Bioelectromagnetics 31:656–663 (2010)

Brief Communication

Protein Changes in Macrophages Induced by Plasma From Rats Exposed to 35 GHz Millimeter Waves

Roza K. Sypniewska,[1] Nancy J. Millenbaugh,[1]* Johnathan L. Kiel,[2] Robert V. Blystone,[3] Heather N. Ringham,[4] Patrick A. Mason,[5] and Frank A. Witzmann[4]

[1]*General Dynamics Information Technology, San Antonio, Texas*
[2]*Counterproliferation Branch, Biosciences and Protection Division, Human Effectiveness Directorate, Air Force Research Laboratory, Brooks City-Base, Texas*
[3]*Department of Biology, Trinity University, San Antonio, Texas*
[4]*Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana*
[5]*Radio Frequency Radiation Branch, Directed Energy Bioeffects Division, Human Effectiveness Directorate, Air Force Research Laboratory, Brooks City-Base, Texas*

A macrophage assay and proteomic screening were used to investigate the biological activity of soluble factors in the plasma of millimeter wave-exposed rats. NR8383 rat macrophages were incubated for 24 h with 10% plasma from male Sprague–Dawley rats that had been exposed to sham conditions, or exposed to 42 °C environmental heat or 35 GHz millimeter waves at 75 mW/cm^2 until core temperature reached 41.0 °C. Two-dimensional polyacrylamide gel electrophoresis, image analysis, and Western blotting were used to analyze approximately 600 protein spots in the cell lysates for changes in protein abundance and levels of 3-nitrotyrosine, a marker of macrophage stimulation. Proteins of interest were identified using peptide mass fingerprinting. Compared to plasma from sham-exposed rats, plasma from environmental heat- or millimeter wave-exposed rats increased the expression of 11 proteins, and levels of 3-nitrotyrosine in seven proteins, in the NR8383 cells. These altered proteins are associated with inflammation, oxidative stress, and energy metabolism. Findings of this study indicate both environmental heat and 35 GHz millimeter wave exposure elicit the release of macrophage-activating mediators into the plasma of rats. Bioelectromagnetics 31:656–663, 2010.

Key words: radiofrequency; oxidative stress; proteomics; two-dimensional gel electrophoresis; 3-nitrotyrosine

Millimeter waves (MMWs) correspond to the range of 30–300 GHz of the electromagnetic spectrum and are currently used for a variety of military and civilian applications. For example, systems that emit radiofrequency radiation at or near 35 GHz are employed by the United States military for land-based and airborne radars [Camacho, 2000] and are being tested for passive MMW imaging of weapons [Appleby, 2004] and use in armored vehicle-mounted multi-functional devices for simultaneous active protection, surveillance, communication, and combat identification [Wehling, 2005]. An increased risk of occupational overexposure to MMWs has been predicted due to the continued development of higher power technologies that may be capable of causing significant temperature increases in superficial body tissues [Ryan et al., 2000].

Roza K. Sypniewska and Nancy J. Millenbaugh contributed equally to this work.

Grant sponsors: U.S. Air Force Office of Scientific Research (AFOSR) and Air Force Research Laboratory (awarded to General Dynamics Information Technology); Grant number: F41624-01-C-7002; U.S. AFOSR (to Dr. R. Blystone); Grant number: F49620-02-1-0372.

*Correspondence to: Nancy J. Millenbaugh, Building 1162, 8262 Hawks Road, Brooks City-Base, TX 78235.
E-mail: nancy.millenbaugh@brooks.af.mil

Received for review 16 December 2009; accepted 22 June 2010

DOI 10.1002/bem.20598
Published online 3 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 DEC 2010		

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Protein Changes in Macrophages Induced by Plasma from Rats Exposed to 35-GHz Millimeter Waves	**FA8650-07-D-6800**
	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER **62202F**

6. AUTHOR(S)	5d. PROJECT NUMBER
R Sypniewska; N Millenbaugh; J Kiel; R Blystone; H Ringham	**7757**
	5e. TASK NUMBER **B3**
	5f. WORK UNIT NUMBER **56**

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
(see section 13),8262 Hawks Road,Brooks City-Base,TX,78235-5147	

