Microwave radiation can alter protein conformation without bulk heating

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Received 15 November 2002; revised 10 March 2003; accepted 20 March 2003

First published online 1 May 2003

Edited by Thomas L. James

Abstract Exposure to microwave radiation enhances the aggregation of bovine serum albumin in vitro in a time- and temperature-dependent manner. Microwave radiation also promotes amyloid fibril formation by bovine insulin at 60°C. These alterations in protein conformation are not accompanied by measurable temperature changes, consistent with estimates from field modelling of the specific absorbed radiation (15–20 mW kg^{-1}). Limited denaturation of cellular proteins could explain our previous observation that modest heat-shock responses are induced by microwave exposure in *Caenorhabditis elegans*. We also show that heat-shock responses both to heat and microwaves are suppressed after RNA interference ablating heat-shock factor function.

Key words: RNA interference; Microwave radiation; Non-thermal; Protein aggregation; Amyloid fibril; *Caenorhabditis elegans*

1. Introduction

We have shown previously that prolonged (2–20 h) exposure of transgenic *Caenorhabditis elegans* to low-intensity microwave radiation (750–100 MHz, 0.5 W) at 25°C induces widespread expression of stress-reporter genes under the control of endogenous *hsp16-1* promoters [1,2]. A temperature of 28°C is required to induce comparable reporter expression purely thermally, yet microwave exposure causes no measurable heating (< 0.2°C) of either *C. elegans* or medium [2]. Such microwave-induced stress responses therefore appear to be non-thermal; indeed, microwave irradiation and mild heating have opposite effects on the subsequent life history of *C. elegans* [3]. Stress-inducible heat-shock protein (HSP) expression is mediated by the heat-shock transcription factor (HSF), which is found complexed to cytoplasmic HSPs (such as HSP90 [4]) in unstressed cells. Under stress, HSF monomers are released and activated in three stages (trimerisation, phosphorylation and migration into the nucleus), before binding to triple inverted arrays of heat-shock elements (consensus –nGAAn–) in the promoters of inducible HSP genes, thereby stimulating their transcription. HSPs act as molecular chaperones to repair and refold damaged proteins within the cell, and also help to dispose of irreparably damaged proteins [5]. On cessation of the stress, excess HSPs rapidly sequester HSF, so turning off transcription of the HSP genes. The major trigger for HSP induction is protein conformational damage, whether this is caused by heat or by a range of chemical agents [6,7]. Different classes of HSPs have different cellular functions, and the small HSPs (12–27 kDa) have a specific role in preventing protein aggregation [8].

Public concern about alleged adverse health effects of microwave radiation from mobile telephone use has focussed largely on the possibility of DNA damage and cancer [9,10]. However, several large-scale epidemiological studies have failed to support any such link [11]. Evidence cited above implies that microwaves may be able to induce a non-thermal heat-shock response, enhancing the expression of small HSPs [1,2,12] in particular. Protein conformational changes are associated with a different group of human diseases, the amyloidopathies (including Creutzfeld–Jakob, Alzheimer's and Parkinson's diseases). We have therefore asked whether prolonged microwave exposure can directly affect protein conformation in vitro, and whether this might underlie the observed induction of HSP expression.

