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Millimeter Wave-induced Modulation of Calcium Dynamics in an Engineered Skin Co-culture Model: Role of Secreted ATP on Calcium Spiking

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Co-culture skin tissue model/ATP/Calcium dynamics/Millimeter wave radiation/Keratinocytes/Neuronal cells.

We have previously designed and characterized a 94 GHz exposure system that allows real-time monitoring of subcellular interactions induced by millimeter wave (MMW) stimulation. For example, studies of the calcium dynamics in neuronal cells in response to 94 GHz irradiation suggested that MMW stimulation increased calcium spiking. In this study, we engineered a 3D co-culture model that represents the major constituents of skin. We used this experimental model along with the custom-designed MMW exposure system to investigate the effects of 94 GHz irradiation in the skin-like tissue construct. Unlike typical non-excitable cells, keratinocytes exhibited calcium spikes in their resting state. Exposure to a 94 GHz irradiation induced a statistically significant increase in the calcium spiking. When co-cultured with neuronal cells in the 3D co-culture skin model, changes in the calcium spiking in neuronal cells depended on the MMW input power. Further, the 94 GHz irradiation caused ATP secretion by keratincytes. ATP is a major factor that modulates the calcium spiking in neuronal cells. Surprisingly, while a 5-fold increase in the ATP secretion enhanced the calcium spiking in neuronal cells, a 10-fold increase significantly hindered the calcium dynamics. Computational simulation of ATP-induced calcium dynamics was in general agreement with the experimental findings, suggesting the involvement of the ATP-sensitive purinergic receptors. The engineered co-culture skin model offers a physiologically relevant environment in which the calcium dynamics is regulated both by the cell-MMW and cell-cell interactions.

INTRODUCTION

Millimeter-wave (MMW) radiation is currently used for a variety of military and civilian applications. Systems are employed by the military for land-based and airborne radars and are being tested for passive MMW imaging of weapons. Moreover, MMW technologies have been developed for armored vehicle-mounted multi-functional devices for simultaneous active protection, surveillance, communication, and combat identification. An increased risk of occupational overexposure to MMWs likely poses a concern due to the continuing development of higher power technologies that may cause significant temperature increases in superficial body tissues. For civilian applications, MMW is also playing an increasingly important role in the modern telecommunication systems (e.g., wireless high-bandwidth data links).

Exposure to MMW radiation is known to cause a temperature rise in biological tissues. The rate and magnitude of temperature rise depends upon the exposure intensity and duration. At a frequency greater than 10^9 Hz (GHz), an MMW radiation penetrates less than a few mm in depth.[1–3] Thus, potential bioeffects to a living host are likely confined to the skin and cause minimal direct effects to the internal tissues and organs. Multiple cell types are found in the skin tissue, including keratinocytes, melanocytes, fibroblasts, and endothelial cells. It is composed of at least three layers, beginning with a superficial barrier layer of inactive cells (stratum corneum, 10 to 20 μm thickness), followed by a epidermis layer of keratinocytes (~ 100 μm thick) and a dermis layer (~ 1 mm) that contains keratinocytes and nerve endings known as nociceptors.[4,5] The nociceptors are nerve fibers that can detect and sense thermal, chemical, and mechanical stimuli and transduce pain sensation. Detailed understanding of nociception has recently been made possible through molecular biology and neurophysiological studies. For example, nociception is mediated by a superfamily

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of receptors currently designated as transient receptor potential (TRP) proteins that are expressed on both keratinocytes and neurons. These channels can conduct ions (e.g., primarily Ca^{2+}) and, upon activation, release neurotransmitters such as ATP, nitric oxide, substance P, and glutamate.[6–9] Interestingly, keratinocytes respond to "warm" temperatures such as ~ 34 to 38°C and ~ 27 to 35°C by activating sub-families of the TRP proteins. These heat sensors are likely coordinated spatiotemporally, perhaps through modulation of calcium dynamics, to regulate reactions to noxious stimuli. Activation of nociceptive signaling pathways in response to external stimuli such as MMW may be primarily confined to keratinocytes due to the limited penetration depth of MMW.

Current advances in tissue engineering offer several advantages to characterize and determine molecular responses induced by external physical stimulations such as 94 GHz irradiation, which the military has utilized to develop the active denial system (www.jnlwp.usmc.mil). First, quantitative molecular benchmarks can be established experimentally without significant sample variation. Second, the engineered tissues can be accurately reproduced and thereby avoid the difficulties associated with using animal or human tissues. For example, sources of tissue non-homogeneity such age or donor health are virtually eliminated. Eliminating donor variability also significantly reduces complications involved in the modeling and prediction of the effects of MMW exposure. Third, biological variation and fluctuation of animal or human tissues is minimized, including thermal regulatory processes such as blood flow and perspiration rate. Fourth, real-time measurements of the nociceptive molecular signatures, including calcium dynamics and generation of neurotransmitters, provide helpful insight for establishing a reliable set of guidelines for intended effects of MMW-based technologies. Lastly, the use of engineered skin tissue models potentially offers a better understanding of the complex biomolecular, cellular and sensory pathways mediating the bioeffects of MMW irradiation.

