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Evidence for non-chemical, non-electrical intercellular signaling in intestinal epithelial cells

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Abstract

Synchrony between mechanically separated biological systems is well known. We posed the question: can cells induce synchronous behavior in neighboring cells which are mechanically separated and which cannot communicate via chemical or electrical mechanisms. Caco-2 cell cultures were divided into three groups. "Inducer" cells were exposed to H_2O_2. "Detector" cells were placed in separate containers near the inducer cells but were not exposed to H_2O_2. Control cells were exposed to fresh media and were kept in a distant laboratory area. Samples were measured for total protein concentration, NFκB activation and structural changes, 10, 30 and 60 min after exposure respectively. Exposing inducer cells to H_2O_2 resulted in a significant reduction in total protein content (−50%), an increase in nuclear NFκB activation (+38%), and structural damage (56%) compared to controls. There was a similar reduction in total protein content (−48%), increase in the nuclear fraction of NFκB (+35%) and structural damage (25%) in detector cells. These findings provide evidence in support of a non-chemical, non-electrical communication. This signaling system possibly plays a role in synchronous, stimulus-appropriate cell responses to noxious stimuli and may explain a number of cellular behaviors that are hard to explain based only on conventional cell signaling systems.

Keywords: Intercellular communication; Cell–cell communication; NFκB; Tight junction; Cell signaling; Biophotonics

1. Introduction

Since the earliest studies of cell signaling systems, chemical and electrical signaling have always been major foci of investigations in cell biology. In contrast, our knowledge of non-chemical, non-electrical (NCNE) signaling remains rudimentary. Nevertheless, there is an increasing body of evidence for other forms of intercellular communication. For example, distant cells align themselves in culture media through cell to cell communication [1]. Also, chemical and electrical signaling system fall short in explaining synchrony between mechanically separated biological units. A recent review by Trushin [2] indicates that experiments on NCNE signaling trace back to 1920, when a Russian scientist showed that chemically isolated onion root cells show increased numbers of mitoses when they are near other actively dividing cells. In later reports Kaznacheev et al. showed that cytopathic effects induced by noxious agents in culture media could be observed in other, chemically and mechanically isolated cells [2]. Another example of NCNE signaling can be inferred form the recent experiment by Ohnishi and colleagues, who observed that cancer cell division in cell culture can be inhibited by ki-energy [3].

To determine whether GI cells can communicate via NCNE signaling, we investigated whether synchrony occurs between distant GI cells. We specifically evaluated whether responses of GI cells exposed directly to oxidants were also exhibited by distant GI cells.

2. Methods

The study was conducted at Rush University Medical Center, Chicago, Illinois, between December 2004 and September 2005. We used exposure to oxidants of an intestinal epithelial

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cell line (Caco-2 cells) as a convenient model because it is one with which we have extensive experience [4–7].

2.1. Cell culture

Caco-2 cells (Rockville, MD) were maintained at 37 °C in complete Dulbecco's minimum Eagles medium (DMEM) in an atmosphere of 5% CCЬ and 100% relative humidity. The experiments were performed when cells reached complete confluency. Cell cultures were separated into three groups. One group, which we labeled *inducer cells*, were exposed to H_2O_2. A second group, which we labeled *detector cells*, were placed near inducer cells but were chemically and mechanically isolated from them; these cells were not exposed to H_2O_2. A third group, which we labeled *control cells*, were not exposed to H_2O_2 and were not placed near inducer cells. They were kept in a distant laboratory, separated by walls and doors; otherwise they were maintained under identical culture conditions.

2.2. Experiments with cells

2.2.1. Experiments with Farhadi's cell containers

This experiment was performed using a newly designed container, called Farhadi's cell container (patent pending). This container is composed of two isolated small containers that are side by side and share a thin common wall or "septum" (Fig. 1). The septum, which is made of a thin layer of glass and separates the inducer cells and detector cells, precludes the passage of any fluid or chemicals between the two containers. Each container could contain 1 mL of liquid and the septum was made of cover glass with thickness of 0.25 mm. The experiment was performed in laboratory bench in presence of daylight and fluorescent light. No dark room or metallic boxes were used.