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**		**8**	

Details on the doses of radiofrequency radiation received by humans during inadvertent overexposures are quite limited, though a few published case studies involving reconstruction of such events are available. These indicate that accidental exposures of military personnel have occurred at 379 mW/cm^2 for approximately 20 min from a 200 W aircraft radar unit operating at a frequency above 10 GHz [Williams and Webb, 1980], and at various unrecorded frequencies and power densities of 1–2685 mW/cm^2 (75–150 mW/cm^2 defined as the medium-dose range) for 20–30 min [Reeves, 2000]. Fourteen of the 34 patients in this latter study reported a warm sensation during the overexposure [Reeves, 2000]. A consensus report based on a review of known radiofrequency radiation hazards concluded that health effects of greatest concern are likely to occur at doses that induce tissue heating, and that the underlying biochemical mechanisms for documented responses in humans are not well understood [IEEE Committee on Man and Radiation, 2002].

The penetration depth of MMW radiation into biological tissue is shallow and, for 35 GHz, is estimated to be about 0.8 mm [Blick et al., 1997]. It is expected, therefore, that absorption of 35 GHz MMW energy should occur mostly within the epidermis and dermis of exposed skin. In agreement with this, prior studies conducted by our laboratory showed intravascular aggregation of leukocytes and upregulation of genes associated with immune cell recruitment and activation in the skin of rats at 6 and 24 h after exposure to 35 GHz MMWs at 75 mW/cm^2 until colonic temperature reached 41–42 °C [Millenbaugh et al., 2008]. Previous investigations also indicate that sustained MMW exposure can cause heating of internal tissues, presumably due to thermal conduction, and effects in peripheral blood cells and tissues distal to the initial site of energy absorption. A series of experiments performed in rats demonstrated that exposure to 35 GHz MMWs at 75 mW/cm^2 for approximately 44–80 min produced elevations in skin and colonic temperatures and changes in heart rate, respiration rate, and blood pressure, with eventual circulatory collapse [Frei et al., 1995; Kalns et al., 2000; Millenbaugh et al., 2006]. In addition, increased levels of the oxidative stress marker, 3-nitrotyrosine (3-NT), were detected in peripheral blood lymphocytes and neutrophils of rats exposed to 35 GHz MMWs at 75 mW/cm^2 for 44–60 min until body core temperature reached 41–42 °C [Kalns et al., 2000]. Based on these results, we hypothesized that 35 GHz MMWs can elicit the release of biologically active mediators into the systemic circulation leading to downstream responses in cells and tissues.

The ability to elicit effects in immune cells has also been observed for other MMW frequencies and power densities. Exposure to 42.2 or 61.22 GHz MMWs at 15 or 31 mW/cm^2 for 20 or 30 min daily for 3 days was found to restore immune system function in mice treated with the immunosuppressant, cyclophosphamide. The MMW exposures enhanced proliferation [Rojavin et al., 1997; Makar et al., 2003, 2006] and interferon-γ (IFNg) secretion in splenic T cells [Makar et al., 2003, 2006], natural killer cell cytolytic activity [Makar et al., 2005], phagocytosis [Rojavin et al., 1997], and tumor necrosis factor-α production in macrophages [Makar et al., 2003, 2006]. These findings provide additional evidence that sustained MMW exposures could lead to secretion of active factors into the circulation.

In the current study, an in vitro bioassay and proteomic screening were utilized to determine if plasma from 35 GHz MMW-exposed rats causes changes in protein expression and levels of 3-NT in a rat alveolar macrophage cell line. Use of an in vitro assay allows delineation of effects induced by plasma-borne compounds from the direct effects of heating that would likely be exhibited by macrophage cells derived from the MMW-exposed rats. A cell line was chosen for this experiment to avoid inter-animal variations in response seen in primary alveolar macrophage cultures [Helmke et al., 1989]. Macrophages were selected for testing because they have a major role in immune system functions, and, as mentioned above, were previously shown to be affected by MMW exposure [Rojavin et al., 1997; Makar et al., 2003, 2006].