Previously, we reported increased intracellular calcium spiking in stem cell-derived neuronal cells in response to 94 GHz irradiation. Both cell surface calcium channels as well as intracellular calcium machinery mediated the increased spiking.[10] Thermal mechanisms alone were not sufficient to account for the amplified calcium spiking activity observed in the exposed neuronal cells. In this study, we developed a 3D co-culture model to mimic the nociception pathways in the skin tissue and examined the effect of 94 GHz irradiation. Our findings suggest that MMW exposure to non-excitable keratinocyes increased the calcium spiking activity and stimulated ATP secretion. Secreted ATP then modulated calcium spiking in neuronal cells, which were highly dependent on the MMW-induced generation of neurotransmitters in keratinocytes.

MATERIALS AND METHODS

Cell cultures

Mouse embryonal carcinoma cells (P19) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and maintained in α-modified Eagle's medium (α-MEM), 2.5% fetal bovine serum (FBS; Atalanta Biologicals, Lawrenceville GA), 7.5% newborn calf serum and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY). Induction of neuronal differentiation was described earlier.[10,11] Briefly, P19 cells were cultured in 10 ml of α-MEM with 0.5 μM all-trans retinoic acid, 5% FBS and 1% penicillin/streptomycin and (Sigma, St. Louis, MO) for four days. Then the small aggregates of cells were isolated and plated onto 24 × 30 mm glass cover slips, and cultured with serum-free neurobasal medium with B-27 supplement (Invitrogen, Grand Island, NY) for additional four days before the experiments. Based on DIC and fluorescent image analysis, approximately 50% or more of P19 cells exhibited a neuronal morphology (neurite extensions) and expressed specific neuronal markers (neurofilaments and β_3-tubulins). Epidermal keratinocytes (HEKn) were obtained from Cascade Biologics (Carlsbad, CA) and cultured in EpiLife® medium supplemented with EpiLife®-defined growth supplement (Cascade Biologics). According to the manufacturer's instructions, culture flasks or glass cover slips were pre-coated with Matrix gel (Cascade Biologics), and keratinocytes used in these studies were passage between 5 and 7.

94 GHz irradiation exposure

A MMW irradiator has been custom-built to enable optical monitoring of the subcellular phenomena in real time (Fig. 1A). A detailed description has been provided elsewhere.[10] Briefly, a Gunn oscillator was used to generate the 94 GHz signal. Millimeter waves were passed through a horizontal waveguide in a TE_{10} mode, and redirected by a 90° E-plane waveguide bend onto the cell sample on a microscope stage. The intensity of the incident beam was measured with a power-calibrated crystal detector. The backward reflection was minimized using an EH-tuner.

The exposure chamber consisted of two 24 × 30 mm coverglasses (nominal thickness 200 μm) separated by a spacer (thickness 200 μm). The chamber was typically sealed with vacuum grease (Dow Corning, Midland, MI), mounted on the stage of an inverted microscope (TE2000, Nikon, Japan), and the applicator's 90° bend was positioned above the chamber. When engineering the two cell type co-culture skin construct, the glass coverslip with keratinocytes was mounted on the top (i.e., directly exposed to 94 GHz irradiation), and the other with neuronal cells was at the bottom (Fig. 1B). When keratinocytes were exposed to 94 GHz irradiation without neuronal cells, keratinocytes were plated on the top coverslip and the bottome one was a blank coverslip

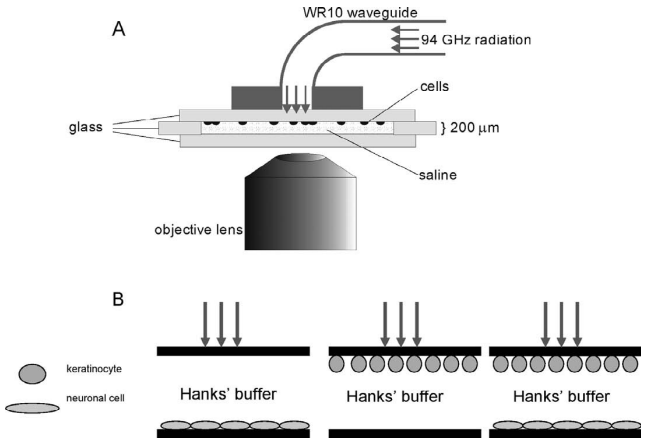

Fig. 1. Schematics of 94 GHz exposure and 3 different cell co-culture models. The MMW exposure apparatus (A) and altered calcium dynamics in neuronal cells in the chamber have been extensively characterized and documented.[10] In the present study, we extended the cell culture models to examine the effects of 94 GHz exposure on keratinocytes alone and in the co-culture model of keratinocytes proximal to the MMW exposure and neuronal cells separated by 200 μm (B). In doing so, interactions between MMW and cells and between major cell types found in the skin are probed in real-time.

