Chapter 5

Phosphorylation-Mediated Control of Stress Responses Induced by Nanosecond Pulsed Electric Fields

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69782

Abstract

Exposure of living organisms to short electric pulses is widely utilized in the life sciences, for example, for DNA transfection. Recent advances in electrical engineering have enabled the production of extremely short electric pulses in the range of nanoseconds, namely, nanosecond pulsed electric fields (nsPEFs). nsPEFs are increasingly recognized as a novel means for cancer therapy, because of their ability to induce cell death. Recent studies have demonstrated that nsPEFs act as cellular stress and activate two independent signaling pathways that involve phosphorylation of translation initiation factors and lead to suppression of general protein synthesis. eIF2α phosphorylation is one of the key reactions in stress-induced translational suppression and is rapidly induced by nsPEFs. Concomitantly, PERK and GCN2, both of which are stress-responsive protein kinases, are activated in nsPEF-exposed cells. Furthermore, nsPEFs cause a reduction in 4E-BP1 phosphorylation, which is controlled by mTORC1 and constitutes an alternative mechanism for translational suppression, independent of eIF2α phosphorylation. In accordance with elevated eIF2α phosphorylation and decreased 4E-BP1 phosphorylation, general protein synthesis is acutely suppressed after nsPEF exposure. These findings demonstrate that nsPEFs induce two independent signaling pathways for translational suppression, further highlighting a unique feature of nsPEFs as a novel means for life sciences.

Keywords: stress response, electroporation, pulsed electric field, eIF2α, PERK, GCN2, 4E-BP1, translational suppression, protein synthesis

1. Introduction

Living cells respond to various environmental stimuli by activating distinct sets of intracellular reactions. Cellular responses to external stimuli generally involve signal transduction that is

mediated by protein phosphorylation and eventually leads to modulation of various cellular events, such as metabolism, gene expression, proliferation, and cell death [1].

Some physical and chemical stimuli have adverse physiological effects and are known as cellular stress. In addition to adverse external stimuli, endogenous deleterious events are also regarded as cellular stress, such as accumulation of misfolded proteins in the endoplasmic reticulum (ER). Cells respond to these cellular stresses by inducing various intracellular reactions, which are collectively referred to as stress responses. Timely induction of stress responses is critical for maintenance of cellular physiology, and its dysregulation is frequently observed in various diseases, such as cancer [2], neurodegenerative disorders [3], and inflammatory diseases [4], indicating the importance of stress responses in physiological and pathological processes.

Currently, diverse forms of physical stimuli are utilized as tools for various biological and clinical applications. Among these physical stimuli, pulsed electric fields (PEFs) have been proven particularly useful, because different biological effects can be achieved, depending on the duration of the electric pulses. PEFs in the range of milliseconds to microseconds primarily act on the cell membrane and generate membrane pores, which are suited for introduction of exogenous macromolecules, such as plasmid DNA, into living cells [5, 6]. Thus, these PEFs are widely used for DNA transfection [7].

Recent advances in electric engineering allow us to generate ultrashort PEFs in the range of nanoseconds, which are referred to as nanosecond PEFs (nsPEFs). Although nsPEFs do not generate membrane pores suitable for DNA transfection, they have been proven to be useful for cancer therapy, because of their ability to induce cell death [8, 9]. Furthermore, nsPEFs have been shown to induce stress responses that are mediated by phosphorylation of multiple translation initiation factors and eventually lead to transient suppression of general protein synthesis. For these reasons, nsPEFs have received considerable attention as a potential therapeutic method with a novel mechanism of action. This review is intended to provide an overview of the stress responses induced by nsPEFs. First, two major mechanisms for stress responses in human cells are explained. Phosphorylation-mediated control of two translation initiation factors, eIF2α and 4E-BP1, is critical in these pathways. Second, an outline of the biological actions of PEFs is provided, with particular emphasis on nsPEFs. Finally, stress responses induced by nsPEFs are described in detail.

2. Stress responses in human cells

2.1. Overview of stress response

The fundamental aspects of cellular stress responses are highly conserved among eukaryotes from yeast to humans. **Figure 1** shows a simplified scheme for eukaryotic stress responses. Under normal physiological conditions, cells continuously undergo protein synthesis, and the rate of protein synthesis is primarily regulated at the translation initiation step. When cells sense stress, they rapidly activate signal transduction that involves phosphorylation-mediated

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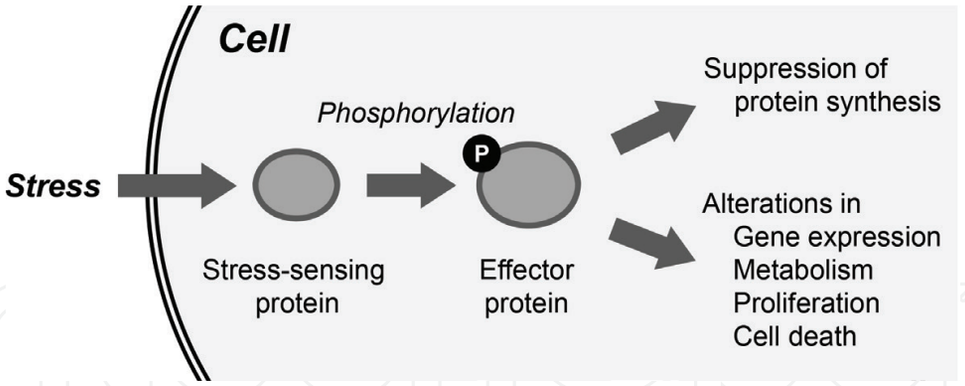