A total of five T-75 flasks were used for this experiment. Adding 4.5mL of fresh media, cells from all flasks were scraped and combined by gentle mixing into one 50mL container. Aliquots from this cell suspension were used throughout the experiment. Each side of the cell container was filled with 1mL of suspension of viable cells ($n=6$). Two different concentrations of hydrogen peroxide (0.5 and 5×10^{-3} mol L^{-1}) were added to inducer cells at room temperature. Only vehicle (fresh media) was added to detector and control cells. After 10 min, the inducer and detector cell suspensions were collected in small conical tubes and kept on ice for biochemical assays. Six Farhadi' s cell containers were used as controls. Experiments on control cells were performed in a different laboratory and samples were always kept in different ice buckets throughout the assays.

2.2.2. Experiments with test tubes

Five 3mL glass test tubes were placed inside a tube rack. Inducer cells were placed in a tube in the middle of four other tubes containing detector cells. The four detector cell tubes were placed at 1, 2, 3, and 4 cm from the inducer cell tube (Fig. 2). Each tube was filled with 1mL of viable cell suspension as described in the first experiment. Hydrogen peroxide was added to the inducer cell suspension to achieve the desired concentration (0.5×10^{-3} mol L^{-1}) at room temperature. Same amount of vehicle (fresh media) was added to detector and control cells. After 10 min the inducer and four detector cell suspensions were collected in small conical tubes and kept on ice for biochemical assays. Three tubes containing 1mL each of the cell suspension served as controls. Experiments on control cells were performed in a different laboratory.

2.2.3. Effect of H_2O_2 on protein solution

As an additional control, the effect of hydrogen peroxide on BSA protein solution was assessed by adding one of two different concentrations of hydrogen peroxide (0.5 and 5×10^{-3} mol L^{-1}) to a 10 g L^{-1} solution of BSA protein. After 10 min, the protein concentration was measured and the data were compared to controls. Only vehicle distilled water was added to control tubes.

2.2.4. Effect of H_2O_2 on NFκB activation

For this experiment, we used twenty T-75 flasks (75 cm^2 tissue culture flasks) containing confluent Caco-2 cell culture monolayers. The flasks were labeled as inducers ($n=6$), detectors ($n=6$), or controls ($n=8$). Cells were washed with Dulbecco's phosphate-buffered saline (DPBS, 4mL/flask). Then 4mL of 0.5×10^{-3} mol L^{-1} H_2O_2 was added to each of the inducer flasks while 4mL of fresh medium (DMEM) was added to control and detector flasks. The eight control flasks were stacked on top of one another (four stacks of two), while six detector cell flasks were positioned on top of the six inducer cell flasks. After half an hour of incubation, the flasks were removed from the incubators and cells were assessed for NFκB activity using a quantitative ELISA assay (Active Motif, Carlsbad, NA) of the nuclear extracts for the NFκB p65 subunit. Manipulations of control cells were the same except that they were done in a separate laboratory and flasks were always kept in a different incubator.

2.2.5. Effect of H_2O_2 on actin cytoskeleton morphology and tight junction structure

Seven-day, post-confluent, Caco-2 cell monolayers were grown on glass coverslips in four separate 6-well cell culture

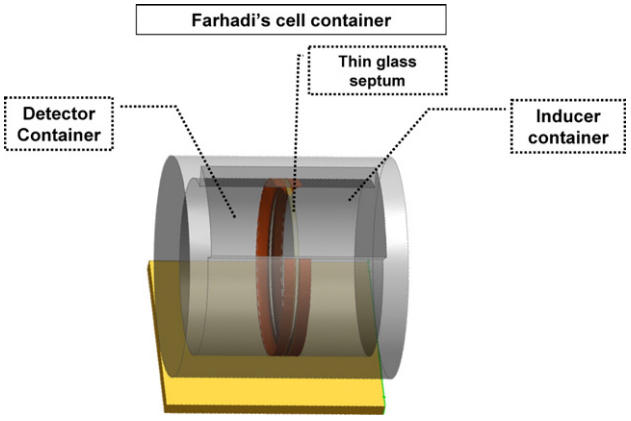

Fig. 1. Farhadi's cell container (patent pending) is composed of two isolated small containers that are side by side and share a thin common wall called septum. Septum is made of thin layer of glass and separates the inducer and detector containers. It precludes the passage of any fluid or chemicals between two containers.