without any cells. The chamber was filled with Hanks' buffer, and then sealed with vacuum grease (Dow Corning, Midland, MI) before being mounted on the stage of an inverted microscope (TE2000, Nikon, Japan). In this paper, either a 30 mW or 60 mW input power was used (for temperature calibration, see ref. 10). The experiments were conducted at room temperature (~22°C) without any forced convection cooling. A thermal camera was employed to detect the temperature. In response to a 60 mW input, the temperature inside the chamber rose rapidly and reached equilibrium in less than 10 s. The maximum temperature rise was estimated 8°C above the ambient. The final steady-state temperature of 30°C in the exposure chamber was achieved quickly and better mimicked the normal skin temperature (32 to 34°C).

Real-time optical microscopy for calcium spike

The details of experimental procedures to identify and record calcium spikes have been provided elsewhere.[10,11] Briefly, cells were loaded with the calcium-sensitive 5 μM Fluo-4 (Invitrogen, Eugene, OR) and incubated at 37°C for 30 min and washed three times with buffer. The active cells demonstrating more than one calcium spike within the 30 min observation period were counted and analyzed for calcium activity with an imaging processor (Metamorph, Universal Imaging). For each of these responding cells, average Fluo-4 fluorescence intensity was plotted as a function of time. After background subtraction, the ratio of the peak and baseline values for each calcium spike was calculated. When this peak-to-base ratio exceeded 1.3, the event was classified as a calcium spike. For each experimental condition, 40 to 70 cells from three independent experiments were analyzed.

ATP release assay

To determine the ATP level released from keratinocytes, the cells seeded on cover glass were incubated for 24 hrs. Each sample was exposed to a 94 GHz irradiation for 30 min and, immediately after exposure, the buffer within the chamber was collected and stored in a –20°C freezer. The ATP level in the buffer was measured using a commercially available kit (Invitrogen, Eugene, OR) and a luminometer (Biotek Luminometer, Winooski, VT). According to the manufacture's instruction, in each 100 μl reaction for ATP measurement, 10 μl sample were mixed with 90 μl recombinant reagents containing 1x reaction buffer, 1mM DTT, 1.25 μg/ml firefly luciferase and 0.5 mM D-luciferin. ATP standard curves were produced using known concentrations of ATP, and the measurements of relative light units were converted into ATP concentrations. The ATP concentrations in the test samples were compared with the baseline ATP level detected in the control sample prepared identically but without 94 GHz exposure.

Statistical analysis

Experimental data are presented as means ± SEM. Each experimental condition was compared to the respective untreated control cells. A group of cells was indentified and the calcium spiking was monitored for a preselected time period (30 or 60 min) before the cells were exposed to a 94 GHz irradiation. Because cells were used to serve as their own control, pairwise comparisons were determined between MMW-exposed and unexposed cell groups. Differences between two selected groups were determined using an unpaired heteroscedastic Student's t test at a significance level of $\alpha = 0.05$. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Rationale for co-culture model to mimic the skin tissue

We have already shown that P19 stem cell-derived neurons normally exhibit spontaneous intracellular calcium spiking, and the number of calcium spikes increased in response to 94 GHz exposure and was dependent upon the input MMW power.[10] However, increased calcium spiking in neuronal cells could not be reproduced by elevating the sample temperature alone, suggesting there were non-thermal interactions driving the response. To extend our previous studies, we have engineered a 3D co-culture model that incorporated the major constituents of skin, i.e., keratinocytes and neuronal cells, to elucidate potential nociceptive signaling. The schematics of the co-culture model are shown

in Fig. 1. The 3D co-culture skin model is designed to accommodate; (1) the effects of 94 GHz irradiation in neuronal cells cultured alone on 2D substrate; (2) the effects of 94 GHz irradiation in keratinocytes cultured alone on 2D substrate; and (3) combination of the two cell types co-cultured but separated by ~ 200 μm, which exceeded the MMW penetration depth (~ 115 μm).[2,12] Since neuronal cells are not in physical contact with keratinocytes and should not experience the 94 GHz-induced effects directly, any altered or modulated changes in the calcium dynamics are likely due to secretion of signaling molecules such as neurotransmitters from the keratinocytes.

