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Nonthermal electromagnetic fields: From first messenger to therapeutic applications

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Nonthermal pulsed electromagnetic fields, from low frequency to pulse-modulated radio frequency, have been successfully employed as adjunctive therapy for the treatment of delayed and non-union fractures, fresh fractures and chronic wounds. Recent increased understanding of the mechanism of action of electromagnetic fields (EMF) has permitted technologic advances allowing the development of EMF devices which are portable and disposable, can be incorporated into dressings, supports and casts, and can be used over clothing. This broadens the use of non-pharmacological, non-invasive EMF therapy to the treatment of postoperative pain and edema to enhance surgical recovery. EMF therapy is rapidly becoming a standard part of surgical care, and new, more significant, clinical applications for osteoarthritis, brain and cardiac ischemia and traumatic brain injury are in the pipeline. This study reviews recent evidence which suggests that calmodulin (CaM)-dependent nitric oxide signaling is involved in cell and tissue response to weak nonthermal EMF signals. There is abundant evidence that EMF signals can be configured *a priori* to increase the rate of CaM activation, which, in turn, can modulate the biochemical cascades living cells and tissues employ in response to external insult. Successful applications in pilot clinical trials, coupled with evidence at the cellular and animal levels, provide support that EMF is a first messenger that can modulate the response of challenged biological systems.

Keywords: calmodulin, first messenger, nitric oxide, pulsed electromagnetic field, radio frequency, signaling, tissue repair

Introduction

The vast majority of pulsed electromagnetic field (PEMF) and pulse-modulated radio frequency (PRF) fields employed clinically do not directly cause a physiologically significant temperature rise in the target cell/tissue area. Furthermore, several experimental and clinical systems have been shown to exhibit preferential responses to differing waveforms that cannot be explained on the basis of energy transfer alone. The observed bioeffects are, therefore, almost certainly due to the messenger (informational) content of each signal type. It was proposed 40 years ago that electromagnetic field (EMF) signals could be configured to deliver information encoded in the amplitude and frequency spectrum of the *in situ* electric field (Pilla, 1972, 1974a,b). This message is "understood" by certain electrochemical

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(voltage-dependent) processes at the electrified interfaces of macromolecules, either free or in the cell membrane. Models have been developed which showed that weak nonthermal EMF could be configured to modulate electrochemical processes such as ion binding (Pilla, 1972, 1974a,b, 2006). Recent refinements of this model have led to the *a priori* configuration of nonthermal PRF signals which have been demonstrated to modulate calmodulin (CaM)-dependent nitric oxide (NO) signaling in many biological systems (Pilla, 2006, 2012; Pilla et al., 2011). The Ca/CaM pathway is kinetically asymmetrical which has led to the proposal that not only could Ca^{2+} binding be modulated by weak electric fields, but Ca^{2+} dissociation, in the same pathway, could also be modulated by weak DC and low frequency AC magnetic fields (Muehsam and Pilla, 2009a,b; Pilla, 2012; Pilla et al., 2011). This study discusses CaM activation as the proposed transduction pathway for EMF signals and shows how this led to the configuration of new PRF signals as first messengers, which could modulate CaM activation as a first responder in a living cell's response to physical and/or chemical challenges. Cellular, animal and clinical results are presented which provide support for Ca/CaM-dependent NO production as an important mediator of EMF signaling that may explain the observed effects of EMF on tissue repair, angiogenesis, pain and inflammation in many studies. It is hoped that the approach presented here can form the basis of a unified approach to EMF therapeutics which could lead to its expanded clinical use.

EMF and the biology of injury and repair

Calcium ions play a pivotal role in signal transduction pathways that include cell growth and division, apoptosis, metabolism, synaptic transmission and gene expression (Bootman, Lipp, & Berridge, 2001; Mellstrom et al., 2008). Regulation of cytosolic Ca^{2+} concentrations is mediated by an elaborate system of pumps, channels and binding proteins found both in the plasma membrane and on intracellular organelles such as the endoplasmic reticulum (Harzheim, Roderick, & Bootman, 2010). High affinity/mediator proteins (e.g. CaM, troponin) are mediators of the multiple physiological responses regulated by changes in intracellular Ca^{2+} concentrations produced by physical and chemical challenges which cause free Ca^{2+} to increase above its normal and well-regulated value of approximately 100 nM (Konieczny, Keebler, & Taylor, 2012). CaM is of particular interest because it is the first responder to changes in cytosolic Ca^{2+} and the many roles it plays in cell signaling and gene regulation pathways once it is activated by bound Ca^{2+} (Faas, Raghavachari, Lisman, & Mody, 2011). As one immediate response to stress or injury, activated CaM binds to its primary enzyme target constitutive nitric oxide synthase [cNOS = neuronal NOS (nNOS) and endothelial NOS (eNOS)], which catalyzes L-arginine resulting in the release of the signaling molecule NO. As a gaseous free radical with an *in situ* half-life of about 5 s (Ignarro, Fukuto, Griscavage, Rogers, & Byrns, 1993), NO diffuses locally through membranes and organelles and acts on molecular targets at distances up to about 200 μm (Tsoukias, 2008). Low transient concentrations of NO activate its primary enzyme target, soluble guanylyl cyclase, which catalyzes the synthesis of cyclic guanosine monophosphate (cGMP) (Cho et al., 1992). The CaM/NO/cGMP signaling pathway is a rapid response cascade which can modulate peripheral and cardiac blood flow in response to normal physiologic demands, as well as to inflammation and ischemia (Bredt & Snyder, 1990). This same pathway also modulates the release of cytokines, such as interleukin-1beta (IL-1β) which is pro-inflammatory (Ren & Torres, 2009), and growth factors such as basic fibroblast growth factor (FGF-2) and vascular