Figure 1. General principle of stress responses in eukaryotic cells. When a cell is exposed to stress, a stress-sensing protein is activated, inducing signal transduction mediated by protein phosphorylation. The stress-induced signal is transduced to downstream effector proteins. Stress responses generally lead to inhibition of cap-dependent translation initiation and consequent suppression of general protein synthesis. Stress responses often cause additional changes, such as alteration of gene expression.

control of translation initiation factors. Stress-induced alterations in the phosphorylation status of translation initiation factors reduce translation initiation and thereby result in attenuation of general protein synthesis. Because protein synthesis requires significant amounts of energy and materials, transient suppression of general protein synthesis conserves cellular resources and is thus beneficial for cells under stress. For these reasons, stress-induced suppression of general protein synthesis serves as a mechanism for survival. Once the stress ends, translation capacity is rapidly recovered by dephosphorylation of the translation factors. Intriguingly, persistent activation of stress responses is often associated with the induction of cell death, suggesting that timely induction and attenuation of stress responses are both critical for cell survival [10–12]. Although eukaryotic cells share a fundamental stress response mechanism [13, 14], as described below, human cells possess more intricate stress responses, including at least two distinct pathways involving phosphorylation of multiple proteins.

2.2. Stress response mediated by eIF2α phosphorylation

2.2.1. Stress-induced eIF2α phosphorylation and translational suppression

Translation initiation is a critical rate-limiting step in protein synthesis, and eukaryotic translation initiation factor 2 (eIF2) plays an essential role in this process [15]. eIF2 binds to guanine nucleotides, such as GDP and GTP. During initiation of translation, an eIF2-bound guanine nucleotide needs to cycle between GDP and GTP (see **Figure 2A** for details of GDP-GTP cycling in translation initiation). eIF2 consists of three subunits, α, β, and γ, and phosphorylation of the α subunit of eIF2 (eIF2α) is induced by various forms of stress [14, 16]. Phosphorylation of eIF2α at serine 51 interferes with GDP-GTP exchange on eIF2 [17] and consequently suppresses translation initiation (**Figure 2B**). The role of eIF2α phosphorylation in inhibition of translation initiation is highly conserved among eukaryotes, and the site of stress-induced phosphorylation (serine 51) is conserved in yeast and humans [13, 14].