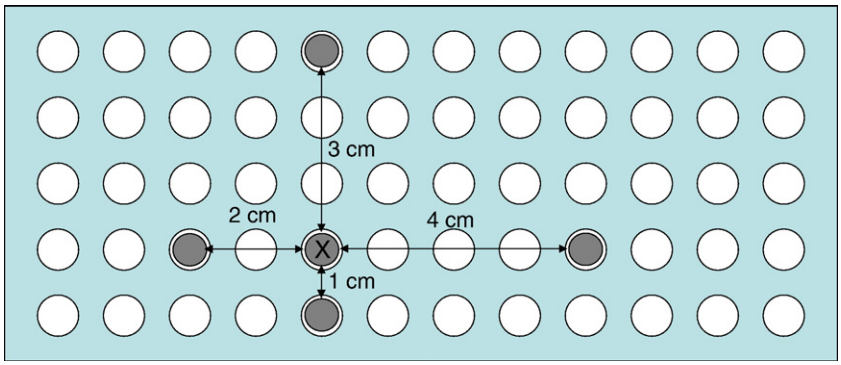

Fig. 2. The schematic view of the tube arrangement in the tube rack (the top view) in distant tube experiment. The tube contained the inducer cells (X) was placed in the middle and was surrounded by four tubes containing detector cells (one on each quadrant), which were placed 1, 2, 3 and 4 cm apart from inducer cell tube. Hydrogen peroxide (0.5×10^{-3} mol L^{-1}) was only added to inducer cell tube.

plates. Randomly selected, non-opaque, six-well plates were labeled as inducer ($n=1$), detector ($n=1$), or control ($n=2$). After incubation, the plates were removed from the incubator and the cells were washed with DPBS, 2 mL/well. This DPBS was replaced with DMEM H_2O_2 solutions (2mL of 0.5×10^{-3} mol L solution in each well) in the inducer plate and with fresh medium (DMEM, 2mL/well) in the control and detector plates. The two six-well control plates were stacked on top of one another, while the inducer cell plates were positioned on top of the detector cell plates. The experiment with control plates was performed in a different laboratory using different incubators (conditions were otherwise the same). After 1 h of incubation, the plates were removed from the incubators and coverslips fixed and stained for assessment of cytoskeleton status and tight junction status using immunofluorescent staining of actin and High-Resolution Laser Scanning Confocal Microscopy, as we previously described [4].

2.3. Protein measurement

Total protein content of each sample was measured using spectrophotometry. Briefly, after adding $0.3\times$mL of RIP A buffer, samples were homogenized by sonication. Samples were then placed in a centrifuge for 90 min at 14,000 RPM (15,996×g) at 4 °C. The pellet was discarded and 2×10^{-6} L of the supernatant sample was added to 38×10^{-6} L of distilled water and 1.2×10^{-6} L of 1:5 diluted Bio-rad protein assay dye (Bio-rad laboratories, CA). Protein content was measured at 595 nm. Samples were kept at 4 °C throughout the procedure. Serial concentrations of BSA were used for creating a standard curve.

2.4. NFκB assay

Cells were assessed for NF-κB activity using nuclear extraction and TransAM NFκB p65 ELISA kits according to manufacturer's protocols (Active Motif, Carlsbad, NA). Nuclear and cytoplasmic extracts were prepared in the presence of phosphatase and protease inhibitors provided with the kit and total protein was determined for each extract (BioRad). A total of 2×10^{-6} g of protein for each nuclear extract was added per well with duplicate wells for each extract. In addition to blank wells using the kit specified buffer, positive control wells containing 2.5×10^{-6} g/well of Jurkat cell lysates (kit positive control) were prepared. Wells were treated as specified by the manufacturer's protocol and developed and absorbance read (colorimetric) at 450 nM at 5 min in an automated plate reader (BioTek). Data are presented as percent absorbance compared to positive controls.

