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# Oxidative effects of nanosecond pulsed electric field exposure in cells and cell-free media

Olga N. Pakhomova [a], Vera A. Khorokhorina [a], Angela M. Bowman [a], Raminta Rodaitė-Riševičienė [b], Gintautas Saulis [b], Shu Xiao [a,c], Andrei G. Pakhomov [a,*]

[a] Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, VA, USA
[b] Department of Natural Sciences, Vytautas Magnus University, Kaunas, Lithuania
[c] Department of Electrical and Computer Engineering, Old Dominion University, Norfolk, VA, USA

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ABSTRACT

Nanosecond pulsed electric field (nsPEF) is a novel modality for permeabilization of membranous structures and intracellular delivery of xenobiotics. We hypothesized that oxidative effects of nsPEF could be a separate primary mechanism responsible for bioeffects. ROS production in cultured cells and media exposed to 300-ns PEF (1–13 kV/cm) was assessed by oxidation of 2′,7′-dichlorodihydrofluoresein ($H_2$DCF), dihidroethidium (DHE), or Amplex Red. When a suspension of $H_2$DCF-loaded cells was subjected to nsPEF, the yield of fluorescent 2′,7′-dichlorofluorescein (DCF) increased proportionally to the pulse number and cell density. DCF emission increased with time after exposure in nsPEF-sensitive Jurkat cells, but remained stable in nsPEF-resistant U937 cells. In cell-free media, nsPEF facilitated the conversion of $H_2$DCF into DCF. This effect was not related to heating and was reduced by catalase, but not by mannitol or superoxide dismutase. Formation of $H_2O_2$ in nsPEF-treated media was confirmed by increased oxidation of Amplex Red. ROS increase within individual cells exposed to nsPEF was visualized by oxidation of DHE. We conclude that nsPEF can generate both extracellular (electrochemical) and intracellular ROS, including $H_2O_2$ and possibly other species. Therefore, bioeffects of nsPEF are not limited to electropermeabilization; concurrent ROS formation may lead to cell stimulation and/or oxidative cell damage.



## Introduction

High-intensity electric pulses of nanosecond duration, also frequently referred to as nanosecond pulsed electric field (nsPEF)[1], have been introduced recently [1,2] as a new agent with potential capability of penetrating plasma membrane and permeabilizing the internal cell structures. Recent studies using widely varied techniques established permeabilization of the mitochondrial membrane [3], endoplasmic reticulum [4], and plasma membrane [5–10] by nsPEF; other studies evaluated pore size, lifetime, ionic permeability, and consequences for cell function [5,8–13]. Membrane permeabilization by nsPEF disrupted ion and water balance across the membrane, leading to isoosmotic volume changes and various types of blebbing [5,8,10]. NsPEF-induced uptake of extracellular $Ca^{2+}$ and its release from intracellular depot could trigger a wide spectrum of $Ca^{2+}$-dependent biochemical cascades [4,14–16]. Cell damage by nsPEF could cause necrotic or apoptotic cell death [17–20], and the method of nanoelectroablation by nsPEF has shown promise in cancer therapy [21–25].

Although membrane permeabilization is a well-established principal mechanism of nsPEF bioeffects, it is not necessarily the only mechanism. Some complex effects of nsPEF, including inhibition of voltage-gated $Na^+$ and $Ca^{2+}$ transmembrane currents [26] and the phenomenon of electrosensitization [27] cannot be simply explained by formation of membrane pores. With the peak electric field of up to hundreds of kilovolts per centimeter and energy absorption rates on the order of gigawatts per gram, other hypothetical mechanisms of nsPEF effects are cell electrodeformation [28,29], mechanical stress due to thermoelastic expansion [30,31], conformational changes in molecular structure [32,33], and electrolysis and electrodissociation of molecules, possibly including ROS formation [18,34].