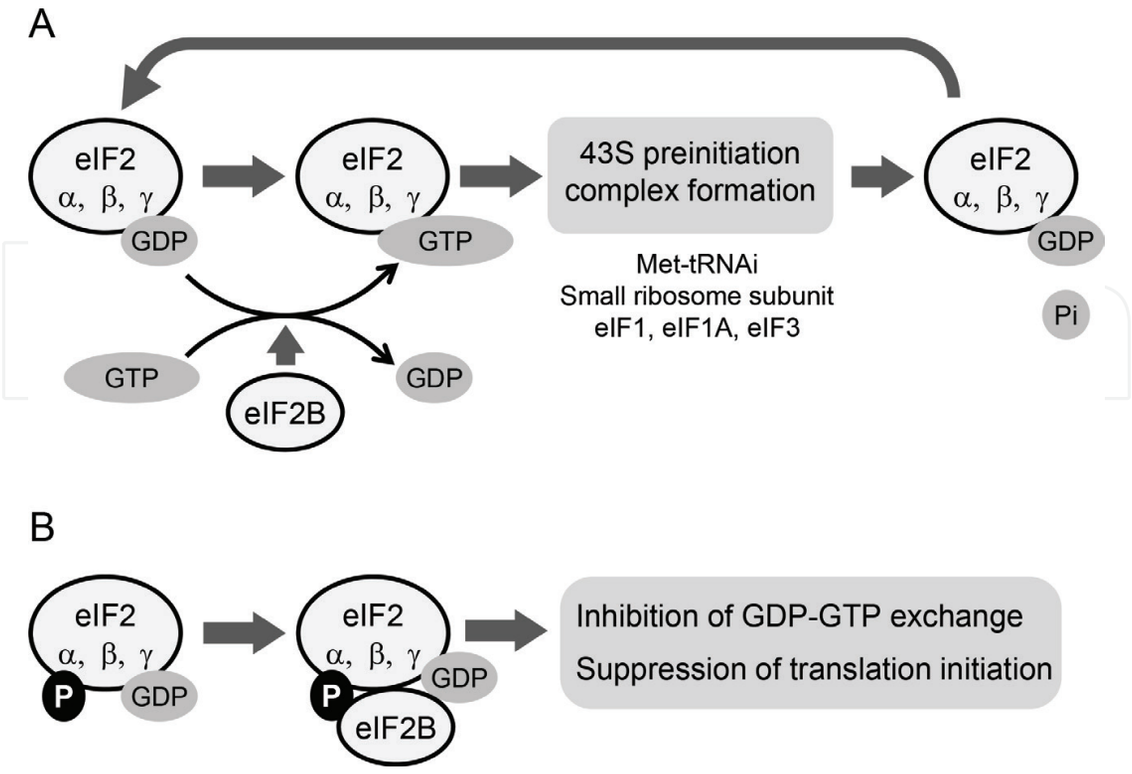

Figure 2. Suppression of translation initiation by eIF2α phosphorylation. (A) Role of GDP-GTP exchange on eIF2 in translation initiation. eIF2 is a trimer composed of three subunits, α, β, and γ, and binds to guanine nucleotides, such as GTP and GDP. For translation initiation, GTP-bound eIF2 recruits the initiator methionyl-tRNA (Met-tRNAi) and in turn forms the 43S preinitiation complex with the small ribosomal subunit and the initiation factors eIF1, eIF1A, and eIF3. The 43S preinitiation complex is recruited to the 5′ end of mRNA, which is marked with a cap structure, and scans the 5′ untranslated region of mRNA for the initiation codon. During this process, GTP on eIF2 is hydrolyzed to GDP, and GDP-bound eIF2 is released from the translation machinery. eIF2B, which has guanine exchange activity, replaces GDP on eIF2 with GTP, and GTP-bound eIF2 enters a new round of translation initiation [18, 19]. (B) Phosphorylation-mediated suppression of GDP-GTP exchange on eIF2. Under stressed conditions, serine 51 of eIF2α is rapidly phosphorylated by a stress-responsive protein kinase. Phosphorylation of eIF2α transforms eIF2 from a substrate into an inhibitor of eIF2B. eIF2B stalls on phosphorylated eIF2 and thus cannot exert its guanine exchange activity. Consequently, eIF2α phosphorylation leads to an increase in the GDP-bound form of eIF2, which is inactive for translation initiation, and results in attenuation of general protein synthesis [14, 16, 17].

Although initiation of translation of most mRNA species is dependent on their 5′ cap and is profoundly affected by eIF2α phosphorylation, a subset of mRNA species is actively translated under stressed conditions via alternative translation mechanisms that are independent of the cap structure. Approximately 2.5% of total mRNA is estimated to be preferentially translated under stressed conditions [20, 21], permitting synthesis of a subset of proteins that play critical roles in control of the stress response. For example, initiation of translation of ATF4 (activating transcription factor 4) mRNA is increased by ER stress caused by accumulation of unfolded proteins in the ER. The 5′ UTR of ATF4 mRNA contains two small open reading frames (upstream open reading frames [uORFs]), which play a critical role in initiation of translation under stressed conditions. ATF4 mRNA encodes a transcription factor that induces gene expression for adaptive responses. Thus, stress-induced eIF2α phosphorylation results in both suppression of general protein synthesis to conserve cellular resources and elevated translation of specific mRNA species for adaptive responses [22, 23].

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In human cells, phosphorylation of eIF2α is induced by a wide variety of exogenous as well as endogenous stresses, such as amino acid deprivation, UV irradiation, and accumulation of unfolded proteins in the ER. Although these stresses are sensed by individual mechanisms, multiple stress-induced events converge on a single reaction, namely, eIF2α phosphorylation. Thus, eIF2α phosphorylation integrates various stress-induced signals. The stress-induced signaling pathway in human and mammalian cells that involves eIF2α phosphorylation and downstream ATF4 induction is referred to as the integrated stress response (**Figure 3**) [12, 24].

2.2.2. Stress-responsive protein kinases

In human and mammalian cells, eIF2α is phosphorylated by four serine/threonine protein kinases, which are differentially activated by stress [14, 25]. These protein kinases show structural homology in their catalytic domains and are considered to exist in a monomeric form under unstressed conditions. Upon stress induction, they undergo homodimerization to become catalytically active, followed by autophosphorylation for full activation [26, 27].

PERK (protein kinase RNA-like endoplasmic reticulum kinase) is an ER transmembrane protein, and its N-terminal domain resides in the ER lumen and plays a role in sensing unfolded proteins. The C-terminal region of PERK is located in the cytoplasm and contains a kinase domain. PERK is activated by accumulation of unfolded proteins in the ER and in turn phosphorylates eIF2α.

GCN2 (general control nonderepressible 2) is critical for translational suppression under amino acid deprivation [28]. GCN2 binds to uncharged transfer RNAs and exerts its catalytic

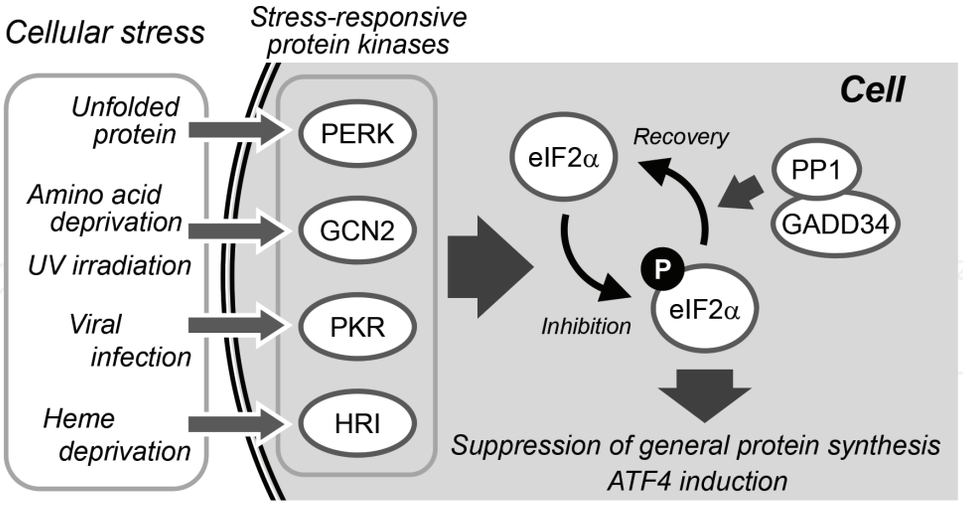

Figure 3. Integrated stress response. Eukaryotic cells respond to various stresses by inducing eIF2α phosphorylation that leads to suppression of general protein synthesis. Human and mammalian cells possess four stress-responsive protein kinases: PERK, GCN2, PKR, and HRI. These protein kinases are differentially activated by stress and in turn phosphorylate eIF2α. eIF2α phosphorylation and downstream ATF4 induction are known as the integrated stress responses, because different cellular reactions induced by various external cues converge to these reactions. eIF2α phosphorylation inhibits cap-dependent initiation of translation and consequently suppresses general protein synthesis. A complex of GADD34 and PP1 dephosphorylates eIF2α and serves as a negative feedback mechanism for the integrated stress response.

activity for eIF2α phosphorylation. In addition to amino acid deprivation, GCN2 has been reported to be activated by UV irradiation [29] and proteasome inhibition [30], although the activation mechanisms remain elusive.