Plasma samples used in the current study were obtained from nine male Sprague–Dawley rats during a prior investigation of 35 GHz MMW effects, and details of the animal care and exposures have been reported elsewhere [Millenbaugh et al., 2006, 2008]. Briefly, all experiments and animal care procedures were approved by the Institutional Animal Care and Use Committee of the Air Force Research Laboratory (Brooks City-Base, TX, USA) and were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals [National Research Council, 1996]. Nine male Sprague–Dawley rats (350–400 g, 3–4 months of age, Charles Rivers Laboratories, Raleigh, NC) were randomly assigned to a sham ($n = 3$), 42 °C environmental heat (EH; $n = 3$), or 35 GHz MMW ($n = 3$) exposure group. The dose of 35 GHz MMWs used in this study is based upon incident power density because the exposure standard for this frequency is also based on this parameter, rather than a normalized specific absorption rate [IEEE International Committee on Electromagnetic Safety, 2006]. An incident power density of 75 mW/cm^2 and a colonic temperature endpoint of 41 °C were chosen for the MMW exposures because these parameters are

associated with MMW-induced immune cell changes in rats [Kalns et al., 2000; Millenbaugh et al., 2008], and this power density has relevance to reported cases of human radiofrequency radiation overexposures [Reeves, 2000]. The EH group was included as a positive control for body core heating. Whole-body environmental heating of rats has been used in prior studies of radiofrequency radiation effects as a more conventional model of hyperthermia [Walters et al., 1998a,b; Millenbaugh et al., 2006, 2008] and is known to elicit release of immunologically active compounds into the plasma [Leon et al., 2006; Leon, 2007]. Warm air at 42 °C has been shown to produce a colonic heating profile similar to that seen in rats exposed to 35 GHz MMWs at 75 mW/cm^2 [Millenbaugh et al., 2006, 2008], and thus this temperature was utilized for the EH exposures in the current investigation. Colonic temperature was used as the endpoint for the EH and MMW exposures in this study because it is not possible to accurately measure the actual dose of energy absorbed in the skin during the experiment [Millenbaugh et al., 2006, 2008]. A pilot study indicated that the average amount of time required to reach the endpoint of 41 °C colonic temperature during MMW and EH exposures, including the 3-min control period, was 45.8 ± 3.8 min (mean $\pm$ SD), and thus 46 min was selected as the duration for the sham exposures.

Animals were anesthetized with isoflurane (Isosol; Vedco, St. Joseph, MO), and hair was removed from the left side of the body using electric clippers. Colonic and skin surface temperatures were recorded during experimentation using a thermistor probe and infrared camera system, respectively. For MMW exposures, continuous-wave 35 GHz radiofrequency radiation was generated by a MMW exposure system (Applied Electromagnetics, Atlanta, GA) and transmitted through a conical horn antenna (6.8 cm diameter aperture) at a forward power of 50 W. The generator output power was monitored throughout exposures with a Model 4-32-B Hewlett-Packard power meter (Agilent Technologies, Santa Clara, CA). The Fresnel distance was calculated to be approximately 53.9 cm, and exposures were conducted under far-field conditions at 110 cm from the antenna. The anesthetized rats were placed on a custom-designed stand with the left, clipped side of the body centered along the boresight of the horn antenna. The incident power density of the MMW field was determined at the exposure site with an isotropic probe (Model 8723; Narda Microwave, Hauppage, NY). The power density distribution produced by the antenna system is Gaussian and was previously estimated to decrease by 50% at 8.5 cm horizontally from the boresight spot on the animal for this experimental set-up [Millenbaugh et al., 2006]. Following a 3-min control period, rats were exposed to MMWs at a peak incident power density of 75 mW/cm^2 until the colonic temperature reached 41 °C.

For EH exposures, rats were subjected to ambient temperature during a 3-min control period followed by warm air at 42 °C in a custom-designed, insulated chamber until the colonic temperature reached 41 °C. For sham exposures, animals were placed in the same experimental set-up used for MMW exposures, but they were not irradiated. At 24 h post-exposure, rats were anesthetized with isoflurane and exsanguinated via cardiac puncture. Blood was transferred to a heparinized vacutainer (BD, Franklin Lakes, NJ) and centrifuged for 15 min at 1100*g*. Plasma was immediately aliquoted, snap frozen, and stored at −80 °C until utilized.