* Corresponding author. Address: Frank Reidy Research Center for Bioelectrics, Old Dominion University, 4211 Monarch Way, Suite 320, Norfolk, VA 23508, USA. Fax: +1 757 314 2397.
*E-mail addresses:* apakhomo@odu.edu, 2andrei@pakhomov.net (A.G. Pakhomov).

[1] *Abbreviations used:* CAT, catalase; DCF, 2′,7′-dichlorofluorescein; DHE, dihidroethidium; DIC, differential-interference contrast; DMSO, dimethylsulfoxide; EP, electric pulses; FBS, fetal bovine serum; $H_2$DCFDA, dichlorodihydrofluorescein diacetate; $H_2$DCF, dichlorodihydrofluorescein; HRP, horseradish peroxidase; IRE, irreversible electroporation; NSCC, nonselective cation channels; nsPEF, nanosecond pulsed electric field; PBS, phosphate-buffered saline; PS, physiological solution; ROS, reactive oxygen species; SOD, superoxide dismutase; TBHP, *tert*-butyl hydroperoxide.

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Specifically, several independent lines of evidence suggested oxidative damage in nsPEF-treated cells. Similar to the effect of sparsely-ionizing radiations, the cytotoxic effect of nsPEF was readily modified by the presence of oxygen, and exposure of cells under hypoxic conditions increased the survival about twofold [34]. Other similarities with ionizing radiations were the dependence of cell survival on the absorbed dose and the typical shape of the survival curve (a shoulder followed by an exponential decline) [34,35]. Cytophysiological manifestations of a chemically-induced oxidative stress [36,37] were amazingly similar to cellular effects of nsPEF-induced damage [5,6]. The current–voltage (*I–V*) characteristics and blocker sensitivity of nonselective cation channels (NSCC) stimulated by a free-radical donor calphostin C [37] were indistinguishable from properties of nanoelectropores, thereby raising a question if these (yet unidentified) NSCC and nanoelectropores might actually be the same entity [5,8]. Finally, findings of DNA damage following nsPEF exposure [38], stimulation of ROS production following traditional electroporation with long (0.1–15 ms) electric pulses [39–41], and demonstration of the production of ROS during electrolysis [42] provide further support to the feasibility of looking at ROS formation as a potential mechanism of nsPEF bioeffects.

In this study, we demonstrate that nsPEF exposures can cause oxidation in cell suspensions, in cell-free media, and in individual cells. NsPEF-induced oxidation is a complex process that involves both electrochemical and biological pathways. The impact of nsPEF-generated oxidants ($H_2O_2$) on cell survival and physiology was only briefly addressed in this study and will be reported separately.

## Methods

### *Cell lines and propagation*

All cell lines were obtained from ATCC (Manassas, VA) and propagated at 37 °C with 5% $CO_2$ in air. Suspension cells, Jurkat clone E6-1 (human T-lymphocytes) and U-937 (human monocytes) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Anchor-dependent cells (CHO-K1, Chinese hamster ovary) were propagated in Ham's F12K medium supplemented with 10% FBS. The media also contained 100 I.U./ml penicillin and 0.1 μg/ml streptomycin. The media and its components were purchased from Mediatech Cellgro (Herdon, VA) except for serum (Atlanta Biologicals, Norcross, GA).

Although we used several cell lines, this study was not focused on comparison of cell line-specific characteristics of nsPEF effect. Instead, we intended to demonstrate that nsPEF effects are not unique for just a single, randomly chosen cell line but can be observed in diverse cell lines, albeit with quantitative differences.

### *Fluorescent dyes, buffers, and chemicals*

Fluorescent dyes dichlorodihydrofluorescein diacetate ($H_2$-DCFDA), carboxy-$H_2$DCFDA, *N*-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red), and dihidroethidium (DHE) were purchased from Invitrogen (Eugene, OR). Other chemicals, including horseradish peroxidase (HRP), *tert*-butyl hydroperoxide (TBHP), superoxide dismutase from bovine erythrocytes (SOD), and catalase (CAT) were obtained from Sigma–Aldrich (St. Louis, MO).