2. Materials and methods

2.1. Protein aggregation assays

Concentrated aqueous solutions of bovine serum albumin (BSA; Sigma type V, 50–100 mg ml^{-1}) were filter-sterilised (0.22-μm Millipore low protein binding filters), and 1.5-ml aliquots dispensed into sterile 24-well plates (Corning Costar) for the temperature profile (Fig. 1B), or 6-ml aliquots into sterile 6-well plates for the time course (Fig. 1A). Plates were wrapped with plastic film to minimise evaporation; controls were further wrapped in aluminium foil as shielding. Exposed plates were placed centrally on the waveguide of the transverse electromagnetic (TEM) cell described previously [1,2]. Microwave exposures were at 1.0 GHz and 0.5 W, with durations ranging from 3 to 48 h and temperatures from 25 to 45°C. Shielded controls were located outside the TEM cell but in the same incubator. Sham-exposed controls (inside TEM cell with power switched off) did not differ measurably from shielded controls. Sample temperatures were checked with a microthermocouple [2] immediately after removal, before reading the optical densities of 1.0-ml samples at 320 nm. For the time course (Fig. 1A), such 1.0-ml samples were taken from each well at the indicated time point in each of four runs. Plastic UV cuvettes were used throughout to obtain the data shown in Fig. 1. Pooled data are expressed as percentages of the initial (time zero or 25°C control) mean value as 100%; differences between control and exposed groups were tested for significance by Welch's *t*-test. For the

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00413-7

time courses, repeated measures ANOVA confirmed a significant effect of microwave exposure within each of four runs. Because the OD_{320} increased linearly over time (Fig. 1A), the slopes of both microwaved and control data sets were compared across all four runs; Student's *t*-test was used in this instance, since the variances were almost identical. For the (non-linear; Fig. 1B) temperature profiles, data from both runs were analysed by multi-way ANOVA (StatGraphics) to separately examine sources of variance in the combined data set.

2.2. Effect of small HSP on protein aggregation

Bacterially expressed HSP16-2 was purified from inclusion bodies as described previously [8], and a 1.0-mg ml^{-1} solution was mixed with 100 mg BSA ml^{-1} to give 0, 50 and 500 μg HSP16-2 ml^{-1} in 50 mg BSA ml^{-1}. Quadruplicate 1.5-ml samples were incubated as controls at 37°C, or exposed to microwaves for 24 h at 37°C (as above), or to mild heat (42°C) for 24 h. Optical densities were again measured at 320 nm, but using quartz cuvettes [8] which gave slightly improved sensitivity.

2.3. Detection of amyloid fibrils

For studies of fibril formation, a 2-mM solution of bovine insulin (Sigma) was adjusted to pH 2.0 with HCl [13]. Twelve replicate 100-μl samples in 0.5-ml microfuge tubes held in polystyrene racks were exposed to microwaves in the TEM cell (as above) for 1–24 h at 60°C, or placed in foil-wrapped plastic boxes outside the TEM cell as shielded controls. All tubes were centrifuged at $1000\times g$ for 1 min to pellet debris, then recentrifuged at $10\,000\times g$ for 2 min onto electron microscopy grids. These were negatively stained with uranyl acetate and examined by electron microscopy (Jeol JEM 1010). Dye binding studies using the fluorometric thioflavin-T assay were performed as described previously [14] on three to eight 5-μl aliquots of all supernatants following the first low-speed centrifugation.

2.4. RNA interference (RNAi) to ablate HSF function

The single *C. elegans* HSF gene is located at Y53C10A on chromosome I, but attempts at feeding RNAi using a genomic construct [15] did not give consistent suppression of heat-induced stress-reporter expression in transgenic *C. elegans* strains (PC72 or PC161 [1,2]). We therefore prepared a feeding RNAi construct in which an 800-bp *Nru*I/*Xba*I fragment of a full-length HSF cDNA (yk663f6) was inserted into the L4440 RNAi feeding vector [16]. Both this RNAi construct and the intact L4440 feeding vector (no insert) were transformed separately into HT115 *Escherichia coli* cells, which were selected, grown up overnight at 37°C, and plated onto 9-cm NGM agar plates supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside and appropriate antibiotics [16]. After incubation for 3 h at 37°C to induce dsRNA expression from the *lac*-regulated T7 promoters flanking the insert, all plates were cooled to 15°C prior to use. Approximately 2000 L1 larvae of the PC161 *C. elegans* strain (which carries twin *hsp16*-regulated stress-reporter genes encoding β-galactosidase and green fluorescent protein (GFP) [2]) were spotted onto both RNAi and vector-only plates, and were grown up to adulthood over 3–4 days at 15°C, with daily transfers onto fresh plates to avoid starvation. At the end of this period, worms were again transferred to fresh plates and kept at 25°C (shielded controls) or 30°C (heat shock) or exposed to microwave radiation (1 GHz, 0.5 W) at 25°C, in all cases for 20 h. Stress reporter induction was assessed by washing worms off the plates and measuring the GFP fluorescence of 1000-worm samples in black non-fluorescent 96-well microplates (Corning-Costar) in a Perkin-Elmer HTS 7000 or Wallac PF2 plate reader. Quantification of β-galactosidase reporter expression in these worms was inaccurate due to gut retention of *lac*-positive HT115 food bacteria which strongly express the same protein.