endothelial growth factor (VEGF) which are important for angiogenesis, a necessary component of tissue repair (Werner & Grose, 2003).

Following an injury, e.g. a bone fracture or a surgical incision, repair commences with an inflammatory stage during which the pro-inflammatory cytokine IL-1β is rapidly released. This, in turn, up-regulates inducible nitric oxide synthase (iNOS), which is not Ca^{2+} dependent, and therefore not directly EMF-sensitive. iNOS produces large sustained amounts of NO in the wound bed (Lee, Efron, Tantry, & Barbul, 2001). Continued exposure to NO leads to the induction of cyclooxygenase-2 and increased synthesis of prostaglandins which also play a role in the inflammatory phase. While this process is a natural component of healing, when protracted it can lead to increased pain and delayed or abnormal healing (Broughton, Janis, & Attinger, 2006). In contrast, CaM/eNOS/NO signaling has been shown to attenuate levels of IL-1β and down-regulate iNOS (Palmi & Meini, 2002). EMF has been reported to down-regulate iNOS at the mRNA and protein levels in monocytes (Reale et al., 2006), and pro-inflammatory cytokines in human keratinocytes (Vianale et al., 2008). Finally, weak electric fields partially reversed the decrease in the production of extracellular matrix caused by exogenous IL-1β in full-thickness articular cartilage explants from osteoarthritic adult human knee joints (Brighton, Wang, & Clark, 2008).

As tissue further responds to injury, the CaM/NO/cGMP cascade, through which EMF rapidly modulates the relaxation of the smooth muscles controlling blood and lymph vessel tone, also reacts to EMF to enhance growth factor release in endothelial cells to modulate angiogenesis. EMF modulation of eNOS activity may, therefore, be a useful strategy to augment angiogenesis for tissue repair and possibly other conditions that require vascular plasticity, such as ischemia (Cooke, 2003). Endothelial cells would therefore be a likely target for EMF modulation of tissue repair. The expectation is that EMF will enhance cGMP formation at the signaling level and increase FGF-2 to enhance angiogenesis. An early study showed that EMF augmented the creation of tubular, vessel-like structures from endothelial cells in culture in the presence of growth factors (Yen-Patton et al., 1988). Another study confirmed a seven-fold increase in endothelial cell tubule formation *in vitro* (Tepper et al., 2004). Quantification of angiogenic proteins demonstrated a five-fold increase in FGF-2, suggesting that EMF modulates angiogenesis by increasing FGF-2 production. This same study also reported that EMF increased vascular ingrowth more than two-fold when applied to an implanted Matrigel plug in mice, with a concomitant increase in FGF-2, similar to that observed *in vitro*. EMF significantly increased neovascularization and wound repair in normal mice, and particularly in diabetic mice, through an endogenous increase in FGF-2, which could be eliminated by using a FGF-2 inhibitor (Callaghan et al., 2008). Similarly, a PRF signal of the type used clinically for wound repair was reported to significantly accelerate vascular sprouting from an arterial loop transferred from the hindlimb to the groin in a rat model (Roland, Ferder, Kothuru, Faierman, & Strauch, 2000). This study was extended to examine free flap survival on the newly produced vascular bed (Weber, Navarro, Wu, Yu, & Strauch, 2005). Results showed 95% survival of PRF-treated flaps compared to 11% survival in the sham-treated flaps, suggesting a significant clinical application for PRF signals in reconstructive surgery. Another study (Delle Monache, Alessandro, Iorio, Gualtieri, & Colonna, 2008) reported that EMF increased the degree of endothelial cell proliferation and tubule formation and accelerated the process of wound repair, suggesting a mechanism based upon an EMF effect on VEGF receptors.

