrocyte suspension was exposed to single electric pulse with intensity 3.0 kV/cm. Scavengers were added before the electro-exposure. 2,5,7,8-tetramethyl-6-chromatol (E₁) was included in 1,2-dipalmitoyl-sn-glycerophosphocholine liposomes. In this case electro-exposure was performed after 30 min incubation of erythrocyte suspension with the E₁-containing liposomes. After the electro-exposure erythrocytes were washed, resuspended in isotonic saline and incubated for 120 min at 37°C. The samples were centrifuged; the clear supernatants were suitable diluted and their absorbance at 525 nm were measured.

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>A₅₂₅</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-treated</td>
<td>0.005 ± 0.001</td>
<td>–</td>
</tr>
<tr>
<td>electro-treated</td>
<td>0.136 ± 0.009</td>
<td>–</td>
</tr>
<tr>
<td>+10 mmol/l uric acid</td>
<td>0.117 ± 0.004</td>
<td>14</td>
</tr>
<tr>
<td>+100 mmol/l uric acid</td>
<td>0.034 ± 0.001</td>
<td>75</td>
</tr>
<tr>
<td>+0.3 mmol/l E₁</td>
<td>0.046 ± 0.008</td>
<td>66</td>
</tr>
<tr>
<td>+0.1 mmol/l E₁</td>
<td>0.087 ± 0.011</td>
<td>36</td>
</tr>
<tr>
<td>under N₂</td>
<td>0.095 ± 0.003</td>
<td>30</td>
</tr>
</tbody>
</table>

As judged by conjugated dienes, electric pulses are able to initiate lipid peroxidation in such highly resistant to peroxidation cells as Ehrlich ascite tumor cells (Fig. 6). In comparison with erythrocytes significant peroxidation was observed at much higher field intensities.

We tried also to investigate the ability of electric pulses to damage E. coli membrane lipids. E. coli does not contain polyunsaturated fatty acids which possibly means that lipid peroxidation can not take place in its membranes. On the other hand, additional experiments (Fig. 7) demonstrated that exposure of E. coli suspension to hydroxyl radicals or to UV-irradiation induced characteristic changes of membrane lipids absorption spectrum. The same changes were observed when E. coli was exposed to electric pulses. Based on this observation we concluded that membrane alterations caused by electric pulses might be similar to those caused by
Figure 6. Conjugated dienes generation in 1 h lethascite tumor cell suspension 1.5 x 10^7 cells/ml isotonic glycerol were exposed to electric pulses. Immediately after the exposure lipids were extracted: chloroform layer was evaporated and the residue was dissolved in ethanol.

Figure 7. UV-absorption spectrum of E. coli membrane lipids 1.5 x 10^7 cells/ml saline were irradiated 20 min with UV-light (curve 3) or were incubated 20 min with 10 μmol/ml H_2O_2 and 5 μmol/ml lСО_4 (curve 2). 1.5 x 10^7 cells/ml isotonic glycerol were exposed to two pulses with intensity 7.5 kV/cm (curve 4). Lipids were extracted with chloroform/methanol; the chloroform layer was evaporated and the residue was dissolved in ethanol. Controls (curve 1) were not exposed to electric pulses.
Figure 8. Relationship between $\Delta A_{220}$, cell survival and field intensity. E. coli ($1.5 \times 10^7$ cells/ml isotonic glycerol) were exposed to varying field strength. Lipids were extracted; chloroform layer was evaporated and absorbance at 220 nm in ethanol was measured. $\Delta A_{220}$ is the difference of absorbance at 220 nm between controls and electro-exposed samples. (Δ) $\Delta A_{220}$, (○) cell survival.

Thus, our experiments demonstrated that exposure of artificial membranes or varying types of cells to electric pulses may cause oxidative damage of membrane lipids. It may be proposed that electric pulses trigger nonspecific redox reactions which possibly generate free radicals or other excited species in the water medium as well as on the membrane surface or even inside the lipid bilayer. Because of the short life-time of these species it has been postulated that only these generated in close proximity to the membrane or inside the membrane can cause damage. On the other hand it has been shown that the membrane voltage induced by external field is a function of the location of the membrane surface (Zimmermann 1983). For this reason it may be expected that excited species are generated at highest concentration in the pole region. If it is so then the concentration of free radical scavengers at this region should be high enough in order to exert measurable protection. This seems to be a reasonable explanation for the need of
high concentrations of water-soluble scavengers and for the much better efficiency of the lipid-soluble scavengers. We believed that electric pulses generate excited species only in limited areas of the membrane lipid bilayer but the local concentration of such damaging species in the site of their formation is relatively high. It seems obvious that in artificial membranes the concentration of such species will be proportional to the strength of the external field. If these membranes contain polyunsaturated fatty acids then peroxidation will start and its intensity, at least in some extent, will depend on the field strength. In cells, however, the concentration of these species and the consequent oxidative damage will depend on some additional factors the most important of which seems to be the antioxidant defence system of the cell (Tretter et al. 1989). If the concentration of the excited species exceeds the capacity of the natural antioxidant systems an oxidative damage of the membrane constituents takes place. Such oxidative alterations may have deleterious consequents (Comporti 1989) including increase of membrane permeability (Witting 1965), decrease of the deformability, etc. resulting in osmotic destruction of the cell. These oxidative damages could be avoided to some extent by addition to the cell-suspending media of compounds such as mannitol, sorbitol, etc., which are radical scavengers and to not interfere with biological reactions. The need of such compounds in the cell media has been empirically established and they are routinely used in electromanipulation procedures (Forster and Neumann 1989).

References

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