Calcium dynamics in keratinocytes

Based on our previous results, we chose a 60 mW input power level that corresponds to a temperature elevation of ~ 8°C. The MMW-induced temperature elevation has been extensively analyzed using numerical simulation, thermographic measurements and a fluoroptic temperature probe with excellent agreement between the three techniques.[10] Figure 2 shows changes in the calcium dynamics in keratinocytes alone in the absence of neuronal cells. Unlike other non-excitable cells, keratinocytes exhibited regular calcium spikes in the resting state. A typical calcium oscillation profile in keratinocytes (Fig. 2A) showed about 4 calcium spikes during the first 30 min without a MMW exposure and doubled to 8.2 ± 0.8 for 60 min observation. Cells exposed to a 94 GHz, 60 mW irradiation for 60 min showed the average number of calcium spikes to increase significantly to 17.7 ± 1.5 ($p < 0.05$) (Fig. 2B). In comparison, when the microscope stage temperature was raised by thermal heating to 32°C, the calcium spikes in keratinocytes increased to 11.8 ± 1.7. Further heating to 42°C increased calcium spiking to approximately 15. When the MMW irradiation or temperature elevation was turned off, the calcium spiking activity retuned to the resting state (data not shown).

Calcium dynamics in neuronal cells in the co-culture model

In our co-culture model, the MMW exposure likely and directly influenced keratinocytes (see Fig. 2). However, the 94 GHz energy decays exponentially in physiological saline with a characteristic penetration depth of ~ 115 μm.[2] A wave propagating through a 200 μm solution should nominally be attenuated by about 5 fold,[12] and thus be unable to directly influence the calcium dynamics in neuronal cells. Instead of directly changing the calcium dynamics by a 94 GHz irradiation, changes of the calcium dynamics in neuronal cells could be mediated by the release of neurotransmitters from keratinocytes. To test this hypothesis, the co-culture skin tissue model (see Fig. 1B) was exposed to a 94 GHz irradiation and changes in the calcium dynamics were monitored in neuronal cells. As expected, the calcium spiking activity in the resting neuronal cells was robust and consistent with our previous results (Fig. 3A; see ref. 10). In response to a 94 GHz, 30 mW stimulation, the number of calcium spikes in neuronal cells appears to increase significantly ($p < 0.05$), as would have been anticipated. Surprisingly, a 60 mW stimulation did not increase the calcium spikes but rather suppressed the calcium spiking activity in neuronal cells. The spiking pattern became irregular within 10 min following the 60 mW exposure (lower trace, Fig. 3A), and the number of calcium spikes decreased to a level (4.3 ± 0.6) that was not significantly different from the control experiments (i.e., cells not exposed to MMW; Fig. 3B). This unexpected observation suggested that the keratinocyte-mediated effect on the calcium dynamics in neuronal cells may be dose-dependent.

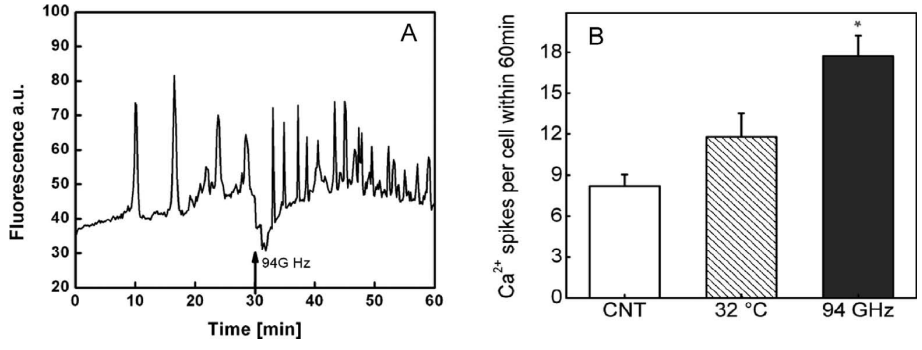

Fig. 2. Real-time calcium dynamics measurements in keratinoctyes. (A) A representative image of Fluo 4-loaded keratinocytes in response to a 94 GHz, 60 mW irradiation. The calcium dynamics in keratinoctyes was observed for the first 30 min without MMW exposure and another 30 min during which the MMW was applied. (B) Average number of calcium spikes in keratinocytes. In one set of experiments, cells were heated by a microscope heating stage and the temperature was elevated to 32°C. The data represents mean ± SEM of 40–70 cells from three to four independent experiments. * indicates a statistically significant difference from respective untreated condition ($p < 0.05$) using pairwise comparison.

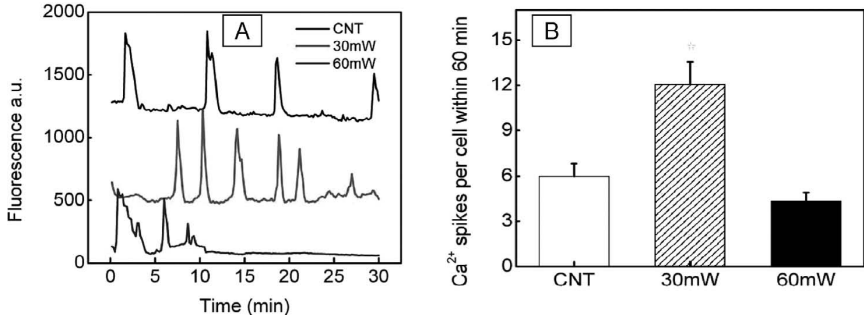

Fig. 3. Effects of keratinocytes on calcium dynamics in neuronal cells. (A) Three traces of calcium dynamics in neuronal cells are shown in control cell (upper trace), exposed to 94 GHz, 30 mW (middle trace) and 60 mW (lower trace) irradiation. These calcium profiles were arbitrary offset for clarity. (B) Average number of calcium spikes in neuronal cells co-cultured with keratinocytes. The data represents mean ± SEM of 40–70 cells from three to four independent experiments. * indicates a statistically significant difference from untreated condition ($p < 0.05$) using pairwise comparison.