PKR (double-stranded RNA-dependent protein kinase) was originally identified as a protein kinase activated by double-stranded RNA, which emerges during viral infection [31]. Phosphorylation of eIF2α by PKR interferes with translation of viral mRNA and thus serves as an antiviral mechanism. In addition, activation of PKR is involved in the pathology of obesity [32] and cancer [33], suggesting various physiological roles of PKR.

Heme-regulated inhibitor (HRI) has physiological roles particularly in erythroid tissues [34]. HRI is activated by heme deprivation and phosphorylates eIF2α to reduce globin synthesis under low-iron conditions [35].

2.2.3. Recovery from eIF2α phosphorylation-mediated translational suppression

Once stressed conditions end, eIF2α must be dephosphorylated to restore general protein synthesis. GADD34 (growth arrest and DNA damage-inducible protein 34) is a critical regulator of eIF2α dephosphorylation, and its activity in the relief of stress responses is controlled at both the transcriptional and translational levels. Expression of *GADD34* gene is low under normal physiological conditions and is activated by various forms of stress. GADD34 mRNA is translated by a cap-independent mechanism, in which uORFs in the 5′ UTR of GADD34 mRNA play critical roles [36]. GADD34 protein forms a complex with protein phosphatase 1 (PP1), yielding a catalytically active protein phosphatase that specifically catalyzes dephosphorylation of eIF2α to relieve translational suppression [37]. Thus, GADD34 constitutes a negative feedback mechanism for eIF2α-mediated translational suppression.

2.3. Stress response mediated by 4E-BP1 phosphorylation

Human cells have an alternative mechanism for stress-induced translational suppression, which involves 4E-binding protein 1 (4E-BP1) [38]. As mentioned above, most mRNA species are translated in a cap-dependent manner. eIF4E binds to the cap structure of mRNA and in turn recruits eIF4G and other translation initiation factors, resulting in formation of an active translation initiation complex on the 5′ end of mRNA. 4E-BP1 serves as a negative regulator of this process. In unstressed conditions, 4E-BP1 is highly phosphorylated at multiple sites, suppressing its inhibitory activity. Under energy deprivation and other stressed conditions, phosphorylation of 4E-BP1 is substantially decreased, and 4E-BP1 competes with eIF4G for binding to eIF4E, thereby inhibiting translation initiation (**Figure 4**) [38].

Phosphorylation of 4E-BP1 is primarily controlled by mTORC1 (mammalian target of rapamycin complex 1), which is a member of the phosphatidylinositol 3-kinase-related family of kinases [39]. Under normal physiological conditions, mTORC1 is catalytically active and suppresses the inhibitory activity of 4E-BP1 by phosphorylation. The kinase activity of mTORC1 is regulated by several cellular proteins, one of which is AMP-activated protein kinase (AMPK). AMPK senses the cellular energy status and negatively regulates mTORC1 activity [40]. AMPK, mTORC1, and 4E-BP1 constitute a stress-responsive mechanism for translational

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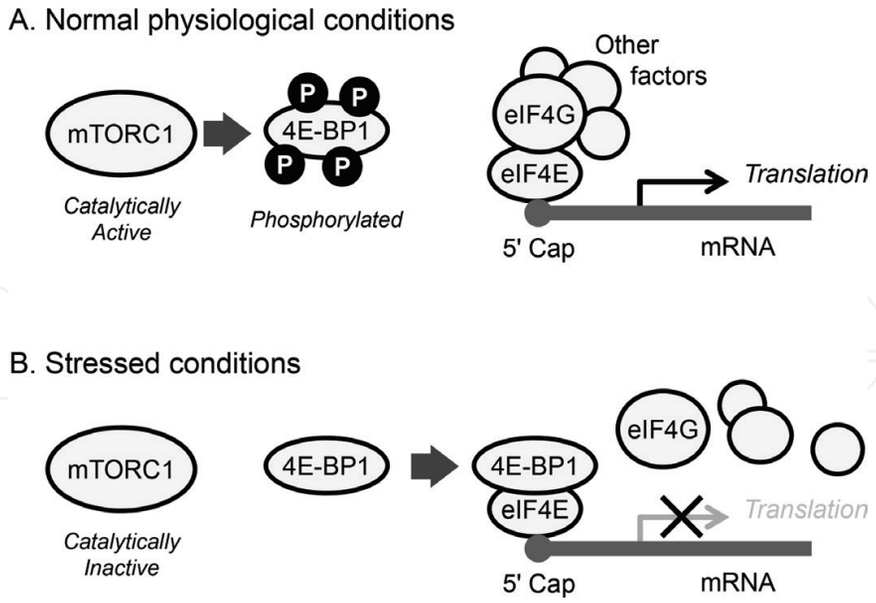

Figure 4. Control of translation initiation mediated by 4E-BP1 phosphorylation. Human cells possess an alternative mechanism for translational suppression, which is distinct from the eIF2α phosphorylation-mediated integrated stress response. eIF4E and other translation initiation factors form an active complex for initiation of translation at the cap structure of mRNA. 4E-BP1 serves as a negative regulator for the complex formation of eIF4E and other factors. (A) Under normal conditions, 4E-BP1 is highly phosphorylated by mTORC1 and sequestered from the translation initiation complex. (B) Under stressed conditions, such as energy deprivation, mTORC1 kinase activity is reduced, resulting in decreased 4E-BP1 phosphorylation, allowing inhibition of eIF4G-eIF4E binding.

suppression, independent of eIF2α phosphorylation. Thus, two mechanisms, the eIF2α-mediated integrated stress response and the 4E-BP1-mediated mechanism, function in translational suppression in human cells (**Figure 4**).