NR8383 rat alveolar macrophages (CRL-2192; ATCC, Manassas, VA) were seeded onto six-well culture plates and incubated for 24 h at 37 °C and 5% CO_2 in RPMI-1640 medium (pH 7.2; without phenol red) supplemented with 1% non-essential amino acids and 15% heat-inactivated fetal bovine serum (all from Sigma–Aldrich, St. Louis, MO). Pilot experiments indicated 10% rat plasma in culture medium was the highest concentration that maintained macrophage cell viability at $\geq$97% after 24 h incubation (data not shown), and therefore, this percentage was selected for further testing. The plated cells were treated for 24 h with fresh culture medium containing 5% fetal bovine serum and 10% plasma from the sham-, EH-, or MMW-exposed rats. An aliquot of plasma was selected from each of the nine animals and used to treat wells in triplicate (27 individual wells). In addition, macrophages were treated in triplicate for 24 h with a mixture of lipopolysaccharide (LPS, 5 μg/ml, *Escherichia coli*, 0:157, Sigma–Aldrich) and recombinant rat IFNg (50 U/ml; R&D Systems, Minneapolis, MN) in culture medium as a positive control for Western blotting. Following treatment, culture medium was removed and cells were washed using phosphate-buffered saline. Cells from one well out of each triplicate set of wells were removed and used to confirm that cell viability was $\geq$97% via the trypan blue exclusion assay. Thus, two wells for each of the nine plasma aliquots plus two wells for the Western blot positive control remained on the plates, and these 20 samples were immediately snap frozen in liquid nitrogen and stored at −80 °C.

Protein sample preparation [Witzmann et al., 2002] and two-dimensional polyacrylamide gel electrophoresis followed by image analysis [Witzmann et al., 2005] were performed as described previously. Briefly, cell lysates were obtained using 400 μl of lysis buffer placed directly on frozen cells on the culture plates.

Protein samples (183.5 μg) from each of the 20 wells were individually loaded onto immobilized pH gradient strips (24 cm, linear pH 3–10; Bio-Rad, Hercules, CA) and isoelectrically focused for 100000 V-h. Strips were placed onto SDS slab gels (20 cm × 25 cm × 0.15 cm; linear 11–17% acrylamide gradient) and proteins were separated in the second dimension at 8 °C for 18 h at 160 V. Gels were stained with colloidal Coomassie blue and scanned under visible light.

For samples from the sham, EH, and 35 GHz MMW plasma treatment groups, gel image data were analyzed by PDQuest software (Bio-Rad) with individual protein quantities expressed as parts-per-million of the total integrated optical density of the gel. Quantities for each spot were first compared between the three treatment groups using the Student's *t*-test within PDQuest ($P \leq 0.01$). Individual abundances for differentially expressed spots were exported to Statistica software (v. 8.0; StatSoft, Tulsa, OK) where the spot values for samples from duplicate wells were averaged. Protein quantities in the EH and MMW treatment groups were then compared to those in the sham group using one-way ANOVA and Dunnett's multiple comparison tests ($n = 3$ per group, $P \leq 0.05$).

All gels from the sham, EH, and 35 GHz MMW plasma treatment groups, plus the gels from the positive control samples treated with LPS and IFNg, were used for immunoblot analysis of nitrated proteins. Two-dimensional Western blotting was performed according to previously published methods [Fultz and Witzmann, 1997]. Briefly, proteins from the gels were transferred onto polyvinylidene difluoride membranes. The membranes were incubated overnight with a mouse monoclonal anti-nitrotyrosine primary antibody, then for 2 h with a polyclonal goat anti-mouse secondary antibody conjugated with horseradish peroxidase (both at 1:10000 dilution; Upstate Biotechnology, Lake Placid, NY). Blots were developed using a 3,3′,5,5′-tetramethylbenzidine reagent kit (Sigma–Aldrich) then scanned under visible light. Protein spots were selected for identification based upon qualitative changes in the signal intensity. Only spots showing apparent changes in signal intensity for all members in the EH or MMW group compared to the sham group were included.

Protein identification using peptide mass fingerprinting was performed as previously reported [Witzmann et al., 2005]. Spots were excised from the gels and processed robotically, including overnight digestion at 37 °C with 6 ng/μl of modified trypsin (Promega, Madison, WI). The resulting peptides were analyzed by matrix-assisted laser desorption ionization mass spectrometry using a Waters MicroMass M@LDI system (MicroMass, Milford, MA). Proteins were identified by comparing the measured peptide mass profiles to theoretical masses using the ProFound search engine (Proteometrics, New York, NY) and the National Center for Biotechnology Information database of non-redundant protein sequences. The *Z*-score estimated by the ProFound software is reported in Table 1 as an indication of the quality of the search result. A *Z*-score of 1.3 corresponds to the 90th percentile.