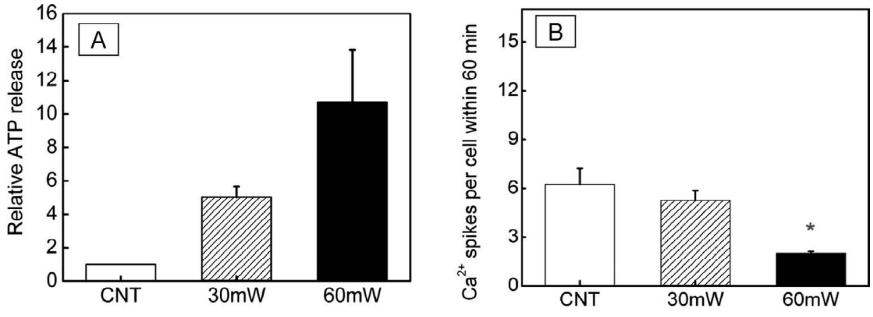

Fig. 4. ATP release. (A) The amount of ATP released from keratinocytes was quantified by collecting the media in the chamber following an exposure of cells to a 94 GHz irradiation and normalized by the amount collected from control cells not exposed. (B) When keratinocytes were treated to block the ATP release, calcium spikes induced by the 94 GHz irradiation was completed prevented. * indicates a statistically significant difference from untreated condition ($p < 0.05$) using pairwise comparison.

ATP release

To identify the source of neurotransmitters that could modulate calcium dynamics, we measured the ATP concentration secreted by keratinoctyes in response to the 94 GHz irradiation. Relative ATP concentrations, presumably secreted by keratinocytes, were quantified and compared. The results indicate that about a 5-fold increase in the ATP secretion was measured in response to a 30 mW exposure, whereas > 10-fold rise in the ATP concentration was induced using a 60 mW exposure (Fig. 4A). Confirmatory experiments were carried out by blocking ATP secretion from keratinocytes and measured the calcium spikes in neuronal cells. Indeed, when keratinocytes were incubated with the ATP channel blocker NPPB,[13] the calcium spiking activity in neuronal cells was prevented (Fig. 4B). A plausible coupling mechanism emerges in which the primary interaction between the skin-like tissue and 94 GHz irradiation appears to be secretion of neurotransmitters (i.e., ATP) from keratinocytes that modulates the calcium dynamics in neuronal cells. An excessive amount of ATP release can prevent but does not enhance the calcium spiking.

Role of purinergic receptors

We next tested the effect of directly adding ATP to neuronal cells to mimic the neurotransmitter secretion. Neuronal cells were cultured alone without keratinocytes and the calcium spiking was recorded in response to externally added ATP. As shown in Fig. 5, addition of ATP up to 0.1 μM induced the calcium spikes to increase to more than 2-fold. However, at 10 μM ATP, the calcium spiking either resembled that found in the resting cells or exhibited a significant reduction. Evidence appears convincing that the calcium dynamics in neuronal cells are regulated in part by the ATP release in the co-culture model and subsequent binding to the purinergic receptors. Indeed, fluorescent immunolabeling of the P2Y2 and P2X2 receptors indicates that these two types of receptors are abundantly expressed both on undifferentiated P19 stem cells and differentiated neuronal cells (Fig. 6). In order to validate the experimental findings, we applied a recently developed mechanistic mathematical model[14] to simulate the effect of ATP on the calcium dynamics. This stimulation model has been designed to examine the ATP-dependent and purinergic receptor-mediated

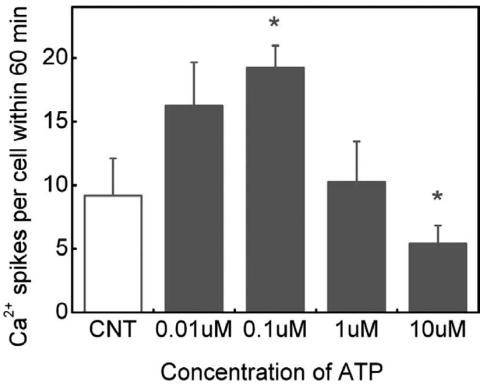

Fig. 5. Effect of externally added ATP on calcium dynamics in neuronal cells. To test directly the involvement of ATP in mediating the calcium spiking in neuronal cells, ATP was added to the cells in the absence of keratinocytes. The calcium spiking activity was enhanced in the presence of ATP < 0.1 μM but reduced to that found in the normal unexposed cells at higher ATP concentrations (> 1 μM). * indicate $p < 0.05$ as compared to control condition with no ATP added.