3. Pulsed electric fields as a novel physical tool in the life sciences

3.1. Effects of pulsed electric fields on living organisms

Pulsed electric fields (PEFs) refer to high-voltage electric pulses, which are milliseconds, microseconds, and nanoseconds in duration. PEFs have different effects on living organisms depending on pulse duration (**Figure 5**). PEFs with duration of milliseconds to microseconds primarily act on the cell membrane and cause pore formation. These membrane pores are suitable for transfer of macromolecules, such as plasmid DNA and drugs [5, 6]. Therefore, exposure of living cells to these PEFs is called electroporation and is commonly used for DNA transfection [7]. In addition, these PEFs are used for the introduction of antitumor drugs, which is called electrochemotherapy [41, 42]. Because PEFs with duration of milliseconds to microseconds primarily act on the cell membrane, these PEFs often induce cell death via cell membrane damage.

Recent advances in electrical engineering have enabled the generation of high-voltage electric pulses for ultrashort periods in the nanosecond range, which are called nanosecond PEFs

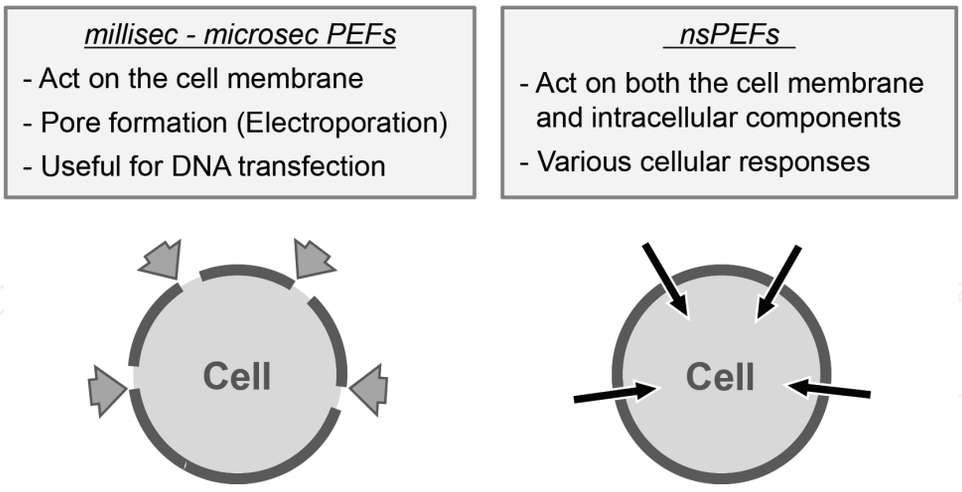

Figure 5. Comparison between electroporation and nsPEF action. PEFs have different effects on living organisms depending on pulse duration. (*Left*) PEFs with duration of milliseconds to microseconds are widely used for electroporation, because these PEFs primarily act on the cell membrane and generate membrane pores suited for macromolecule transfer. (*Right*) nsPEFs generate small membrane pores that permeate small molecules, such as ions and water. Furthermore, nsPEFs have been suggested to directly affect intracellular components. Although nsPEFs are unsuitable for DNA transfection and cancer electrochemotherapy by electroporation, these PEFs can induce various cellular responses, including cell death induction.

(nsPEFs). It has become increasingly evident that nsPEFs have unique biological actions distinct from electroporation. The pulse duration of nsPEFs is too short to generate membrane pores large enough for entry of macromolecules. Thus, nsPEFs are generally unsuitable for DNA transfection and cancer electrochemotherapy. Instead, nsPEFs produce very small membrane pores that allow passage of small molecules, such as ions and water [43–45]. Accordingly, exposure of cultured human cells to nsPEFs causes Ca^{2+} influx and membrane blebbing due to ion imbalance across the cell membrane [46–48].

Whereas millisecond-to-microsecond PEFs primarily act on the cell membrane, previous theoretical studies have strongly suggested that nsPEFs exert their effects on both the cell membrane and intracellular components [49, 50]. In accordance, extensive biochemical analyses have proven that nsPEFs elicit various intracellular responses, as described below.

3.2. Cellular responses to nsPEFs

Recent studies have revealed that nsPEFs elicit different intracellular responses in a manner dependent on nsPEF intensity. **Figure 6** summarizes the relationship between nsPEF intensity and intracellular responses in human cells. Relatively weak nsPEFs do not cause morphological changes observable under a microscope, growth retardation, or cell death. However, cells rapidly respond to such stimuli by activating multiple intracellular signal pathways, including MAPK pathways [51, 52] and AMPK pathway [53]. Intracellular signaling is mediated by sequential phosphorylation of proteins in these pathways, leading to expression of downstream genes [51, 52]. When moderate-intensity nsPEFs are used, two independent mechanisms for stress responses are activated [54]. Intense nsPEFs efficiently induce cell death in vitro [8] as well as in vivo [9].