Colonic and left skin surface temperatures of the rats during the 3-min control period followed by sham, EH, or 35 GHz MMW exposure are shown in Figure 1A. For EH and MMW exposures, individual animals reached the 41 °C colonic temperature endpoint at different times. Exposure duration, including the control period, ranged from 42.7 to 44.0 min and 46.0 to 47.0 min for the EH and MMW groups, respectively. Data points in Figure 1A are shown only for those times when all three subjects in each of these two groups were still undergoing exposure. Colonic and skin surface temperatures were maintained at relatively constant levels of 36.9–37.3 and 33.4–34.6 °C, respectively, throughout the 46 min sham exposures. In the EH- and MMW-exposed rats, the colonic and skin temperature profiles are similar to those seen in our previous studies [Millenbaugh et al., 2006] and are consistent with an initial deposition of energy at the skin followed by transfer of heat to internal tissues. The average colonic temperatures differed by up to only 0.4 °C between these two groups at any time point during exposures. However, the average maximum skin surface temperature of 41.7 ± 0.4 °C (mean ± SD) reached during MMW exposures was greater than the maximum of 39.7 ± 0.1 °C achieved during EH exposures (Student's *t*-test, $P = 0.0009$).

The MMW-emitting system used in this study allows only one side of the animal to face the oncoming energy field, whereas the entire rat, except for the nose, was placed within the EH chamber. Figure 1B shows an infrared thermogram of the left side of a rat captured just prior to termination of a 35 GHz MMW exposure. As reported in an earlier study, the temperature distribution across the skin during MMW exposures is not homogeneous [Millenbaugh et al., 2008], and, in this example, temperatures varied by approximately 6.1 °C within the clipped region. This thermal pattern may be due in part to the Gaussian distribution of the incident MMW power density, and irregularities in the shape of the rat that perturb the pattern of energy absorption in the skin. In contrast, the infrared thermograms captured at the end of the EH exposures showed temperature variations of only 1.1–1.4 °C across the clipped region of skin (thermogram images not shown).

Approximately 600 protein spots from the NR8383 macrophages were matched in the two-dimensional gel

TABLE 1. Proteins Identified in NR8383 Rat Macrophages Treated With Plasma From Sham-, EH-, or 35 GHz MMW-Exposed Rats

Spot no.[a]	Protein	Accession no.[b]	Mass (kDa)	p*I*	No. peptides	%C[c]	*Z*-score[d]	Abundance[e]		
								Sham	EH	MMW
1	Heterogeneous nuclear ribonucleoprotein C isoform a (HNRPC)	XP_222697	12.9	4.8	5	48	1.16	168 ± 37	433 ± 38*	472 ± 79*
2	Prohibitin (PHB)	NP_114039	29.9	5.6	13	45	2.43	650 ± 24	1135 ± 171*	938 ± 67
3	Glutamate dehydrogenase 1 mitochondrial precursor (GLUD1)	P10860	61.8	8.3	14	25	2.43	75 ± 17	116 ± 24	181 ± 13*
4	Enolase 1α (ENO1)	NP_036686	47.5	6.2	22	45	2.29	1723 ± 349	3279 ± 612	4057 ± 433*
5	Malate dehydrogenase 1 NAD (soluble) [*Mus musculus*] (MDH1)	AAH50940	39.1	6.2	4	8	1.10	2096 ± 437	4228 ± 805	5422 ± 580*
6	Aldo-keto reductase family 1 member A1 (AKR1A1)	NP_112262	36.7	6.8	12	35	1.14	154 ± 28	299 ± 20*	423 ± 26*
7	**Annexin 1 (ANXA1)**	NP_037036	39.2	7.0	30	58	2.43	1490 ± 259	2598 ± 397	3284 ± 496*
8	**Aldo-keto reductase family 1 member B8 (AKR1B8)**	NP_775159	36.5	7.1	12	38	2.43	562 ± 49	1090 ± 292	1543 ± 297
9	**Glyceraldehyde-3-phosphate dehydrogenase [*Mus musculus*] (GAPDH)**	XP_484436	36.1	8.7	10	28	2.43	2836 ± 421	9401 ± 2361*	8993 ± 332*
10	**Transaldolase 1 (TALDO1)**	NP_113999	37.6	6.6	12	36	1.46	771 ± 145	1841 ± 328	2645 ± 382*
11	**Superoxide dismutase 2 (SOD2)**	NP_058747	24.9	9.3	9	38	2.43	3111 ± 372	6357 ± 367*	7807 ± 732*
12	**Vimentin (VIM)**	NP_112402	53.8	5.1	20	35	2.23	2301 ± 41	3556 ± 188*	3462 ± 435*
13	**Protein disulfide-isomerase A3 precursor (PDIA3)**	P11598	57.1	5.9	14	22	2.43	909 ± 222	1719 ± 346	1329 ± 149