An important action of NO is modulation of nociception (Cury et al., 2011), perhaps via a feedback loop between NO and cytokines which modulates inflammatory pain (Chen et al., 2010). Clinically, NO enhances the actions of narcotics for postoperative analgesia (Lauretti et al., 1999), which may play a role in

the reported effects of PRF signals on reduction of postoperative narcotic usage (Hedén and Pilla, 2008; Rawe, Lowenstein, Barcelo, & Genecov, 2012; Rohde, Chiang, Adipoju, Casper, & Pilla, 2009). It has also been shown that addition of an NO donor to nonsteroidal anti-inflammatory drugs and aspirin enhances analgesia (Borhade et al., 2012; Velazquez, Praveen Rao, & Knaus, 2005). Modulation of the endogenous opioid pathway by physical modalities has previously been described for extremely low frequency magnetic fields (Kavaliers & Ossenkopp, 1991; Prato et al., 1995) and transcutaneous electrical nerve stimulation (Sluka et al., 1999). It has recently been reported that PRF signals upregulate the expression of endogenous opioid precursors in human epidermal keratinocytes and dermal fibroblasts both at the mRNA and protein levels (Moffett, Fray, & Kubat, 2012). The same PRF signal was also used in a rat pain behavior model, wherein pain reduction was, in part, related to EMF modulation of the release of β-endorphin (Moffett et al., 2010, 2011).

EMF and CaM-dependent signaling

Ca/CaM binding has been well characterized, with a binding time constant reported to be in the range of 1–10 ms (Blumenthal & Stull, 1982), whereas the dissociation of Ca^{2+} from CaM requires the better part of a second (Daff, 2003). Thus, Ca/CaM binding is kinetically asymmetrical, i.e. the rate of binding exceeds the rate of dissociation by several orders of magnitude ($k_{on} \gg k_{off}$), driving the reaction in the forward direction according to the concentration and voltage dependence of Ca^{2+} binding. The asymmetry in Ca/CaM binding kinetics provides an opportunity to configure any EMF waveform to induce an electric field that can produce a net increase in the population of bound Ca^{2+} (activated CaM) (Pilla, 2006, 2012; Pilla et al., 2011; Pilla, Muehsam, Markov, & Sisken, 1999). However, this is only possible if pulse duration or carrier period is significantly shorter than bound Ca^{2+} lifetime. Thus, Ca^{2+} binds to CaM as the voltage at the binding site increases; however, Ca^{2+} does not immediately dissociate from CaM when the voltage decreases as the waveform decays or the sinusoidal wave changes polarity, because the Ca^{2+} just bound in the initial phase of the waveform is sequestered for the better part of a second to permit activated CaM to activate its target enzyme. Thus, the Ca/CaM signaling pathway can exhibit rectifier-like properties for any EMF signal because ion binding kinetics are asymmetrical, not because there is a non-linearity in electrical response, as has already been discovered for RF signals (Kowalczuk et al., 2010).

Initial configuration of an EMF signal that can modulate NO signaling in real-time by increases in the population of bound Ca^{2+} to CaM, thereby increasing the concentration of activated CaM, is achieved by analysis of the kinetic equations describing the two step Ca/CaM/cNOS binding process in terms of dielectric properties using the electrochemical information model (Pilla, 1972, 1974a,b, 2006, 2012; Pilla et al., 1999, 2011). Considering a linear forward binding step, there results a two time constant electrical equivalent circuit analog which represents the kinetics of Ca^{2+} binding to CaM, and of activated CaM binding to cNOS. This allows analysis of signal-to-noise ratio (SNR), which compares the effective voltage induced in the Ca^{2+} binding pathway to thermal noise voltage in the same pathway. This is the essential first step because a net detectable increase in voltage must be induced at the binding site in order for more Ca^{2+} to bind to CaM according to its voltage dependence than would have been bound in the absence of EMF. Note that a net increase in bound Ca^{2+} via the induced electric field can only occur if the ion-binding step is kinetically asymmetrical, as it is for Ca/CaM (Pilla, 2012; Pilla et al., 2011). What follows will summarize how a nonthermal PRF signal can be configured

a priori to optimally modulate Ca^{2+} binding to CaM. Focus is on the PRF waveform in this study because of the easier regulatory pathway to clinical devices; however, any EMF waveform may be configured using the approach outlined below.

As shown in detail elsewhere (Pilla, 1974, 2006; Pilla et al., 2011), the kinetics of Ca^{2+} binding to CaM may be represented by a series R_A–C_A electrical equivalent circuit, where R_A is the equivalent resistance of binding, inversely proportional to k_{on}, and C_A is the equivalent capacitance of binding, proportional to bound Ca^{2+}. The time constant for Ca^{2+} binding is, thus, $\tau_A = R_A C_A$. To evaluate SNR for the Ca/CaM target, the quantity of interest is the effective voltage, $E_b(s)$, induced across C_A, evaluated for simple ion binding using standard circuit analysis techniques (Cheng, 1959):