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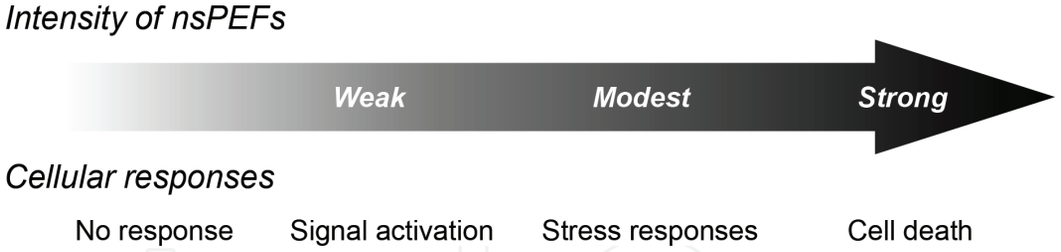

Figure 6. Relationship between nsPEF intensity and cellular responses. Exposure of human cells to nsPEFs causes different cellular responses, depending on intensity of nsPEFs. Relatively weak nsPEFs activate several signal transduction pathways, such as MAPK pathways, and their downstream gene expression without affecting on cell viability. Moderate-intensity nsPEFs elicit stress responses and cause growth retardation. Intense nsPEFs induce either apoptotic or necrotic cell death in a cell-type-dependent manner and can be used for cancer therapy.

When cultured human cells are exposed to intense nsPEFs, cell-type dependency of cell death modes has been observed. For example, apoptosis is induced in HL-60 and Jurkat cells by intense nsPEFs [8, 55, 56], whereas necrotic cell death is elicited in several cell lines, including U937, K562, and HeLa S3 [56, 57], demonstrating that the cellular context determines the mode of cell death. Induction of necrosis by intense nsPEFs is a Ca^{2+}-dependent process [48, 58], while nsPEF-induced apoptosis is largely unaffected by the presence or absence of Ca^{2+} [58].

Because of the ability of nsPEFs to induce efficient cell death, many studies have attempted to apply nsPEFs to cancer therapy and have demonstrated their effectiveness in several mouse models [9, 59, 60]. Furthermore, a human clinical trial of nsPEFs for cancer therapy has started [61]. For these reasons, nsPEFs have attracted much interest and are increasingly recognized as a novel method of cancer therapy.

4. Induction of stress responses by nsPEFs

Previously, nsPEFs have been shown to activate two independent stress-responsive pathways, both of which are mediated by protein phosphorylation and result in translational suppression. First, nsPEFs induce eIF2α phosphorylation via two protein kinases, PERK and GCN2. Concomitant with eIF2α phosphorylation, GADD34 expression is elevated. Second, nsPEFs cause decreased phosphorylation of 4E-BP1, which is presumably controlled by mTORC1. Following induction of these phosphorylation-mediated stress responses, general protein synthesis is markedly reduced in nsPEF-exposed cells. **Figure 7** represents a summary of nsPEF-induced stress responses.

4.1. eIF2α phosphorylation induced by nsPEFs

Most mRNA species are translated in a cap-dependent manner, and eIF2 plays a critical role in this process. Under stressed conditions, the activity of eIF2 for translation initiation is suppressed by phosphorylation of its α subunit (eIF2α) [14]. eIF2α phosphorylation is considered to be a hallmark of induction of the integrated stress response and can be examined by

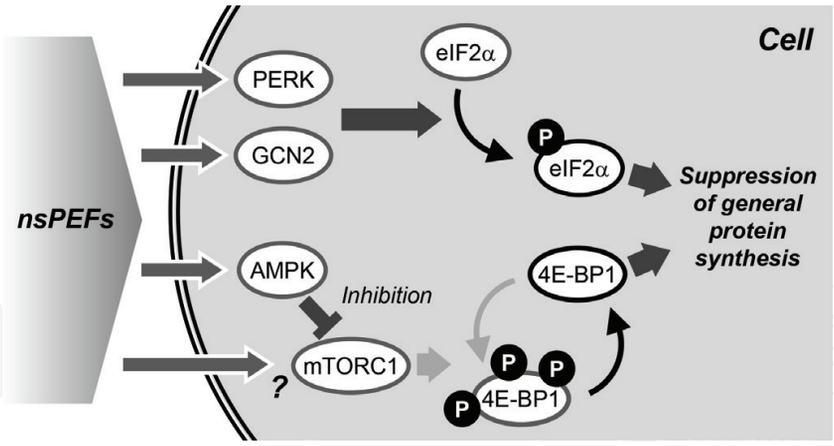

Figure 7. Stress responses induced by nsPEFs. nsPEFs affect multiple stress-responsive proteins in the two independent signaling pathways, both of which result in suppression of general protein synthesis. The first signaling pathway induced by nsPEFs is mediated by eIF2α phosphorylation. nsPEFs activate PERK and GCN2, which in turn phosphorylate eIF2α. eIF2α phosphorylation interferes GDP-GTP exchange in translation initiation, resulting in suppression of general protein synthesis. The second pathway induced by nsPEFs involves a reduction in 4E-BP1 phosphorylation. 4E-BP1 is highly phosphorylated by mTORC1 under normal conditions. nsPEFs cause a decrease in 4E-BP1 phosphorylation, leading to inhibition of eIF4G-eIF4E complex formation and consequent suppression of translation initiation. nsPEFs are known to activate AMPK, which is known to function as a negative regulator of mTORC1 under energy deprivation conditions. AMPK activation may account for the decrease in 4E-BP1 phosphorylation following nsPEF exposure. Direct effects of nsPEFs on mTORC1 will be examined by future research. Increased eIF2α phosphorylation and decreased 4E-BP1 phosphorylation serve as two independent mechanisms for the suppression of general protein synthesis.