[a]Protein spot number in two-dimensional gel image in Figure 1A. Bold font indicates anti-nitrotyrosine immunopositive proteins.
[b]Accession number from National Center for Biotechnology Information database.
[c]Percent protein sequence coverage.
[d]*Z*-score calculated by ProFound search engine as an indicator of search result quality. A *Z*-score of 1.3 corresponds to the 90th percentile.
[e]Abundances are in parts-per-million of total optical density of the gel. Values are mean ± SEM.
*$P \leq 0.05$ compared to sham group determined by one-way ANOVA and Dunnett's tests, $n = 3$.

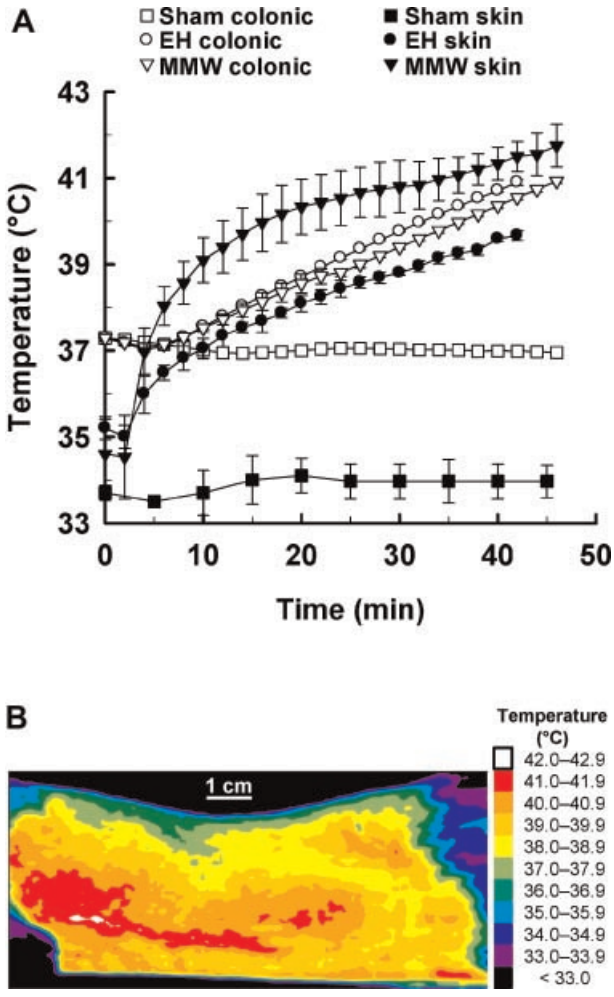

Fig. 1. **A**: Colonic and skin surface temperatures of rats during exposure to sham conditions, 42 °C EH, or 35 GHz MMWs at 75 mW/cm^2. Values are mean ± SD, $n = 3$ per group. The 3-min pre-exposure control period is included in all plots. Duration of the sham exposure was 46 min. For EH and MMW exposures, the time required to reach the 41 °C colonic temperature endpoint, including the control period, varied for individual animals. Exposure durations ranged from 42.7 to 44.0 min and 46.0 to 47.0 min, respectively, in the EH and MMW groups. Data points are shown only for those times when all three subjects in the groups were still undergoing exposure. **B**: Example of an infrared thermogram showing the left, clipped region of rat skin during exposure to 35 GHz MMWs at 75 mW/cm^2. The temperature scale is at the caudal end of the rat.