3. Results and discussion

There is evidence that microwave radiation may speed up rates of folding and unfolding for globular proteins in solution [17]. We reasoned that this should increase the chances of collision between partially unfolded molecules, leading to irreversible aggregation and hence increased light scattering [8], which would be most easily monitored in concentrated solutions of protein. This would be prohibitively expensive using purified intracellular enzymes, hence we chose to use the very soluble secreted protein, BSA. As shown in Fig. 1A, the above prediction was confirmed for sterile BSA solutions at 37°C, with an increasing difference in absorbance over time between microwaved and shielded control samples. Light scattering in both shielded control and microwave-exposed groups increased linearly over time at 37°C, but the gradient was steeper in the latter case. Over four runs the mean slopes were 0.260 ± 0.055 (S.D.) for shielded controls but 0.372 ± 0.053 (S.D.) for exposed samples, a difference which is statistically significant ($P=0.0257$; $n=4$). Measurements with a sensitive microthermocouple at the end of the exposure period [2] detected no difference (<0.2°C; $P>0.05$) in temperature between the two groups.

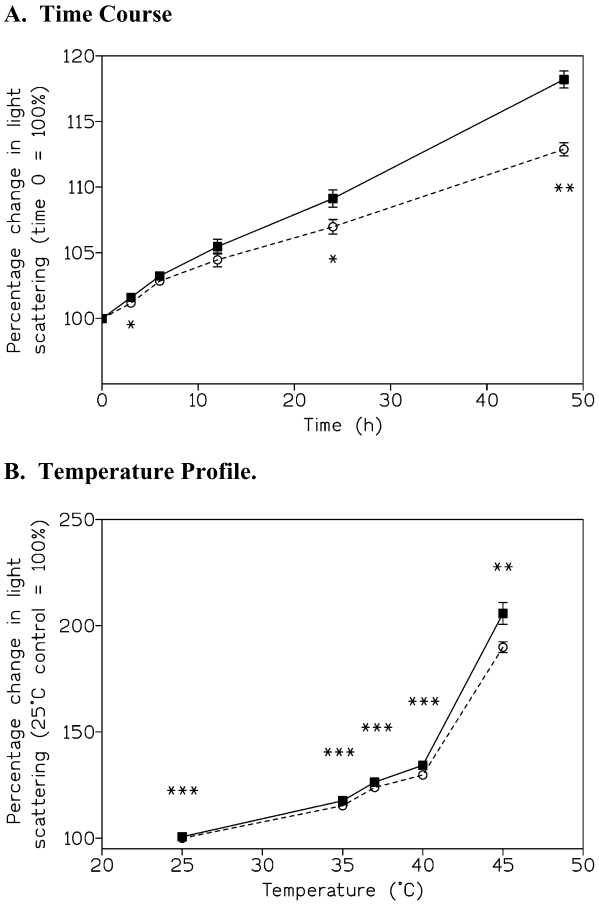