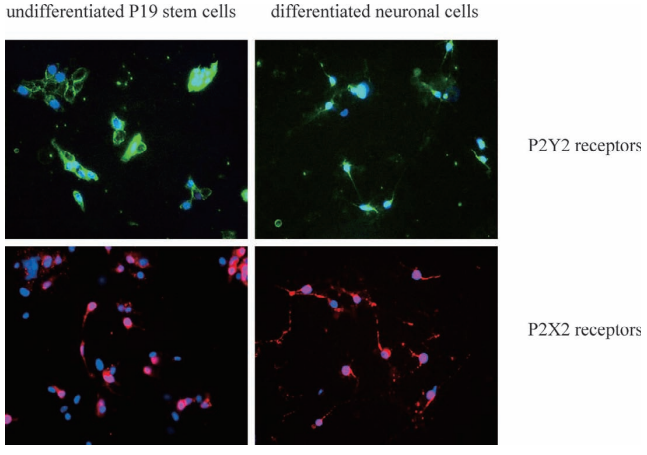

Fig. 6. Immunostaining of ATP receptors: P19-differentiated neurons were fixed and incubated with primary antibody (Rabbit anti-P2X2 or Goat anti-P2Y2; Santa Cruze Biotechnology, CA) and kept at 4°C overnight. Cells were further incubated with either TRITC-conjugated Goat anti-Rabbit IgG or FITC-conjugated Mouse anti-Goat IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were finally stained with DAPI (Molecular Probe, Invitrogen). Images were recorded using a 20x objective.

calcium dynamics. For example, the model was able to quantitatively reproduce altered calcium dynamics in dorsal root ganglion neurons as a function of extracellular ATP, offering insights into interactions between ATP and calcium dynamics. The simulation results, shown in Fig. 7, suggest that changes in the calcium dynamics exhibited a pattern that is qualitatively consistent with the experimental findings. Increase in the calcium spikes was observed in response to

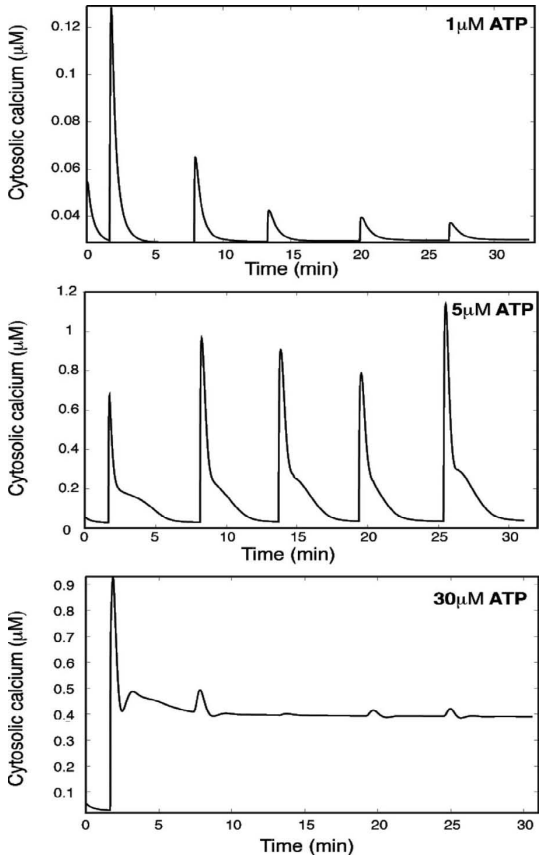

Fig. 7. Simulation of intracellular calcium dynamics in the presence of extracellular ATP. The P2X/P2Y model[14] was used to calculate the cytosolic calcium levels as a function of extracellular ATP. Three extracellular ATP concentrations were simulated. The calcium spiking initially increased as a function of the ATP concentration (e.g., 5 μM). However, consistent with the experimental results, beyond a threshold ATP concentration, the calcium spiking was abolished. Effects of other neurotransmitters such as nitric oxide, substance P or glutamate have not been examined in this simulation.

adding higher ATP concentrations, but above a threshold ATP concentration, the calcium spikes disappeared. It should be noted that while the simulated results do not exactly predict the experimentally observed calcium spiking, they are in general agreement. Moreover, the simulated results do not account for possible effects of other neurotransmitters (e.g., nitric oxide and substance P) that might have contributed to the altered calcium dynamics in neuronal cells. For example, we observed generation of nitric oxide (NO) in keratinocytes (Fig. 8). Exploiting advantages of our unique exposure system, real-time quantification of the NO level was determined using NO-specific fluorescent dyes. Within a 30 min MMW exposure, the fluorescently monitored NO level increased by ~ 2-fold in keratinocytes, suggesting that neurotransmtters other than ATP can also participate in the MMW-mediated activation of neciception.