$$E_b(s) = \frac{(1/s\,C_A)E(s)}{(R_A^2 + (1/s\,C_A)^2)^{1/2}}, \tag{1}$$

where $E(s)$ is the induced electric field, and s is the real-valued frequency variable of the Laplace transform (Cheng, 1959; Pilla, 1970). Default (1) clearly shows that $E_b(s)$ is dependent upon $E(s)$, which can be defined for any waveform, e.g. rectangular, sinusoidal, arbitrary and chaotic. In every case, only that portion of the applied electric field at the Ca/CaM binding pathway, $E_b(s)$, can increase surface charge (bound Ca^{2+}). Thus, as long as it is nonthermal, the total applied energy in $E(s)$ is not the dose metric. Rather, the frequency spectrum of $E_b(s)$, for any $E(s)$, is taken as a measure of biological reactivity. The calculation of SNR has been described in detail elsewhere (Pilla, 2006). Briefly, thermal noise in the binding pathway may be evaluated as follows (DeFelice, 1981):

$$\mathrm{RMS_{noise}} = \left[4\,kT\int_{\omega_1}^{\omega_2} \mathrm{Re}\,[Z_A(\omega)]\,\mathrm{d}\omega\right]^{1/2}, \tag{2}$$

where $\mathrm{RMS_{noise}}$ is the root mean square of the thermal noise spectral density; Re is the real part of the total binding impedance, Z_A; $\omega = 2\pi f$; and the limits of integration (ω_1, ω_2) are determined by the bandpass of binding, typically 10^{-2}–10^7 rad/s. SNR is evaluated using the following equation:

$$\mathrm{SNR} = \frac{\mathrm{E}_b(s)}{\mathrm{RMS_{noise}}}, \tag{3}$$

where $E_b(s)$ is given by the Laplace transform of $E(s)$ applied to the overall target impedance, which may be distributed for tissue or neuronal targets (Pilla, Nasser, & Kaufman, 1994).

Nonthermal PRF signals, consisting of 1–5 ms bursts of 27.12 MHz sinusoidal waves repeating at 2–5 bursts/s with 1–5 μT peak amplitude were developed, *a priori*, using the approach just described (Pilla, 1972, 2006; Pilla et al., 2011). These signals, referred to hereinafter as simply PRF, were tested on CaM-dependent myosin light chain kinase activation in cell free assays (Markov, Muehsam, & Pilla, 1994; Pilla, 2006); dendrite outgrowth from avian neural explants; bone repair in a rabbit model (Pilla et al., 1999); CaM-dependent cGMP production at the cellular level, wherein trifluoro perazine, a CaM antagonist blocked the PRF effect (Pilla et al., 2011); and on cutaneous wound and Achilles' tendon repair in rat models (Strauch, Patel, Navarro, Berdischevsky, & Pilla, 2007; Strauch et al., 2006).

A recent study specifically examined the effect of PRF on CaM-dependent NO signaling. Dopaminergic cells (MN9D) were challenged acutely with a nontoxic concentration of lipopolysaccharide (LPS), which causes an immediate increase in cytosolic Ca^{2+}. As reviewed above, any free cytosolic Ca^{2+} above approximately 100 nM instantly activates CaM, which, in turn, instantly activates cNOS. The result is

immediate NO production. PRF was applied during LPS challenge. The results (Figure 1A) showed that PRF approximately tripled NO release from challenged MN9D cells within seconds, as measured electrochemically in real-time using a NO selective electrode (Pilla, 2012).

PRF has been reported to augment CaM-dependent NO release from human articular chondrocyte, human umbilical vein endothelial cells (HUVEC) and fibroblast cultures (Pilla et al., 2011; Pilla, 2012). These studies showed that an EMF effect could be obtained only if chemical (e.g. serum depletion) or physical (e.g. temperature shock) imbalances were large enough to cause sufficient increases in cytosolic Ca^{2+} to satisfy the Ca/CaM dependence of cNOS (Bredt & Snyder, 1990). Direct measurement of cytosolic Ca^{2+} binding to CaM under physiological conditions in living cells or tissue has not yet been successfully performed under EMF exposure. However, the CaM antagonist N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) was able to block the EMF effect on NO release, supporting CaM activation as a principle EMF target for the modulation of tissue repair. A typical example of the effect of PRF on NO release in challenged fibroblast cultures is summarized in Figure 1B, which shows that a single 15-min PRF exposure produced a nearly two-fold increase in NO, which could be blocked with W-7.

Clinical applications of PRF

Prior to performing any clinical studies, the PRF signal was extensively tested in a full thickness cutaneous wound model in the rat, as described in Strauch et al. (2007). Dosimetry was assessed by varying burst duration and burst repetition rate of a 27.12 MHz carrier at constant amplitude such that SNR ranged from 0.3 to >1. The results showed that wound tensile strength at 21 days was directly proportional to SNR in the Ca/CaM binding pathway, consistent with an EMF effect on CaM-dependent signaling for wound repair.