Western blotting using an antibody specific to phosphorylated eIF2α. When cultured cells are exposed to nsPEFs, eIF2α phosphorylation can be detected, indicating that nsPEFs activate the integrated stress response [54]. nsPEF-induced eIF2α phosphorylation can be detected in all cell lines examined so far, which include HeLa S3, HCT116, Jurkat, and mouse embryonic fibroblasts (MEFs). In HeLa S3 cells, eIF2α phosphorylation is detectable within 1 min after nsPEF exposure, persists at high levels for 30 min, and decreases thereafter [54]. GADD34 is known to play a critical role in recovery from the integrated stress response. Under stressed conditions, GADD34 is positively controlled at the transcriptional and translational levels. Consistently, expression of GADD34 is significantly activated in nsPEF-exposed cells, suggesting that GADD34 is involved in recovery from the nsPEF-induced stress response [54].

As described above, cellular responses to nsPEFs are dependent on nsPEF intensity (**Figure 6**). Relatively mild nsPEFs activate several signal transduction pathways, such as MAPK pathways, but are insufficient to induce eIF2α phosphorylation. Moderate levels of nsPEF intensity are required for induction of eIF2α phosphorylation. Such nsPEFs also cause retardation in cell proliferation but not cell death [54]. Intense nsPEFs induce eIF2α phosphorylation and cell death. Currently, it remains unknown whether the nsPEF-induced stress response positively affects cell survival or facilitates cell death induction.

4.2. Participation of PERK and GCN2 in nsPEF-induced eIF2α phosphorylation

Human and mammalian cells have four stress-responsive protein kinases for eIF2α phosphorylation. These kinases differentially respond to various forms of stress, and at least one

of them is activated for eIF2α phosphorylation [14]. Autophosphorylation is critical for the activation of these kinases and can be analyzed by Western blot analysis using antibodies against phosphorylated forms of these kinases. In nsPEF-exposed cells, PERK and GCN2 are activated, as shown by their autophosphorylation [54]. Experiments using PERK and GCN2 knockout cells suggest that these kinases play mutually compensatory roles in nsPEF-induced eIF2α phosphorylation. MEFs lacking either *PERK* or *GCN2* gene display nsPEF-induced eIF2α phosphorylation comparable to that in wild-type cells. However, double-knockout cells lacking both *PERK* and *GCN2* genes exhibit a significant reduction in nsPEF-induced eIF2α phosphorylation [54]. These observations suggest that PERK and GCN2 perform redundant functions in nsPEF-induced eIF2α phosphorylation.

Because PERK is well known to be activated by ER stress, the observation on the nsPEF-induced PERK activation raised the possibility that nsPEFs cause ER stress. To clarify this point, downstream events in the ER stress pathway were analyzed [54]. The signal pathway induced by ER stress generally leads to alterations of gene expression [62]. Transcription of *CHOP* (CCAAT-enhancer-binding protein homologous protein) gene is a major downstream event in the ER stress response. In addition, mRNA for XBP1 (X-box-binding protein 1) is known to undergo alternative splicing after ER stress induction [63]. However, quantitative RT-PCR analysis of *CHOP* and *XBP1* mRNAs demonstrated that nsPEF-exposed cells showed neither elevated CHOP expression nor altered XBP1 splicing. Furthermore, UV irradiation has been reported to induce activation of GCN2 and transcription of downstream genes, such as *GADD45* (growth arrest and DNA damage-inducible 45) [64, 65], but quantitative RT-PCR analysis showed no substantial changes in these mRNAs in nsPEF-exposed cells. These observations indicate that nsPEFs exert their effects in a manner that is distinct from ER stress or UV irradiation, although nsPEFs activate PERK and GCN2 [54].

4.3. Decreased 4E-BP1 phosphorylation by nsPEFs

In addition to the eIF2α-mediated response, a distinct mechanism involving 4E-BP1 phosphorylation is known to play a critical role in stress-induced translational suppression [38]. Under normal physiological conditions, 4E-BP1 is highly phosphorylated by mTORC1 [39], and hyperphosphorylation suppresses its inhibition of cap-dependent translation initiation. Stressed conditions, particularly energy deprivation, reduce mTORC1 activity and result in decreased 4E-BP1 phosphorylation. 4E-BP1 at low phosphorylation status interferes with assembly of translation factors on the cap structure of mRNAs and thereby suppresses general protein synthesis (**Figure 4**). In nsPEF-exposed cells, 4E-BP1 phosphorylation is rapidly decreased [54], suggesting that nsPEFs activate stress responses mediated by 4E-BP1 phosphorylation. The decrease in 4E-BP1 phosphorylation is indistinguishable between wild-type cells and *PERK*/*GCN2* double-knockout cells, supporting the idea that nsPEFs activate two independent mechanisms.

The decrease in 4E-BP1 phosphorylation following nsPEF exposure suggests that nsPEFs cause a reduction in the catalytic activity of mTORC1. A previous study demonstrated that AMPK is rapidly activated by nsPEFs [53]. AMPK functions as an energy sensor and is activated by elevated intracellular AMP levels, which are primarily caused by energy deprivation

[40]. The catalytic activity of mTORC1 is well known to be negatively regulated by AMPK [39]. A previous study has demonstrated that, concomitant with AMPK activation, nsPEFs induce phosphorylation of AMPK substrates, such as acetyl-CoA carboxylase-2 [53], suggesting that nsPEF-activated AMPK also phosphorylates other substrates, including mTORC1. Although mTORC1 catalytic activity has not been examined in nsPEF-exposed cells yet, the above observations suggest that AMPK downregulates mTORC1, leading to reduced 4E-BP1 phosphorylation in nsPEF-exposed cells (**Figure 7**).