2.5. Assessment of cytoskeletal and tight junction structure

After removing the plates from the incubators, the cells were fixed in a standard cytoskeletal stabilization buffer (2mL/well) and incubated for 2 more h in separate incubators as we previously described [4]. Cells were then washed with fluorescein isothiocyanate–phalloidin (specific for F-actin staining; Sigma-Aldrich, St. Louis, MO), 1:33 dilution for 1 h at 37 °C. After staining, cells were observed in a microscope using an argon laser ($\kappa=488$ nm), a 63× oil immersion plan-apochromat objective, and a numerical aperture of 1.4 (Carl Zeiss, Jena, Germany). Five random points were then selected from each well plate slide (six slides per plates) and images were obtained for evaluation by two observers in a blinded manner. In these images, the cytoskeletal elements were examined for their overall morphology, orientation, and disruption and the percentage of cells displaying cytoskeletal morphology or tight junction abnormalities were calculated.

2.6. Statistics

Data are presented as mean±SEM. Comparisons among the experimental groups were performed using ANOVA and post hoc analysis, $p<0.05$ was deemed significant.

3. Results

3.1. Experiment with Farhadi's cell containers

We first wished to determine whether cells that were chemically and mechanically separated by a thin glass septum

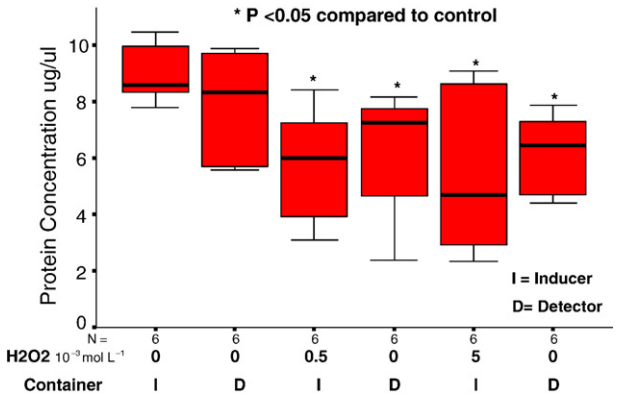

Fig. 3. Protein concentration of cell lysate dropped significantly in inducer cells 10 min after being exposed to hydrogen peroxide (0.5 and 5×10^{-3} mol L^{-1}). There was a significant parallel drop in protein content in detector cells compared to control cells.

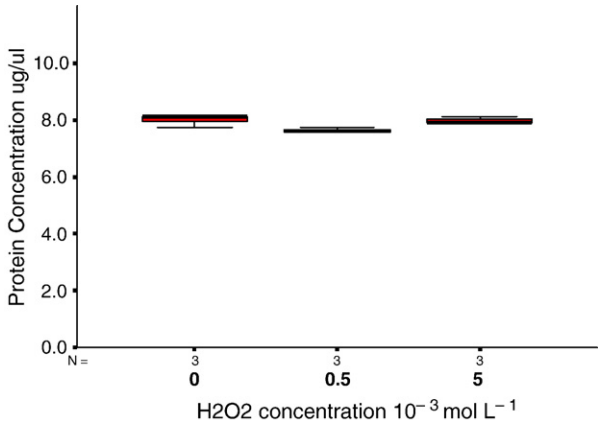

Fig. 5. Protein concentration of BSA solution did not drop when it was exposed to hydrogen peroxide (0.5 and 5×10^{3} mol L^{-1}).

can communicate. Oxidant stress was chosen as a stimulus because it is a validated, potent inducer of the survival pathways. Our data show, as expected, that when cells (inducer cells) are exposed to hydrogen peroxide the protein concentration drops significantly (Fig. 3). There was a parallel and significant drop in protein concentration in cells (detector cells) which had not been exposed to hydrogen peroxide. No significant changes were observed in control cells.

3.2. Experiment with distant tubes

We next wished to determine whether this effect could be reproduced in groups of cells that were even further separated. Similar to the experiments with cell containers, changes in detector cells paralleled those in inducer cells. The effect could be seen in detector cells as far as 4cm away from inducer cells (Fig. 4). This finding shows that NCNE signals pass through air between two test tubes.