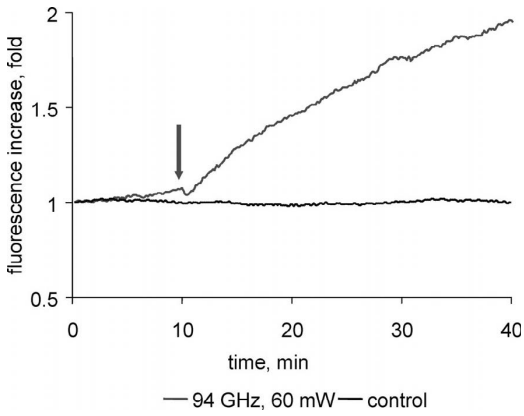

Fig. 8. Increase in the nitric oxide (NO) level. Keratinocytes were loaded with the NO-specific dye, DAF/AM, and fluorescently monitored in real-time. In control cells, the NO level remained virtually unchanged. In response to a 94 GHz, 60 mW exposure, at least a 2-fold increase in the NO level was measured inside the cell. The arrow indicates the start of the MMW irradiation.

However, it should be noted that the fluorescent dye does not diffuse out of the cell and therefore the amount of NO secreted cannot easily be estimated.

DISCUSSION

Real time assessment of subcellular interactions using a 3D co-culture model has been performed. We applied the custom-built 94 GHz exposure system[10] to monitor high-resolution subcellular effects in 3D microenvironment. In the present study, we engineered a 3D co-culture tissue construct that includes two major constituents of the skin; keratinoctyes and neuronal cells. Activation of the epidermis by heat or MMW exposure may release diffusible signaling molecules which, in turn, activate neurons. Our results using this 3D co-culture model suggested that keratinocytes respond to 94 GHz irradiation by increasing calcium spiking and secreting neurotransmtters such as ATP in a dose-dependent manner. As expected, at a nominal 94 GHz input power of 30 mW the calcium spiking activity in neuronal cells increased and induced a 5-fold ATP secretion. However, an excessive amount of ATP was secreted when a 60 mW power was applied, which virtually prevented calcium spiking in neuronal cells. Altered calcium spiking in neuronal cells was reversed by blocking the ATP channels on keratinocyte or reproduced by adding ATP externally.

The dominant effects associated with MMW irradiation are expected to be thermally induced. We therefore carefully measured and reported the temperature rise in our custom-built 94 GHz exposure system using finite element modeling, thermographic measurement and using a fluoroptic temperature probe. Although these techniques measure an average temperature increase, all three measurements are in good agreement.[10,12] A 60 mW input power induced an average temperature increase of ~ 8°C, and rapid thermal equilibration was achieved < 10 s. Since all experiments were performed at room temperature (~ 22°C), the expected temperature in the exposure chamber would rise to ~ 30°C (60 mW input). However, thermal heating of the microscope stage alone to 32 or 42°C caused about 12 or 15 calcium spikes in keratinocytes, respectively, but a 94 GHz, 60 mW stimulation increased the number to ~ 18 calcium spikes ($p < 0.05$). The mechanistic basis for this statistically significant increase in calcium spiking remains uncertain. It is potentially possible that "localized hot spots" may have developed due to spatially non-homogeneous distribution of electromagnetic field inside the chamber.[10,12] Due to heterogeneous distribution of MMWs across the cell layer, the localized hot spots with a temperature elevation higher than average may exist on the MMW-exposed cell-saline interface. Dielectric scattering by groups of cells may further complicate non-uniform energy deposition pattern in the cell layer and could contribute to formation of local microscopic heating sites. Alternatively, keratinocytes interact with MMW in ways that are both thermal and non-thermal in nature. The issue of thermal versus non-thermal mechanisms of MMW-induced bioeffects is still controversial. There are no conclusive and reliable data unequivocally validating or refuting the hypothesis of putative non-thermal effects of MMWs on cell. However, many of the phenomena reported in the literature were notably different from those caused by thermal heating alone,[15–18] and therefore strongly suggestive of non-thermal mechanisms.

