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Calcium-mediated pore expansion and cell death following nanoelectroporation

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ABSTRACT

Opening of long-lived pores in the cell membrane is the principal primary effect of intense, nanosecond pulsed electric field (nsPEF). Here we demonstrate that the evolution of pores, cell survival, the time and the mode of cell death (necrotic or apoptotic) are determined by the level of external Ca^{2+} after nsPEF. We also introduce a novel, minimally disruptive technique for nsEP exposure of adherent cells on indium tin oxide (ITO)-coated glass coverslips, which does not require cell detachment and enables fast exchanges of bath media. Increasing the Ca^{2+} level from the nominal 2–5 μM to 2 mM for the first 60–90 min after permeabilization by 300-nsPEF increased the early (necrotic) death in U937, CHO, and BPAE cells. With nominal Ca^{2+}, the inhibition of osmotic swelling rescued cells from the early necrosis and increased caspase 3/7 activation later on. However, the inhibition of swelling had a modest or no protective effect with 2 mM Ca^{2+} in the medium. With the nominal Ca^{2+}, most cells displayed gradual increase in YO-PRO-1 and propidium (Pr) uptake. With 2 mM Ca^{2+}, the initially lower Pr uptake was eventually replaced by a massive and abrupt Pr entry (necrotic death). It was accompanied by a transient acceleration of the growth of membrane blebs due to the increase of the intracellular osmotic pressure. We conclude that the high-Ca^{2+}-dependent necrotic death in nsPEF-treated cells is effected by a delayed, sudden, and osmotically-independent pore expansion (or de novo formation of larger pores), but not by the membrane rupture.

1. Introduction

Effects of intense nsPEF treatments in mammalian cells include permeabilization of the plasma membrane, endoplasmic reticulum (ER), and mitochondria [1–7]; Ca^{2+} uptake from the outside and release from the ER [2,6,8–10]; destruction of the cytoskeleton [11–13]; cell swelling and blebbing [14–16]; and activation of signaling and cell death pathways [10,17–21]. The cytotoxic effects of nsPEF have attracted particular attention as a novel and promising modality for cancer treatment [22–26].

With the complexity of the cellular response to nsPEF, the mechanisms and specific pathways leading to cell death have only been partially understood. The early studies focused on the apoptotic response, and only recently early necrosis was reported by several groups as a separate or even a predominant mode of nsPEF-induced cell death [15,18]. The primary cause of necrosis was the persistent plasma membrane permeabilization to small solutes (<1 nm), which resulted in the osmotic imbalance, water uptake, and cell swelling culminating in the membrane rupture. When the uptake of water was blocked by an isoosmotic addition of a pore-impermeable solute (sucrose [14]), cells were rescued from the necrotic death, but nonetheless died later on by apoptosis [15]. However, the cause of the apoptosis in cells rescued from the necrosis was not identified.

Ca^{2+} signaling is critically involved, in many ways, in both the initiation and effectuation of the cell death (see [27] for review). Several studies pointed to the role of mitochondria and Ca^{2+} increase in the initiation and execution of the nsPEF-induced apoptosis [3,28], and the increase in the cytosolic Ca^{2+} is one of the best known nsPEF effects [2,6,8,9,29,30]. In human pancreatic cancer cells, nsPEF caused Ca^{2+}-dependent production of reactive oxygen species, indicating that overloading mitochondria with Ca^{2+} and their destruction were part of the apoptotic mechanism [28]. Finally, cell lethality increased with increasing extracellular Ca^{2+} for either nsPEF or conventional electroporation [31,32]. The injection of Ca^{2+} into a tumor prior to its electroablation ("Ca^{2+} electroporation") has increased the treatment efficiency in animals and humans [31].

Abbreviations: BPAE, bovine pulmonary artery endothelial cells; ER, endoplasmic reticulum; nsPEF, nanosecond pulsed electric field; Pr, propidium; ROS, reactive oxygen species

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Therefore, our experiments were initially aimed at defining the role of Ca^{2+} in nsPEF-induced apoptosis in cells rescued from the early osmotically-driven necrosis. Instead, we found that the increased Ca^{2+} facilitated the early necrosis and thereby decreased the cell population that could potentially enter the apoptotic process. The Ca^{2+}-mediated necrotic pathway did not rely on the osmotic swelling, and overrode any protection rendered by the blockage of water uptake. Below we demonstrate that Ca^{2+}-mediated necrosis results from a delayed, abrupt, irreversible, and osmotically-independent expansion of pores in the cell membrane.

2. Materials and methods

2.1. Cells and media

We utilized suspension cells U937 (human monocytes), and adherent cells CHO-K1 (Chinese hamster ovary) and BPAE (bovine pulmonary artery endothelial). U937 and CHO cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and propagated as described previously [1,15,33]. BPAE were a kind gift from Dr. J. Catravas (Center for Bioelectrics, ODU). They were grown at 37 °C with 5% CO_2 in air in a low glucose DMEM with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml amphotericin B. The media and its components were purchased from Thermo Scientific (Waltham, MA), Sigma-Aldrich (St. Louis, MO), and Atlanta Biologicals (Norcross, GA).

2.2. nsPEF exposure and viability of suspension cells

To control for the extracellular Ca^{2+} during and after nsPEF, we utilized RPMI 1640 medium formulated without Ca^{2+} ("no Ca^{2+}" medium). Ca^{2+} chelators were not used, as their possible entry into electropermeabilized cells could affect the cytosolic free Ca^{2+} level and the physiological consequences of electroporation. The actual level of free Ca^{2+} in this medium was checked by ratiometric fluorescence with Fura-2 [2] and equaled 1–2 μM.