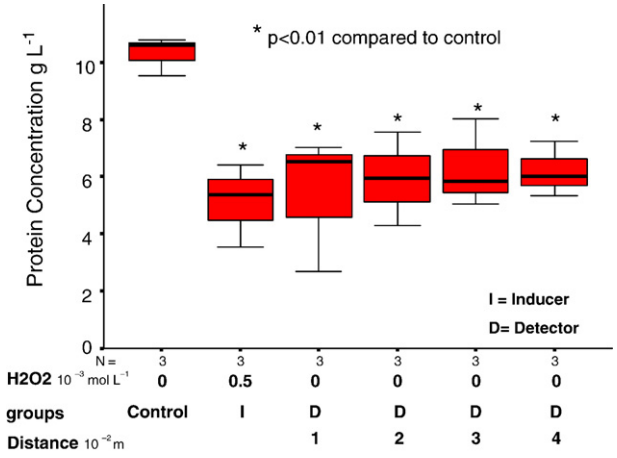

Fig. 4. Protein concentration in distant tubes of cell lysate dropped significantly in inducer cells 10 min after being exposed to hydrogen peroxide (0.5×10^{-3} mol L^{-1}). There was a significant parallel drop in the protein content in detector cells in all distant tubes (D=distance of the detector cell tube from inducer cell tube).

3.3. Effect of H_2O_2 on pure protein solutions

Exposure of BSA protein solutions to two different concentrations of hydrogen peroxide (0.5 and 5×10^{-3} mol L^{-1}) did not change the protein concentration (Fig. 5).

3.4. Effect of H_2O_2 on NFκB activation

We next wished to determine whether NCNE signaling interacts with known, intracellular, oxidative stress pathways. We chose NFκB activation because this transcription factor is a key regulator of cellular gene expression in response to oxidant stress and because a successful experiment could provide strong evidence for stimulus specific (e.g., oxidant specific) gene regulation by NCNE cell signaling. As expected, exposure of inducer cells to hydrogen peroxide activated NFκB compared to control cells as evidenced by movement of the p65 subunit into the nuclear fraction (Fig. 6). There was a parallel and significant increase in NFκB activation in detector cells compared to control cells.

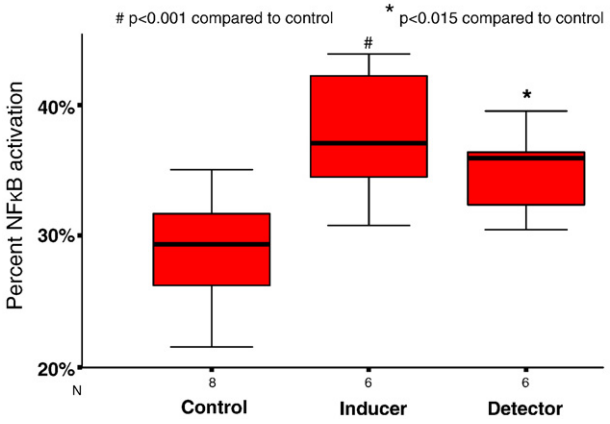

Fig. 6. NFκB activation percent in nuclear fraction of cells increased significantly in inducer cells 30 min after being exposed to hydrogen peroxide (0.5×10^{-3} mol L^{-1}). There was a significant parallel increase NFκB activation in detector cells in the distant nearby cell culture flasks.

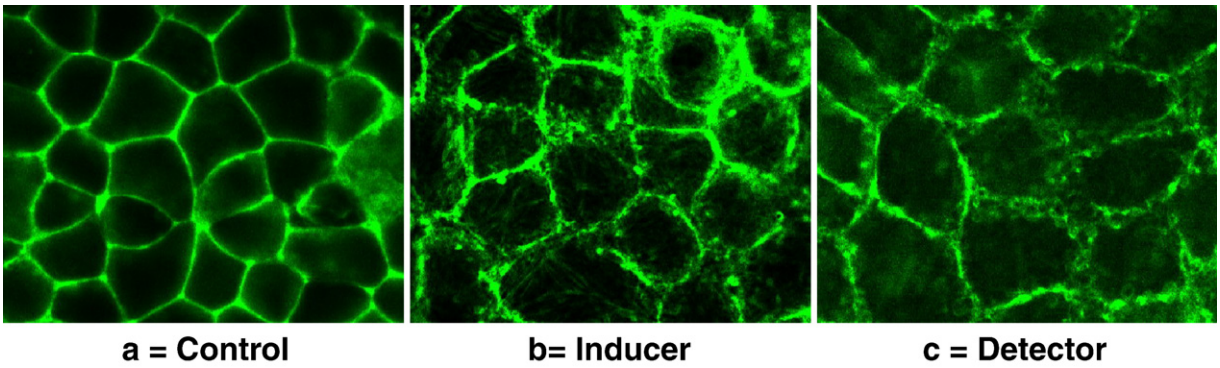

Fig. 7. Representative immunofluorescent staining images of actin cytoskeleton show the cell cytoskeletal and tight junctions structure. The sharp lines divide normal cells in control slides (a). There is obvious derangements and irregularities in inducer cells (b). The detector cells also shows similar pattern of outline irregularities (c).