Chemicals and dyes were diluted in RPMI medium without phenol red or serum, or in phosphate-buffered saline (PBS). In some experiments, we also used a physiological solution (PS) composed of (in mM): 136 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4) [5,9,27].

### *Oxidative response in cell suspensions*

$H_2$DCFDA dye is perhaps the most popular selection for non-specific ROS detection and as an oxidative burst indicator [43–46]. The nonfluorescent $H_2$DCFDA crosses the cell membrane and, under the action of intracellular esterases, is deacetylated into 2′,7′-dichlorodihydrofluorescein ($H_2$DCF). This substance is also non-fluorescent, but has relatively low ability to escape from the cell through the plasma membrane. Oxidation of $H_2$DCF produces highly fluorescent 2′,7′-dichlorofluorescein (DCF).

$H_2$DCF is highly sensitive to a broad spectrum of ROS, but, depending on the environmental conditions, can also undergo fast self-oxidation and photooxidation, which must be carefully taken into account. The state of knowledge about this dye, its utility and caveats for ROS detection have been reviewed recently [43].

$H_2$DCFDA was stored at −20 °C as 10-mM aliquots in anhydrous DMSO. For dye loading, cells were collected by centrifugation and resuspended at 1–1.4 × $10^6$ cells/ml in RPMI without FBS. Following a 30-min incubation at 37 °C with 20–50 μM of the dye, cells were separated by centrifugation and resuspended in a fresh RPMI medium without phenol red and without FBS. The final cell density was adjusted to 0.6–1.2 × $10^6$ cells/ml unless stated otherwise. The cell suspension was dispensed in 1- or 2-mm gap electroporation cuvettes (BioSmith Biotech, San Diego, CA) and subjected to nsPEF exposure or sham exposure, or was used for parallel control. Upon completion of all scheduled exposures, the samples were dispensed in triplicates into a 96-well black-wall plate and read with a Synergy 2 microplate reader (BioTEK, Winooski, VT), with ex./em. settings at 485/528 nm. Depending on the specific protocol, the readings could be performed repeatedly at different time intervals after the exposure. Matched parallel control samples accompanied each exposed sample and were treated in precisely the same manner, excluding only the exposure procedure itself.

As a positive control for ROS production, dye-loaded cells were incubated at 37 °C with 200 μM TBHP. The incubation started before the first nsPEF exposure, and the sample was read together with all other samples.

The dye and dye-loaded cell samples were at all times protected from light. Overhead illumination in the experimental lab was turned off for the duration of experiments.

### *Oxidative response in cell-free media*

As a first step, $H_2$DCFDA was converted into $H_2$DCF by alkaline deacetylation as recommended by the supplier. The 10-mM stock of $H_2$DCFDA was mixed with an equal volume of methanol and 1/2 volume of 2 M KOH. Following 1-h incubation, pH was adjusted to 7.0 using 1 M HCl, and the mixture was diluted with a test medium (either RPMI without phenol red and FBS, or PS).

The final concentration of $H_2$DCF in the medium was 20 or 70 μM. In some series of experiments, ROS scavengers were added to the medium prior to nsPEF exposure: mannitol (10 mM), SOD (50 μg/ml), CAT (0.5 mg/ml, which is approx. 1000–2500 units/ml), or a cocktail of the latter two enzymes.

Experiments began immediately once all the solutions were prepared. The samples were protected from light at all times. Once an exposure regimen was chosen, the test solution was aliquoted in two identical electroporation cuvettes; one of the cuvettes was exposed to nsPEF (or sham exposed, i.e., placed in the exposure set-up for a certain time, but no pulses were triggered) and the other one served as a parallel control. Immediately after the completion of exposure, the exposed and control samples were dispensed in triplicates into a 96-well plate and read with the plate reader. The interval between exposure and reading was kept to the minimum (about 1 min) unless stated otherwise. After reading, the samples were discarded, and the next experiment started using