U937 cells were spun and rinsed twice, resuspended in no Ca^{2+} RPMI at 3×10^6 cells/ml, and then split into two aliquots. In one of them, Ca^{2+} level was raised to 2 mM ("2 Ca^{2+}" medium).

The samples were transferred into 1-mm gap electroporation cuvettes and exposed to 300 pulses of 300-ns duration, at 700 V amplitude and 10 Hz repetition rate from an AVTECH AVOZ-D2-B-ODA generator (AVTECH Electrosystems, Ottawa, Canada) as described earlier [15,33]. Parallel controls were "sham" exposed. Immediately following either nsPEF or sham treatment, all samples were mixed with a 6× volume of the same medium (0Ca^{2+} or 2Ca^{2+}) and a 3× volume of an isoosmotic (290 mOsm/kg) water solution of either sucrose or NaCl [15]. As a nanopore-impermeable solute, sucrose was shown to prevent the osmotic water uptake in nsPEF-treated cells [14,15], thereby rescuing them from the osmotically-mediated necrosis. [15]. NaCl did not render such protection and served as a control for the equivalent dilution of the medium [15].

The samples in tested media (no Ca^{2+} + sucrose; no Ca^{2+} + NaCl; 2 Ca^{2+} + sucrose; and 2 Ca^{2+} + NaCl) were aliquoted into a 96-well plate at 100 μl/well and incubated at 37 °C for 1 h. This time interval was chosen to allow either for electropore resealing, or for cell rupture due to the osmotic imbalance if (most) pores failed to reseal. Next, all samples were supplemented with 10 μl of FBS and 10 μl of RPMI properly enriched with Ca^{2+}, in order to bring the Ca^{2+} level in all samples to 2 mM.

At 1.5, 4, or 24 h after nsPEF, 10 μl of the Presto Blue reagent (Life Technologies, Grand Island, NY) were added to the wells. After 1 h of incubation at 37 °C with the reagent, the wells were scanned with a Synergy 2 microplate reader (BioTek, Winooski, VT), using excitation at 530 nm and detection at 590 nm.

2.3. Caspase 3/7 activity

We utilized a Caspase-Glo 3/7 Assay from Promega (Madison, WI) according to the manufacturer's instructions. The buffer compositions, exposure, and sample handling protocols were the same as described in the above Section 2.2. In 1.5, 4, or 24 h after nsPEF exposure, cells were aliquoted at 50 μl/well into a 96-well plate and 50 μl of Caspase-Glo 3/7 reagent was added. The luminescence was measured by the Synergy 2 reader after 40 min of incubation at room temperature.

2.4. A novel concept of nsPEF exposure of adherent cells: the use of indium tin oxide (ITO)-coated glass coverslips

Electroporation cuvettes are designed for cell suspensions, so adherent cells need to be removed from the substrate prior to nsPEF treatment. This step alters cell physiology and may affect the survival rate, along with the re-attachment after nsPEF. The greatest challenge is the replacement of media in electroporated cells, which are too fragile to be spun (that is why we use only media dilutions and avoid centrifugation in Section 2.2).

Here, we introduce a novel method of nsPEF exposure of adherent cells in electroporation cuvettes, which utilizes ITO-coated glass coverslips and is devoid of stressful cell handling. ITO is a biologically inert material which uniquely combines high electrical conductance with optical transparency. If cells grown on a "regular" glass coverslip are pulsed in a cuvette, the glass layer shields the cells from the E-field. However, the ITO layer cancels this protection, resulting in an efficient and uniform nsPEF exposure of cells over the entire coverslip surface. The method does not require detachment or reattachment of cells, and changes of media are accomplished simply by moving the coverslip into the new medium. NsPEF treatments of cells on ITO coverslips were highly efficient, requiring about 20-fold fewer pulses than for cells in suspension. Of note, cells grown on a wrong (non-ITO) surface of the coverslip were fully shielded and could not be damaged by any number of pulses.

2.5. Buffers for nsPEF exposure and post-exposure incubation of adherent cells

For nsPEF exposure, a 1-mm cuvette was filled with a buffer containing either 2 mM Ca^{2+} or no added Ca^{2+}. Both buffers contained (in mM) 136 NaCl, 5 KCl, 2 $MgCl_2$, 10 HEPES, and 10 Glucose, and were supplemented with either 2 mM $CaCl_2$ or extra 3 mM NaCl, respectively.

For post-exposure incubation and cell imaging, we either used the same buffers, or mixed them 7:3 with an isoosmotic sucrose solution ("no Ca^{2+} + sucrose" and "2 Ca^{2+} + sucrose"). For the latter buffer, the sucrose also contained 2 mM Ca^{2+} to prevent Ca^{2+} dilution. The presence of sucrose prevented the colloid-osmotic swelling and membrane rupture of nanoporated cells [14,15].

In addition, the post-exposure incubation buffers contained 3 dyes, namely Hoechst (0.5 μg/ml), YO-PRO-1 (1 μM), and propidium (Pr) iodide (50 μg/ml). Cell-permeant Hoechst was used to label nuclei in all cells, whereas poorly permeant YO-PRO-1 and impermeant Pr served as markers of cell permeabilization. The buffers that were used during nsPEF exposure were always formulated without the dyes.

The osmolality of all buffers was at 290–310 mOsm/kg, as verified with a freezing point microosmometer (Advanced Instruments, Inc., Norwood, MA), and their pH was adjusted to 7.4. The chemicals and dyes were purchased from Sigma-Aldrich and from Life Technologies.

2.6. Experiment protocol and monitoring of permeabilization of adherent cells

Standard glass coverslips (#0 thickness, 8 mm diameter) were covered with ITO by Diamond Coatings (Halesowen, UK). For better cell