3.5. Effect of H_2O_2 on actin cytoskeleton morphology and tight junction structure

Finally, we sought to determine whether NCNE signaling is capable of eliciting oxidant-induced changes in cell morphology and tight junction structure in distant cells. As we expected, exposure of cells to hydrogen peroxide results in disruption of normal actin cytoskeletal morphology and tight junction structure in 56% of exposed inducer cells (Fig. 7a–c). There were parallel and significant changes in detector cells compared to control cells (25%, $p<0.0001$).

4. Discussion

Scientific experiments related to the principle of non-chemical non-electrical communication between biological systems can be traced back to 1920 and the work of Gurvitch [2]. He showed that chemically isolated onion root cells show increases in the number of mitoses when they are near other actively dividing cells. This effect was abolished after using a UV-opaque filter between these cells. Unfortunately, that work, and other similar studies, were not published in English and, therefore, they did not provoke a lot of attention in the western world. Since then, several additional reports have provided evidence of distant, non-chemical, non-electrical cell-to-cell communication [2]. In a series of experiments, Kaznacheev et al. in 1985 observed cell morphology by staining cells and showed that cytopathic effects induced by radiation, chemical agents, or viral agents could be induced in other, chemically isolated cells [2]. In another report, Kirkin showed that the rate of cellular proliferation in a group of cells would be enhanced if the cells were non-chemically exposed to another group of proliferating cells [2]. This effect disappeared if the inducer cells were fixed by methanol. These phenomena suggest the existence of a NCNE signal that conveys messages between distant cells. They further show that these messages can elicit complex consequences, such as cell damage and cell proliferation. In 1992 Albrecht-Buehler observed another form of NCNE cell-to-cell communication [1]. He also observed that cultured cells move toward infrared light and proposed that centrosomes are infrared detectors (cell eyes) and that microtubules are cables carrying signals between subcellular organelles (cell nerves) [8–12]. Neither of these studies explored the underlying mechanism for this phenomenon, or whether this type of communication involves conventional cell signaling systems.

We chose three markers to investigate NCNE signaling. Protein synthesis (total cell protein) is a general indicator of cell function and the disturbance of protein synthesis has already been reported in cells being exposed to oxidants [13–15]. Our data reproduce the prior reports that oxidant-exposed cells enter a state of emergency and decrease their protein content by half in a matter of minutes [14,15]. However, our data provide direct and compelling evidence that an oxidant-induced drop in protein synthesis occurs through changes in intracellular signaling because (1) protein concentration did not change when pure protein solution was exposed to oxidant. Although BSA may not be an ideal representative of cell proteins, absence of changes in its concentration suggests that the observed decreases in total protein even in the inducer cells is not a pure inorganic chemical reaction caused by H_2O_2. In fact this reaction requires cell environment and most likely is caused by activation of specific cell signaling and (2) cells not being exposed to oxidants (detectors) showed the same behavior that those cells that were exposed to oxidant (inducer).

We used NFκB transcription factor activation as our second marker to show whether NCNE signaling interacts with known intracellular signaling systems, such as oxidant-induced intracellular cascades. The transcription factor NFκB is a key regulation point for cell stress signaling. Several studies have shown that this signaling system is activated through various cell damaging processes (e.g., oxidative stress) and this signaling system can initiate several key signaling cascades in cells [16–19]. We found that activation of this signaling system in inducer cells in response to oxidative stress is observed in mechanically separated detector cells through an NCNE signaling mechanism. Our data are consistent with a model in which, NCNE and “conventional” signaling can interact with one another.