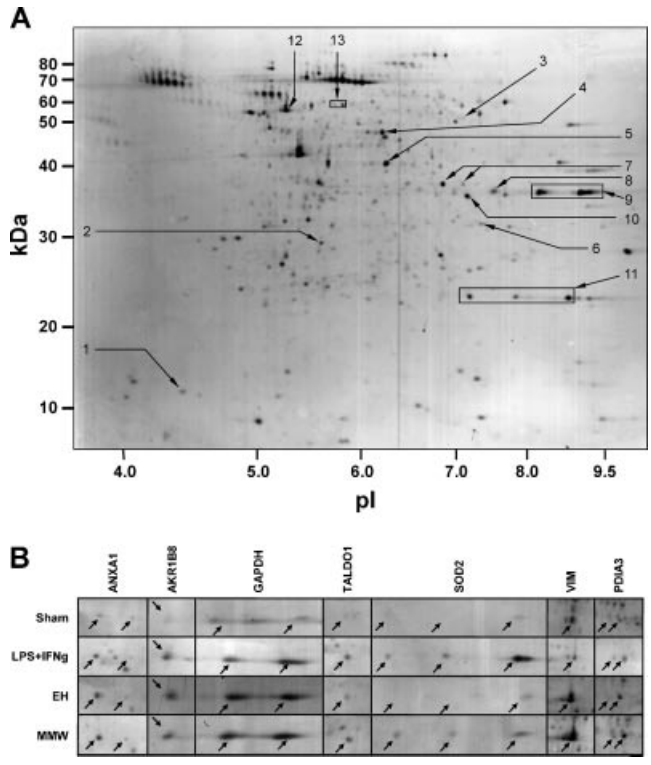

Fig. 2. **A**: Representative two-dimensional gel image of proteins from NR8383 rat macrophage cells treated for 24 h with plasma from a 35 GHz MMW-exposed rat. Thirteen proteins that showed changes in expression or increases in anti-nitrotyrosine immunoreactivity were analyzed by peptide mass fingerprinting. Identifications are listed in Table 1 according to the corresponding spot numbers on the gel. Axes on the gel were calibrated based on isoelectric point (p*I*) and molecular weight calculated from identified proteins using the Compute pI/MW Tool (http://us.expasy.org/tools/pi_tool.html). **B**: Examples of 3-nitrotyrosine immunopositive proteins from NR8383 macrophage cells stimulated for 24 h with plasma from sham-, EH-, or 35 GHz MMW-exposed rats, or with a mixture of 5 μg/ml lipopolysaccharide and 50 U/ml interferon-γ (LPS + IFNg). Individual panels were cropped from the two-dimensional Western blot images. Protein spots were selected for identification based upon qualitative changes in the signal intensity. Only spots showing apparent changes in signal intensity for all members in the EH or MMW group compared to the sham group were included. Protein identification information is listed in Table 1.

images and compared between treatment groups. Thirteen proteins, which were found to be differentially expressed or to contain increased levels of 3-NT in cells treated with plasma from EH- or MMW-exposed rats compared to cells treated with plasma from sham-exposed rats, were identified (Table 1). Figure 2A shows an example of a two-dimensional gel image of macrophage proteins with the locations of the identified spots marked by arrows and numbers. Proteins found to be present as multiple spots, indicating the presence of post-translational modifications, were grouped under a single spot number.

Ten of the 11 differentially expressed proteins were increased 1.5- to 3.4-fold in the MMW group compared to the sham group, 5 of which were also upregulated in the EH group by 1.5- to 3.3-fold. One protein, prohibitin, was increased only in the EH group compared to the sham group. Many of these proteins have been associated with macrophage activation during experimental models of inflammation and

oxidant-induced cellular stress. For example, superoxide dismutase 2 (SOD2) is an antioxidant that reduces superoxide radical (O_2^-) to H_2O_2, and O_2 and is induced by LPS or pro-inflammatory cytokines in multiple cell types including human monocytes [Gadgil et al., 2003]. Another upregulated enzyme, aldo-keto reductase family 1 member A1, protects DNA and proteins from damage by reducing highly reactive aldehydes generated by the peroxidation of lipids. Cytoprotection may also be provided by elevations in annexin 1 (ANXA1), a protein shown to inhibit nitric oxide release in stimulated murine macrophages [Ferlazzo et al., 2003].

Macrophage activation requires increased energy production to drive biochemical reactions involved in the generation of the respiratory burst and the compensatory mechanisms that limit cell damage. Several of the proteins upregulated in the macrophage cells by plasma from EH- or MMW-exposed rats are metabolic enzymes previously associated with cellular response to oxidative stress, namely, enolase 1α (ENO1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamate dehydrogenase 1 (GLUD1), malate dehydrogenase 1 (MDH1), and transaldolase 1 (TALDO1). In addition to increased energy yields, some of these proteins could provide enhanced cytoprotection during activation. For example, it has been postulated that ENO1 may function as a molecular storage site for the antioxidant, glutathione [Fratelli et al., 2002], and elevations in TALDO1, a key enzyme of the pentose phosphate pathway, may provide increased levels of reducing equivalents for inhibition of oxidant-induced injury and apoptosis [Cha et al., 2007].