Postoperative pain management

Postoperative pain is one of the principal reasons for extended post-surgical hospital stays. Double-blind, placebo-controlled, randomized clinical studies have reported that PRF significantly accelerates post-op pain reduction and, concomitantly,

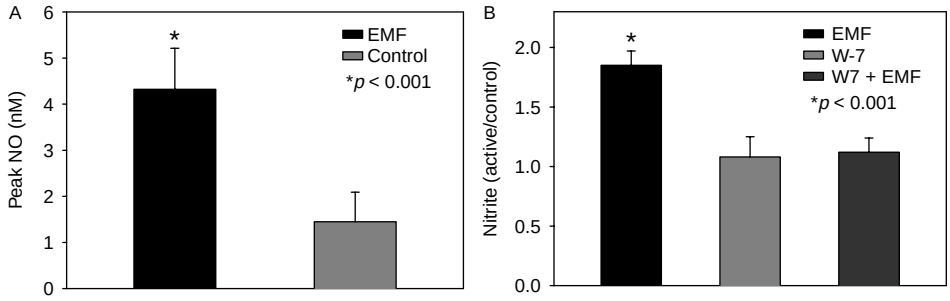

Figure 1. Effect of a nonthermal PRF signal in clinical use on NO release at the cellular level. **A**: MN9D dopaminergic cells during challenge with LPS (100 ng/mL). NO was measured in real-time with a NO selective electrode. Results show that PRF produced an immediate (<5 s) nearly three-fold increase in peak NO. **B**: 15 min PRF exposure on NO in conditioned medium from human fibroblasts challenged with depleted serum. PRF increased NO (via Griess determination of nitrite) by nearly two-fold. The CaM antagonist W-7 blocked the PRF effect, but had no effect on baseline NO already present from the serum depletion challenge which existed at plating. Data from Pilla (2012).

narcotic requirements (Hedén & Pilla, 2008; Rawe et al., 2012; Rohde et al., 2009). The availability of wound exudates in one study (Rohde et al., 2009) allowed the time course of the inflammatory cytokine IL-1β to be quantified in the wound bed. In that study, the PRF signal from a disposable device placed in the surgical dressing and activated immediately after breast reduction surgery was programmed to automatically provide a 20-min treatment every 4 h. Wound exudates were assessed for IL-1β, and pain was self-assessed using a Visual Analog Scale (VAS) hourly in the immediate postoperative period. The results showed that concentrations of IL-1β were approximately 2.5-fold higher in wound exudates of sham-treated patients at 5 h post-op compared to those of the active group ($p < 0.001$). Concomitantly, in the active group, PRF produced a 2.5-fold decrease in pain by 5 h post-op ($p < 0.001$), persisting to 24 h post-op. No significant changes in VAS scores were observed in the control group over this same time period. Sham-treated patients used 2.2-fold more narcotics over the first 24 h post-op ($p = 0.002$). The time course for both pain and IL-1β reduction were concomitant, suggesting that PRF therapy produced rapid endogenous changes in the dynamics of IL-1β availability in the wound bed by modulation of CaM-dependent NO signaling. The results are summarized in Figure 2. This application of PRF therapy accelerates surgical healing which reduces patient morbidity and could reduce the cost of health care via shorter hospital stays and faster return to function.

Degenerative joint disease

Mechanical degradation of articular cartilage, particularly in knee and hip joints leads to osteoarthritis (OA) which affects nearly 10% of the adult population. Cartilage degeneration causes pain which can become so severe that joint replacement is the only relief. In a recent double-blind, placebo-controlled, randomized pilot clinical study, PRF was self-applied for 15 min twice daily for 42 days in adults with early knee OA (Nelson, Zvirbulis, & Pilla, 2012). The results showed mean VAS pain score decreased in the active cohort by 50% ± 11% versus baseline starting at day 1 and persisting to day 42 ($p < 0.001$). There was no significant decrease in VAS versus baseline at any time point in the sham cohort ($p = 0.227$). The overall decrease in mean VAS score for the active cohort was nearly three-fold that of sham cohort ($p < 0.001$). The rapid reduction of pain in the active group is similar to that observed in the previous post-op pain study, suggesting that PRF enhanced CaM-dependent NO signaling. Indeed, one source of pain in knee OA is joint effusion (swelling) which can be rapidly reduced by blood and lymph vessel dilation modulated by NO/cGMP signaling. It was also speculated that PRF down-regulated IL-1β with its consequent attenuation of inflammation. The results are summarized in Figure 3.