Fig. 1. Effects of microwave radiation on the aggregation of BSA. A: Sterile BSA samples (75 mg ml^{-1}) were shielded or exposed to microwaves (1.0 GHz, 0.5 W) at 37°C for 0–48 h, then light scattering was measured at 320 nm. Open circles, dashed line: shielded controls; filled squares, solid line: microwave-exposed. Points show mean ± S.E.M. of pooled data from four independent runs ($n=24$), each normalised as a percentage of the initial OD_{320} value (time 0 = 100%). B: Sterile BSA samples (75 mg ml^{-1}) were similarly shielded or exposed (same conditions) for 24 h at temperatures ranging from 25 to 45°C, and light scattering was measured at 320 nm. Open circles, dashed line: shielded controls; filled squares, solid line: microwave-exposed. Points again show mean ± S.E.M. of pooled data from two independent runs ($n=48$), each normalised as a percentage of the 25°C control value (= 100%). In both parts, significant differences (Welch's *t*-test) between exposed and shielded control groups are shown by asterisks: $*0.05\geq P\geq0.01$; $**0.01\geq P\geq0.001$; $***0.001\geq P\geq0.0001$.

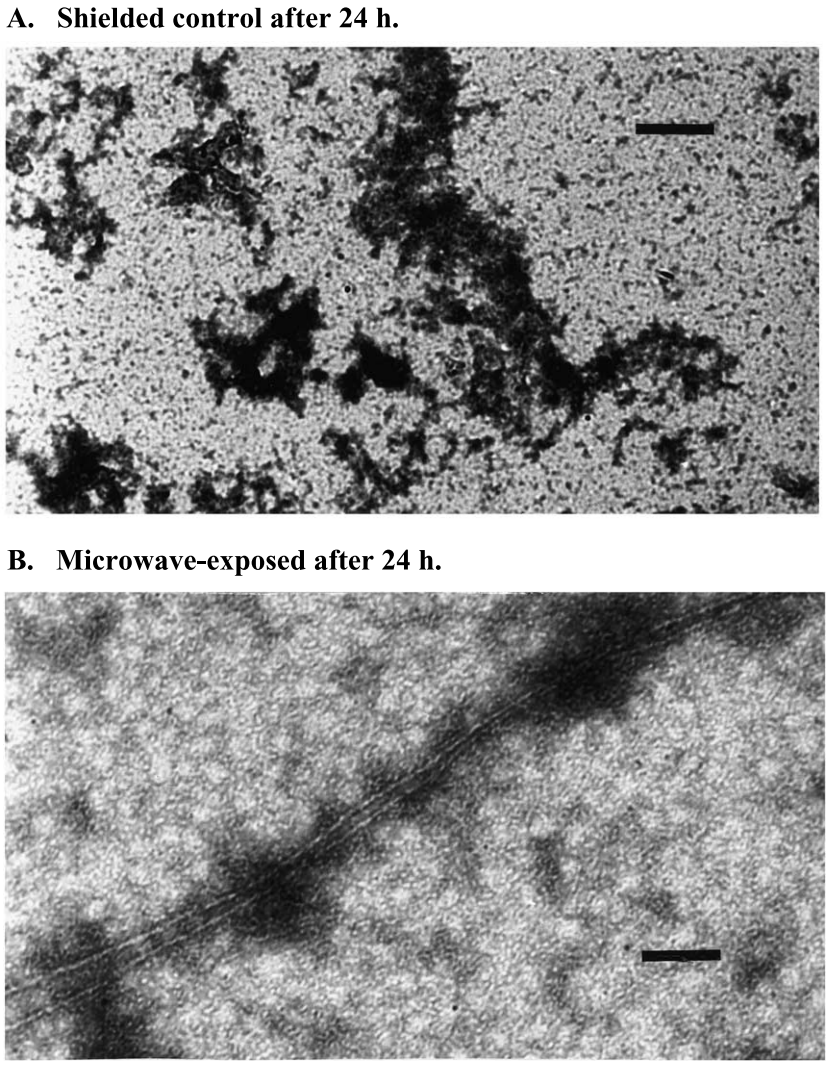

Fig. 2. Effect of microwaves on amyloid fibril formation by bovine insulin at 60°C. Bovine insulin (2 mM, pH 2.0) samples were shielded or microwave-exposed (1.0 GHz, 0.5 W) for 24 h at 60°C, then fibrils and aggregates were centrifuged onto grids for negative staining and electron microscopy. Typical fields are shown for control (A) and exposed (B) samples. Bars in each part show 100 nm. Fibril diameter in B is approximately 10 nm.