It is interesting to note that while robust calcium spiking is expected in excitable cells (neurons and myocytes), non-excitable cells (e.g., keratinocytes) were presumed to be less sensitive to calcium dynamics. In neurons calcium spiking no doubt represents a complex dynamical process that reflects ion transport across the cell membrane, regulation of internal calcium store buffering mediated by protein binding.[19–21] One of the major pathways for calcium influx in excitable cells is the voltage-gated calcium channels (VGCCs). Specifically, at least three subtypes of VGCCs are preferentially expressed in sensory neurons, including L-, T, and N-type.[22] The fast responding T-type channels are activated by a relatively smaller change in the membrane potential. Recent findings indicate that inhibition of the VGCC channels suppressed thermal hyperalgesia and allodynia in rat model study.[23] Moreover, in both neurons and keratinocytes, specific proteins that function as ion channels are expressed that readily respond to the external physical, chemical and thermal stimuli. For example, the family of TRP channels constitutes sub-families of receptors that are designed to delineate the type and magnitude of external stimuli. Thermo-sensitive TRP channels (designated as TRPV3) demonstrate a unique threshold and can be activated by warm temperature and also shows an increased response at noxious temperatures. Since TRPV3 are found

to be specifically expressed in keratinocytes,[9] these skin cells are capable of detecting and responding to heat in the similar manner to that observed in heat-sensing neurons. Indeed when TRPV3 receptors are transfected in Chinese hamster ovary cells, neither capsaicin, low pH nor hypotonic osmotic treatment activated the cells, but a temperature elevation activated the TRPV3 receptors. Several studies have suggested that direct chemical signaling from keratinocytes to dorsal root ganglia neurons[9,24] and in central nervous system[25] could be mediated by ATP. In response to a MMW exposure, the altered calcium dynamics for modulation of nociceptive signals via the TRP channels is also presumed to be involved. Moreover, multiple subcellular processes are clearly dependent on the intracellular calcium dynamics. Elucidation of intricate interplay between the stimulated TRP channels, VGCCs and intracellular calcium store that regulates the overall calcium dynamics and is manifested in the detectable fluorescent calcium signals would be significantly advance the current understanding in the MMW-mediated nociception. The use of pharmacological reagents is not likely sufficient to examine such an interplay. The VGCC inhibitors applied to resting neuronal cells[10,11] and keratinocytes (current study; data not shown) induce significant changes in the calcium dynamics even without heating or 94 GHz irradiation. Powerful molecular biology tools are available, however, to directly address this issue. Our laboratory is currently designing mechanism studies in which transient small interfering RNA techniques will be applied to suppress specific calcium channels and therefore unravel complex interactions between various calcium influx/efflux pathways.

In addition to eliciting calcium spikes in keratinocytes, a MMW exposure appears to mediate ATP secretion. Nucleotide release has been observed in response to mechanical stimulation, osmotic pressure, oxidative stress, and microbial products.[26–32] The released nucleotides bind to purinergic receptors and regulate multiple cellular processes such as cell death, growth, differentiation, migration, and cytokine production.[33–36] Activation of purinergic receptors has been shown to modulate the calcium dynamics and therefore likely to significantly impact the calcium-dependent cellular processes. It is worth noting that simulation studies (Fig. 7) qualitatively predict the effect of extracellular ATP on the calcium dynamics in neuronal cells. Implication is that ATP release may trigger nociceptive signaling by interacting with the purinergic receptors (see Fig. 6) involved in calcium transport. The role of ATP as a proinflammatory mediator is also known. Specifically, the ATP release from keratinocytes has been identified as a cause for skin inflammation. When the skin is exposed to irritants, keratinoctyes release a significant amount of ATP rapidly (< 10 min) and causes dermatitis.[37] While not fully captured in the co-culture model, it appears evident that the MMW-induced secretion of ATP from keratinocytes is involved in nociception which can be elicited by heat, cold, mechanical and electrical events or pathogens.

The exact mechanisms of ATP secretion in response to a 94 GHz irradiation are yet to be fully elucidated. However, when incubated with an anion channel blocker (NPPB), the calcium spiking was completely inhibited (Fig. 4). It should be noted that NPPB is a cell volume-sensitive ATP inhibitor, suggesting that the 94 GHz irradiation may have caused changes in the cellular morphology and subsequently altered the cell volume. Indeed, our observation is in agreement that the 94 GHz irradiation remodels the cytoskeleton[10] and also induces morphological changes in keratinocytes, which is particularly noticeable at higher input power levels (unpublished observation). The mechanism(s) of ATP release in response to a MMW exposure is probably more complicated than involvement of the NPPB-sensitive channels. ATP can be released through exocytosis mechanisms (e.g., secretory vesicles), specific ATP-transporting system such as anion channels, hemi-gap-junction channels, or even transient electroporetic membrane damage.[38,39] Both ATP-dependent channels[40] and morphologically sensitive stretch-activated cation channels[41,42] can also contribute to the modulation of calcium dynamics. Using the quinacrine fluorescent dye, we labeled and indentified multiple ATP-containing vesicles in keratinocytes (images available but not shown). These ATP-rich vesicles are likely the primary candidate responsible for the release of ATP in response to various external stimulations. Overall, the effects of MMW exposure on the skin tissue are regulated by intricate interplay of events involving the release of ATP and other neurotransmitters from keratinocytes and potential direct coupling between neuronal cells and MMW irradiation.

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