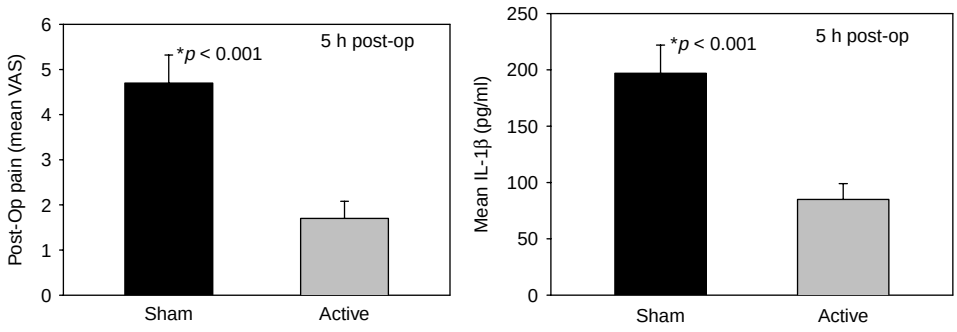

Figure 2. Rapid PRF effect on post-op pain in breast reduction patients. Left: mean maximum VAS pain score was about 2.5-fold higher in the sham cohort at 5 h post-op. Right: IL-1β in wound exudates was concomitantly 2.5-fold higher in the sham cohort. Results suggest that PRF acted through the CaM/NO signaling pathway. Data from Rohde et al. (2009).

Angina and angiogenesis

PRF has a rapid effect on NO release via CaM-dependent signaling, suggesting that an immediate effect on vasodilation *in vivo* could be expected (Nelson et al., 2012). This was tested in a double-blind, placebo-controlled, randomized pilot clinical study in inoperable ischemic cardiomyopathy patients with chronic angina (Durán, Breslin, & Sánchez, 2010). Patients in the active cohort received PRF treatment with a 20 cm diameter circular applicator coil placed directly over the heart for 30 min twice daily for 3 months. Measurements were continued, in a wash-out period, for 5 months. Patients in the sham cohort received identical inactive devices and remained blinded because indicator lights on devices were identical for both sham and active groups. In addition, the PRF signal could only be detected with specialized laboratory equipment not available to patients or health care personnel. The Seattle Angina Questionnaire (SAQ) was employed to assess anginal severity and frequency, as well as physical activity. Results given in Figure 4 show that anginal severity was reduced nearly two-fold at 5 months in the active group (higher numbers are better), with no significant change in the sham group ($p < 0.001$). Similar results were obtained for anginal frequency and physical activity. Overall, PRF produced results equivalent to angioplasty in patients who were not candidates for this intervention. In a related study designed to assess the effect of PRF on cardiac angiogenesis, a reproducible thermal myocardial zone of injury was created in the region of the distal aspect of the Left Anterior Descending Artery at the base of the heart in a blinded rat model (Patel, Factor, Wang, Jana, & Strauch, 2006; Strauch, Herman, Dabb, Ignarro, & Pilla, 2009; Strauch, Patel, Rosen, Casper, & Pilla, 2006). PRF exposure was 30 min twice daily for 3, 7, 14 or 21 days. Sham animals were identically exposed, but received no PRF signal. A separate group of animals treated for 7 days received L-nitrosoarginine methyl ester (L-NAME), a general NOS inhibitor, in their drinking water. Upon sacrifice, myocardial tissue specimens were stained with CD-31 and the number of new blood vessels was counted on histological sections at the interface between normal and necrotic muscle at each time point. The results showed mean new vessel count was not significantly increased by PRF at day 3, but was significantly increased at day 7 (+50%, $p = 0.006$), day 14 (+67%, $p = 0.004$) and day 21 (+99%, $p < 0.001$). The results shown in Figure 5 for day 7 indicate that L-NAME completely blocked the PEMF effect on

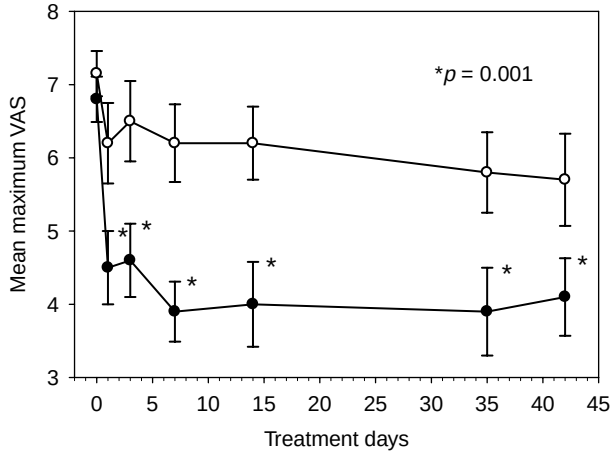

Figure 3. Effect of PRF on pain from knee osteoarthritis in a randomized double-blind clinical study. Mean maximum pain (VAS) in the active cohort was 50% of its baseline value within the first 24 h, which persisted to day 42. There was no significant decrease in pain in the sham cohort at any time point. Data from Nelson et al. (2012).