The microwave-induced difference in light scattering was also strongly temperature-dependent, being slight at 25°C (though highly significant, due to very low variance) but much larger at 45°C (Fig. 1B). The effects of microwave exposure seem less striking here, due to the steep increase in light scattering caused by heat above 40°C. Multi-way ANOVA analysis of the sources of variance within this data set confirms a highly significant microwave effect at all temperatures (F ratio = 7.13, $P = 0.0078$ over two runs; Fig. 1B). These simple in vitro experiments demonstrate a small but consistent effect of microwaves on protein aggregation.

Several proteins share a propensity to undergo conformational changes leading to amyloid fibril formation [13,18]. One such protein is bovine insulin [18], which used to be injected into human diabetic patients and occasionally caused adverse immune reactions (though it has now been almost entirely replaced by cloned human insulin). Amyloid fibril formation, although extremely slow under physiological conditions, is greatly accelerated at low pH (2.0) and high temperatures (70°C) [13]. As independent confirmation of our BSA findings, we therefore asked whether microwave irradiation might also promote amyloid fibril formation. Characteristic fibrils were present following microwave exposure of bovine insulin for 24 h at 60°C (Fig. 2B), but none were seen in parallel shielded controls (Fig. 2A). After negative staining for electron microscopy (EM), such fibrils appeared as pale strands bounded by dark tramlines (diameter c. 10 nm; Fig. 2B [13,18]), whereas irregular dark masses of aggregated protein were widespread under both conditions. Microthermocouple measurements again failed to detect any consistent temperature difference between exposed and control samples. In a blinded experiment, an EM technician correctly identified two exposed samples (fibrils present) from among seven controls (fibrils absent). Because fibril formation cannot be readily quantified by EM, we used a dye binding assay specific for amyloid fibrils [14]. We found a linear increase in dye binding over 6 h in microwave-exposed samples, but no such increase in shielded controls (Fig. 3). Multi-way ANOVA analysis of data from three independent runs confirms a significant effect of microwaves (F ratio = 13.2, $P = 0.0005$). We conclude that

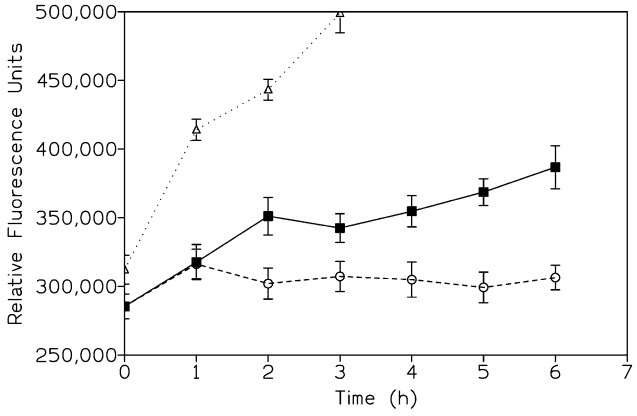

Fig. 3. Rate of amyloid fibril formation. Bovine insulin (2 mM, pH 2.0) samples were shielded or microwave-exposed for 0–5 h at 60°C or 70°C (shielded controls only), then fibril formation was measured using the thioflavin-T binding assay [14]. Open triangles, dotted line: 70°C positive controls; open circles, dashed line: 60°C shielded controls; filled squares, solid line: microwave-exposed at 60°C. All points are means ± S.E.M. from three to eight samples in each of three independent runs (data pooled from all runs).

microwave irradiation can also promote amyloid fibril formation in vitro, at least under the non-physiological conditions used here.