4.4. Suppression of general protein synthesis by nsPEFs

As described above, nsPEFs cause elevated eIF2α phosphorylation and decrease 4E-BP1 phosphorylation, both of which are known to be involved in suppression of general protein synthesis. To test whether exposure to nsPEFs actually leads to translational suppression, measurement of protein synthesis rates was required. To this end, metabolic labeling of newly synthesized proteins with radioactive amino acids was employed [54]. **Figure 8** shows an outline of metabolic labeling using ^{35}S-labeled amino acids. Using this method, suppression of general protein synthesis in nsPEF-exposed cells was demonstrated [54]. After nsPEF exposure, overall protein synthesis quickly decreased, and maximum suppression of protein synthesis was observed at 30 min. Protein synthesis in nsPEF-exposed cells recovered to approximately 80% within 2 h. When cells were treated with UV irradiation, general protein synthesis decreased gradually for several hours. Compared to UV irradiation, nsPEFs cause acute translational suppression, and recovery is more rapid than in UV-irradiated cells.

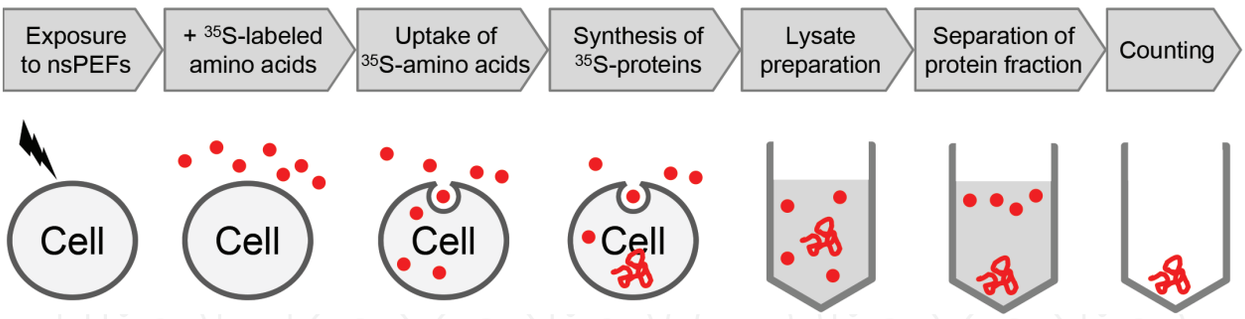

Figure 8. Measurement of protein synthesis rates by metabolic labeling of newly synthesized proteins with radioactive amino acids. A rate of protein synthesis can be measured as incorporation of radioactive amino acids into cellular proteins. Following appropriate treatment, such as nsPEF exposure, cells are incubated in culture medium containing ^{35}S-labeled methionine and cysteine. During incubation, cells use radioactive amino acids to synthesize proteins, yielding ^{35}S-labeled proteins. Following preparation of whole-cell lysate, the protein fraction is separated from the free amino acids, and the radioactivity incorporated into the proteins is quantified by liquid scintillation counting.

5. Conclusion

Exposure of cultured human cells to nsPEFs elicits two distinct stress responses, both of which are controlled by phosphorylation of translation initiation factors. nsPEFs rapidly induce eIF2α phosphorylation and concomitant activation of the stress-responsive kinases,

PERK and GCN2. In addition, nsPEFs cause decreased 4E-BP1 phosphorylation and AMPK activation, which appear to constitute a stress response pathway involving mTORC1. nsPEFs elicit acute suppression of general protein synthesis via two reactions for inhibition of translation initiation. Collectively, these findings clearly indicate that nsPEFs act as a novel form of cellular stress and suppress general protein synthesis.

Although the identification of key events in nsPEF-induced stress responses has significantly advanced our understanding of the biological effects of nsPEFs, several critical questions remain to be elucidated. First, the site of action of nsPEFs for eIF2α-mediated stress response is currently unclear. Because PERK and GCN2 are the most upstream molecules in their signaling pathways, nsPEFs may act directly on these kinases, causing eIF2α phosphorylation. Second, the decrease in 4E-BP1 phosphorylation and the activation of AMPK strongly suggest that mTORC1 participates in the nsPEF-induced stress response, because energy deprivation sequentially causes AMPK activation, reduced mTORC1 activity, and consequent decreased phosphorylation of 4E-BP1. To test this idea, the relationships among AMPK, mTORC1, and 4E-BP1 in nsPEF-exposed cells should be investigated in detail. Furthermore, analysis of effects of nsPEFs on cellular energy levels is also important for understanding how nsPEFs control 4E-BP1 phosphorylation.

Finally, the significance of nsPEF-induced stress responses for cell survival should be determined. Suppression of general protein synthesis under stressed conditions conserves biological resources and is regarded as an important mechanism for cell survival [2]. However, prolonged activation of stress responses often has an opposite effect, facilitating the induction of cell death, presumably because elimination of overstressed cells is beneficial for the body [10, 14]. Currently, it remains unclear whether nsPEF-induced stress responses serve as a prosurvival mechanism or serve to facilitate cell death induction. Future efforts will focus on understanding the contribution of nsPEF-induced stress responses to cell survival and death. Previous studies have revealed the unique effects of nsPEFs as a novel form of cellular stress. More detailed understanding of the molecular mechanisms and biological importance of nsPEF-induced stress responses will pave the way toward more effective applications of this novel technology in a wide range of biomedical sciences.

Acknowledgements

This work was supported by JSPS KAKENHI grant numbers 26350540 and 16H02311 and by a grant from the SEI Group CSR Foundation.

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