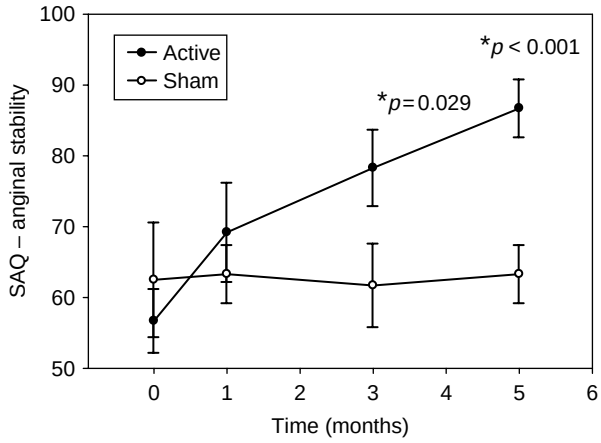

Figure 4. Effect of PRF for 30 min 2 × daily for 3 months, followed by 2 month wash-out, on cardiomyopathy patients with inoperable chronic angina. Results show that anginal severity was reduced nearly two-fold in active patients versus no change in shams (larger numbers are better). Outcome is similar to that produced by angioplasty. Data from Shen et al. (2009).

angiogenesis, suggesting that the transduction pathway for PRF effects in this study involved CaM-dependent NO signaling.

Discussion and conclusion

Experimental and clinical evidence reviewed here strongly support the role of Ca/CaM-dependent NO production as an important mediator of EMF signaling for cell proliferation, differentiation, tissue repair, angiogenesis, pain reduction and anti-inflammation in cell, animal and clinical studies. The experimental results provide support for the proposal that PRF signals can be configured, *a priori*, to increase the rate and amount of Ca^{2+} binding to CaM. This, in turn, can modulate cNOS activation, producing a rapid and transient increase in NO production in various cell types. Disruption of tightly regulated free cytosolic Ca^{2+} in cells is a

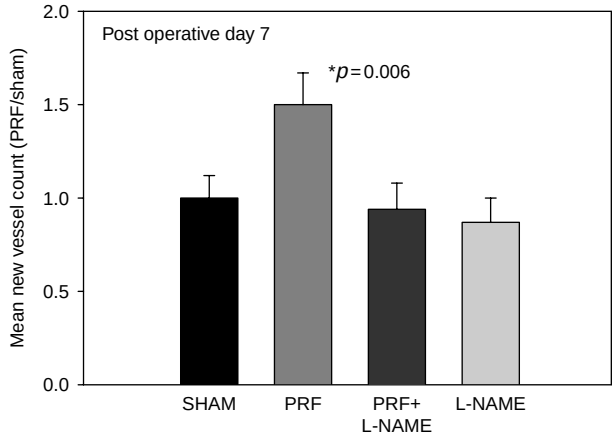

Figure 5. Effect of PRF in a thermal myocardial necrosis rat model. The results show that PRF for 30 min twice daily increased angiogenesis by 50% versus shams. L-NAME, a NOS inhibitor, blocked the PRF effect on vessel regrowth in this model, suggesting that Ca/CaM/NO signaling is the transduction pathway for these PRF effects. Data from Strauch et al. (2009).

signal for endogenous tissue repair and regeneration mechanisms, which opens the Ca/CaM binding EMF-sensitive pathway. Cytosolic Ca^{2+} is in homeostasis for uninjured or unchallenged cells and tissues, and thus EMF would not be expected to have a physiologically significant effect. This is consistently observed with the PRF signal employed in the studies reviewed here, providing one explanation for the reports of no known side effects from current therapeutic applications of EMF.

It is important to realize that all of the biological and clinical results presented here were obtained with a nonthermal radio frequency signal (PRF). Indeed, it has been shown that maximum SAR for a 27.12 MHz carrier (short wave radio band), which is pulse modulated with a 2 ms burst repeating at 2 bursts/s, inducing a mean electric field amplitude of 30 V/m in a cylindrical saline phantom target is about 10^{-4} W/kg (Pilla et al., 2011). Temperature rise for any RF exposure duration may be calculated from SAR × duty cycle (0.4% for the PRF signals employed in the studies reviewed here) $= 4 \times 10^{-5}$ W-s/kg $= 4 \times 10^{-5}$ J/s-kg. From this, temperature rise for 1 h PRF exposure is readily calculated using the heat capacity of the saline phantom. The worst case result, not taking into account the decay of induced E with target depth, and assuming no heat exchange, shows that a maximum undetectable temperature rise of 0.00034 °C/h can be expected.