In vitro, and presumably also in vivo, protein aggregation is inhibited by small HSPs [8], which include the *C. elegans* HSP16 family that is known to be induced by microwave exposure [1,2]. We therefore purified bacterially expressed *C. elegans* HSP16-2 [8] in order to study its effect on the aggregation of BSA induced by microwaves at 37°C and by mild heating at 42°C. As shown in Fig. 4, both conditions caused substantial protein aggregation after 24 h (relative to 37°C controls), but this was inhibited partially by HSP16-2 at 50 μg ml^{-1} and more strongly so at 500 μg ml^{-1}. Although the ratio of HSP16-2 to BSA was far less than stoichiometric

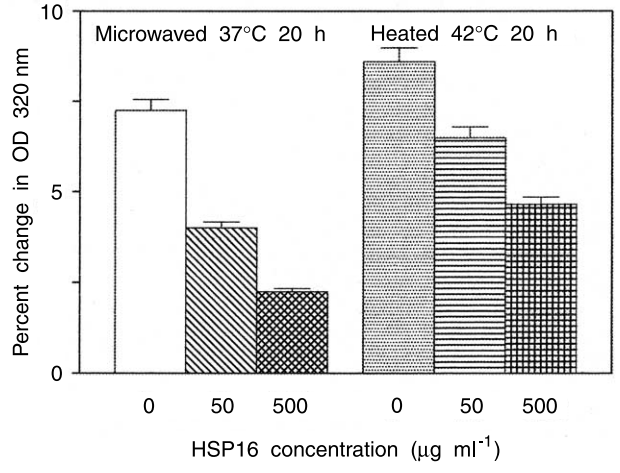

Fig. 4. Effect of HSP16-2 on BSA aggregation. Different concentrations of HSP16-2 (0, 50 and 500 μg ml^{-1}) were prepared in 50 mg ml^{-1} BSA, and were then either shielded or exposed to microwaves (1.0 GHz, 0.5 W) at 37°C for 24 h, or else subjected to mild heat (42°C) for the same period. Light scattering was measured in all samples and the percentage change from the initial value ($t = 0$) calculated. Bars show mean and S.E.M. from four replica samples under each condition tested.

[8], the former apparently sufficed to cope with the limited extent of BSA aggregation observed under these mild exposure conditions.

Accurate dosimetry is clearly crucial for the interpretation of these findings, particularly in relation to human exposure levels from mobile phones. Modelling of specific absorbed radiation (SAR) distributions within our exposure system (data not shown) indicated an average SAR of 15–20 mW kg^{-1} across most of the sample area, with peak SARs of up to 50 mW kg^{-1} (averaged over the 1-g well contents) confined to the corners. This pattern was approximately matched by the spatial distribution of optical densities in the BSA light-scattering experiments (Fig. 1), where the highest values were always recorded in corner wells (data not shown). Although these SARs are higher than those estimated previously [2], they still fall within the lower end of the range experienced by mobile phone users [19]. Thermal effects of microwaves only become apparent at SARs $\geq$100-fold greater than those experienced within our exposure system, confirming our conclusion (from microthermocouple measurements) that these effects are non-thermal.

The key link between conformational damage to cellular proteins and the activation of HSP gene expression is provided by the master transcriptional regulator, HSF [4]. Since there is only a single HSF gene in the *C. elegans* genome, it is possible to suppress HSF function using RNA interference (RNAi [15,16]). As shown in Fig. 5, PC161 worms whose HSF function has been ablated (by RNAi with the yk663f6 construct) are unable to mount a stress response to either heat (20 h at 30°C) or microwave radiation (1 GHz and 0.5 W for 20 h at 25°C). By contrast, control worms fed with bacteria carrying the vector only show modest induction of the stress-