The third marker was the cytoskeletal structure. Actin is one of the major cell cytoskeletal filaments. The morphology of the cytoskeleton is an indicator of cell structure and more importantly tight junction structure. Several reports [4–7] including our own show that oxidant-activated NFκB can result in downstream cytoskeletal and tight junction damage in cells.

Thus, our data provide evidence that not only do redox-sensitive pathways (e.g., NFκB) get activated in detector cells, the pathways elicit the similar fate, i.e., cytoskeletal damage in these cells. It remained to be seen whether NCNE signaling act at several level in the detector cells or it activate the redox-induced signaling pathway at one level (NFκB or upstream) in the detector cells and the observed cytoskeletal damage is the Domino effect of the activation of this pathway. However, the latter possibility is more plausible.

Over all, our data support the possibility that NCNE (unconventional) communication between cells exists. This unconventional signaling could cause stimulus-appropriate responses in distant cells that could result in synchrony between cells within a single body organ or even between organs.

The mechanism of this NCNE signaling system is unknown. Our data show that NCNE signals pass through air between two test tubes. Ultrasonic waves are easily deflected from interface between liquid/solid and this scattering could create a significant impediment in passage of this wave from one container to other container because the wave has to pass through two such interfaces. This makes the possibility of ultrasonic waves as the source of this messaging system less likely. There are several theories. Electromagnetic waves (biophotons) are perhaps the most appealing candidate. Living cells are continuously and spontaneously emitting biophotons in the form of ultra weak electromagnetic radiation in the range of 260–800 nm — which covers the spectrum from UV to infrared [20,21]. Cells that are exposed to stressful situations show altered biophotonic responses depending on the type of cell and/or type of toxin exposure [22]. It seems that dying or stressed cells emit intense biophotonic bursts [22]. Changes in the characteristics of this radiation during changes in the chemico-physical status of emitter cells could be used for differentiation of healthy and diseased tissue for example [23,24]. The intensity of the emission could be used as a measure of tumor viability after chemotherapy [24]. On the other hand, there are several reports that show evidence that biological systems can be affected by electromagnetic energy [25–28]. It has been shown that this form of energy affects a wide variety of cellular functions, including cellular proliferation and differentiation [29,30], apoptosis [31,32], DNA synthesis [26,33], RNA transcription [34], protein expression [25], ion transportation [35], cell shape and orientation [36] and even redox-mediated rises in NFκB and cell damage [37].

Evidence also suggests that the effect of electromagnetic fields on biological systems may be receptor mediated [38]. In fact, many cells in the body – not just specialized retinal and pineal cells – can detect electromagnetic waves [39,40] through a sensing organ for this ultra weak radiation [8–11]. Whether cells which are able to emit and receive electromagnetic waves (photons) actually use them for intercellular communication needs to be established.

If the existence of NCNE cell communication proves to be true, the impact of this phenomenon on biological experiments and research could be substantial. Our data show that this effect can be exerted on cells in adjacent culture flasks in the same incubator, or on tubes as far away as 4 cm. For example, in a typical cell biology experiment where we expose cells to a specific chemical or physical condition, NCNE signaling may result in synchronous responses in adjacent cells, and thus could constitute a potential confounding factor.

Our study has several limitations including using cancer cell line instead of normal cell line, using only noxious stimuli and measuring dependent end points. However, it provides supportive evidence for the existence of NCNE signaling. It remains to be determined whether this form of signaling is distance dependent, whether this signaling can be modified by different filters or magnetic fields, whether this signaling directly results in damages to the cytoskeleton or it only activates NFκB or its upstream mediators; whether this signaling is limited to intercellular signaling or has a role in intracellular signaling as well. As future direction, experiment to obtain distance response curve and effect of various filters, black paper, and material that are permeable to coherent radiation (polarizing filters) may help to better understand the nature of this communication. These and many other questions should be answered in future research aimed at recognizing and decoding this unconventional signaling system. This might be a situation for using nanotechnology, highly sensitive detectors, and computer-assisted pattern analysis. Knowledge of this novel signaling system would result in better understanding of cell physiology and provide a starting point for using this signaling system for controlling cell behavior and function. This may open a new horizon in non-chemical non-electrical manipulation of cells in the body without the use of conventional chemical or physical agents. This way there could be a possibility of affecting the body organs or disease process through this mode of cellular communication.

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