Given the evidence presented that PRF can modulate CaM-mediated NO signaling, it is of interest to examine whether similar nonthermal bioeffects could be produced from the RF signals emitted by cellular phones. It is reasonable to assume that Ca^{2+} binding to CaM is a potential target pathway for a Global System for Mobile Communications (GSM) signal. Thus, the rectifier-like property of Ca/CaM binding in the CaM/NO signaling pathway provides a sufficient condition for a nonthermal RF effect to occur. The necessary condition for a nonthermal bioeffect is that the GSM signal produces sufficient SNR in the Ca/CaM pathway. SNR has been evaluated for a single 577 μs burst of an 1800 MHz GSM signal and a single 2000 μs burst of a 27.12 MHz sinusoidal PRF signal configured to modulate the CaM/NO signaling pathway (Pilla and Muehsam, 2010). The results showed that SNR in the Ca/CaM pathway for the 1800 MHz GSM signal at 20 V/m (Parazzini et al., 2007) is similar to that of a single 2000 μs pulse of the 27.12 MHz PRF signal at 12 V/m [within the therapeutic range (Hedén & Pilla, 2008; Nelson et al., 2012; Rawe et al., 2012; Rohde et al., 2009; Shen et al., 2009)]. Peak SNR for the GSM signal is in the same range as that for the PRF signal and occurs in a frequency range consistent with published values for k_{on} for Ca/CaM. Therefore, it is reasonable to expect brief exposure to a GSM signal to result in modulation of the CaM/NO signaling pathway, just as has been shown throughout this study for the PRF signal. As for PRF, a physiologically significant effect may only occur if the cell/tissue target is challenged, as evidenced in a study which showed no effect on cerebral blood flow when a 900 MHz GSM signal was applied to healthy human volunteers (Kwon et al., 2012). On the other hand, GSM and other RF signals used in communication may have nonthermal bioeffects, including therapeutic effects, in a challenged biological system. Indeed, recent reports show that long-term exposure to a GSM signal protects against and reverses cognitive impairment in a mouse model of Alzheimer's disease (Arendash et al., 2010, 2012a,b; Dragicevic et al., 2011). Both IL-1β and iNOS are up-regulated in cerebral inflammation which enhances the deposition of β-amyloid causing cognitive impairment (Chiarini, Dal Pra, Whitfield, & Armato, 2006). If a GSM signal can act via the Ca/CaM pathway, it could down-regulate both factors, which may prevent or reverse the effects of Alzheimer's disease and any other neurodegenerative disease with an inflammatory component.

While the results presented here support an electric field effect on CaM signaling via the asymmetrical voltage-dependent kinetics of Ca^{2+} binding to CaM (k_{on}), it is

important to emphasize that its slow dissociation kinetics (k_{off}) can be responsive to weak DC and combined DC/AC magnetic (B) fields. Indeed it has been reported that weak B fields can affect NO signaling (Reale et al., 2006; Vianale et al., 2008). Of the many models proposed to explain the bioeffects of weak magnetic fields, those involving modulation of the bound trajectory of a charged ion by classic Lorentz force (Chiabrera & Bianco, 1993; Edmonds, 1993; Muehsam & Pilla, 2009a,b) are most relevant to asymmetrical ion binding kinetics, suggesting that weak B-field effects on the trajectory of the ion within the binding site itself could affect reactivity. One interpretation of this is that weak DC and certain combinations of weak AC/DC magnetic fields could enhance or inhibit the exit of the target ion from the binding site, thereby accelerating or inhibiting the overall reaction rate by manipulating dissociation kinetics (k_{off}), even in the presence of thermal noise. This has been tested with success for CaM-dependent myosin phosphorylation (Muehsam & Pilla, 2009), suggesting that analysis of B-field effects on bound ion trajectories could be used to explain and predict weak magnetic field effects on CaM-dependent NO signaling via modulation of Ca/CaM dissociation kinetics.

It is clear that EMF can instantaneously modulate NO release in injured tissue. At the cellular level, EMF-mediated NO signaling could be the common transduction mechanism in studies that report up- and down-regulation of anti-inflammatory genes (Brighton et al., 2008; Moffett et al., 2012; Wang, Clark, & Brighton, 2006), and modulation of adenosine pathways (De Mattei et al., 2009; Varani et al., 2011). At the clinical level, there is strong support that EMF-mediated signaling is anti-inflammatory and can enhance angiogenesis. NO release via cNOS is dynamic (Batchelor et al., 2010) and closely linked to the negative feedback provided by phosphodiesterase inhibition of cGMP (Mo, Amin, Bianco, & Garthwaite, 2004). This causes ensuing dynamic consequences in the rates of up- or down-regulation of growth factors and cytokines. For example, iNOS activity in the inflammatory stage of healing can be rapidly down-regulated by inhibition of nuclear factor-kappa B in a negative feed-back mechanism (Chang et al., 2004). However, the interplay among the many up- and down-regulations necessary to provide an enhanced biological or clinical outcome places requirements on the overall dosimetry of PRF in which SNR analysis must be complemented with predictions of effective regimens. It is, nonetheless, tempting to unify the results presented here via EMF modulation of NO signaling; however, many further studies are required to establish a clear relationship between EMF effects on NO signaling and clinical outcomes.

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Declaration of interest

The author report no conflicts of interest. The author alone are responsible for the content and writing of the paper.

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