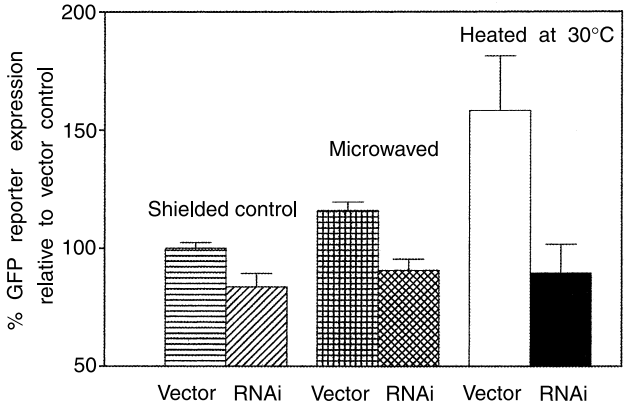

Fig. 5. Effect of RNAi against HSF on stress responses in PC161 *C. elegans*. L1 larvae of the PC161 strain were grown to adulthood at 15°C on HT115 bacteria carrying either the yk663f6-derived RNAi construct or the L4440 feeding vector only, and were then exposed to microwaves at 25°C or to temperatures of 25°C (shielded) or 30°C (heat shock) for 20 h. Resultant GFP reporter expression was monitored in aliquots of 1000 worms. Left-hand pair of bars, shielded controls at 25°C; central pair of bars, microwave-exposed at 25°C; right-hand pair of bars, heat-shocked at 30°C. In each pair of bars, that on the left shows GFP expression in vector-only controls (normal stress responses), while that on the right shows GFP expression in RNAi worms (HSF function ablated). Each bar shows the mean and S.E.M. from four to eight replicates in each of four independent runs, after normalising the mean 25°C control value for each run to 100%. Vector-only responses to microwaves and heat are both significantly different from shielded control values ($P < 0.05$).

reporter gene following microwave exposure and higher induction by heat shock. It is worth pointing out that RNAi ablates even the very low levels of heat-induced reporter expression seen in vector-only controls at 25°C (compare the left-hand pair of bars in Fig. 5; see also [2]). The fact that stress reporter (GFP) expression is reduced to a similarly low plateau by RNAi, independent of the stressor applied (heat at 25 or 30°C, or microwaves at 25°C), carries the implication that any heat-shock response induced by microwaves [2] must be mediated largely if not wholly through HSF activation and is probably triggered by conformational damage to cellular proteins.

It would be premature to extrapolate from these preliminary in vitro observations to any link between mobile phone use and human amyloidopathies. We have shown that microwave irradiation activates the expression of small HSPs as part of a modest stress response [2], and that HSP16-2 can inhibit microwave-induced changes in protein conformation in vitro (Fig. 4). Taken together with evidence that a mild heat-shock response can provide long-lasting protective effects and so extend lifespan in *C. elegans* [20,21], it is conceivable that moderate microwave exposures might even prove beneficial rather than harmful. What is clear is that microwaves can exert non-thermal effects in biological systems, at least partially arising from alterations in the conformation of cellular proteins.

Acknowledgements: The authors wish to thank the Leverhulme Trust for a Study Abroad Fellowship to D.d.P., Dr Mireille Dumoulin (Department of Chemistry, University of Cambridge) for advice on the dye binding assay, Professor Yoji Kohara (National Institute for Genetics, Mishima, Japan) for the yk663f6 cDNA clone, Dr Francis Gilbert (School of Life and Environmental Sciences, University of Nottingham) for advice on appropriate statistical analysis, and Drs Mays Swicord and Giorgi Bit-Babik (Motorola Research Laboratories, Plantation, FL, USA) for modelling the SAR distributions within our TEM cell. Research funding sources included the UK Defence Evaluation Research Agency and Mobile Telephone Health Research initiative (to D.d.P.), and the Canadian Natural Sciences and Engineering Research Council and Canadian Institutes of Health Research (to E.P.M.C.).

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