To further test for cell activation in our model, two-dimensional Western blotting was used to evaluate macrophage proteins for changes in 3-NT, a commonly used marker of enhanced generation of reactive oxygen and nitrogen species during inflammation. Results indicated increased 3-NT in seven proteins in the EH or MMW groups compared to the sham group (Table 1 and Fig. 2B). These included ANXA1, GAPDH, TALDO1, SOD2, vimentin (VIM), aldo-keto reductase family 1 member B8 (AKR1B8), and protein disulfide-isomerase A3 precursor (PDIA3). Treatment of macrophages with LPS and IFNg appeared to also cause elevated levels of 3-NT in all seven proteins except VIM and PDIA3. Increased tyrosine nitration in the EH and MMW groups is in agreement with the other data reported here that suggest plasma from the EH- and MMW-exposed rats induced oxidative stress in the macrophage cells. Also, increased levels of 3-NT in ANXA1, GAPDH, TALDO1, SOD2, VIM, and PDIA3 have been detected in multiple human and rodent models during inflammation or oxidative stress indicating that these proteins are common targets of nitration [Aulak et al., 2001, 2004; Miyagi et al., 2002; Xiao et al., 2005; Ghosh et al., 2006; Ghesquiere et al., 2009].

In summary, the current study suggests that plasma from EH- or 35 GHz MMW-exposed rats can induce changes in protein expression and 3-NT in a rat alveolar macrophage cell line. Thus, in our animal model [Millenbaugh et al., 2008], exposure to either EH or MMWs may cause release of active mediators into the systemic circulation. The data presented here are in line with prior reports of MMW-induced effects in rodents that indicate involvement of secondary molecular signals in downstream immune responses such as increased production of cytokines by macrophages, natural killer cells, and T cells [Makar et al., 2003, 2005, 2006], neutrophil recruitment to skin vessels [Millenbaugh et al., 2008], and increases in oxidative stress in peripheral leukocytes [Kalns et al., 2000]. Also, our findings provide a panel of cellular proteins for further investigation of the biochemical mechanisms underlying the effects of 35 GHz MMW overexposure. Studies to identify specific plasma-borne mediators of macrophage activation and thresholds for elicitation of their release by MMW exposure are in progress.

ACKNOWLEDGMENTS

We thank Dr. J. Kalns for useful discussions related to experimental design, and Mr. C. Cerna and Ms. B. Brott for technical assistance.

REFERENCES

Appleby R. 2004. Passive millimetre-wave imaging and how it differs from terahertz imaging. Philos Transact A Math Phys Eng Sci 362:379–392.

Aulak KS, Miyagi M, Yan L, West KA, Massillon D, Crabb JW, Stuehr DJ. 2001. Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. Proc Natl Acad Sci USA 98:12056–12061.

Aulak KS, Koeck T, Crabb JW, Stuehr DJ. 2004. Dynamics of protein nitration in cells and mitochondria. Am J Physiol Heart Circ Physiol 286:H30–H38.

Blick DW, Adair ER, Hurt WD, Sherry CJ, Walters TJ, Merritt JH. 1997. Thresholds of microwave-evoked warmth sensations in human skin. Bioelectromagnetics 18:403–409.

Camacho JP. 2000. Federal radar spectrum requirements. NTIA Special Publication 00-40, National Telecommunications and Information Administration. http://www.ntia.doc.gov/osmhome/reports/ntia00-40/index.htm (last accessed April 1, 2010).

Cha MH, Rhim T, Kim KH, Jang AS, Paik YK, Park CS. 2007. Proteomic identification of macrophage migration-inhibitory factor upon exposure to TiO_2 particles. Mol Cell Proteomics 6:56–63.

Ferlazzo V, D'Agostino P, Milano S, Caruso R, Feo S, Cillari E, Parente L. 2003. Anti-inflammatory effects of annexin-1: Stimulation of IL-10 release and inhibition of nitric oxide synthesis. Int Immunopharmacol 3:1363–1369.

Fratelli M, Demol H, Puype M, Casagrande S, Eberini I, Salmona M, Bonetto V, Mengozzi M, Duffieux F, Miclet E, Bachi A, Vandekerckhove J, Gianazza E, Ghezzi P. 2002. Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. Proc Natl Acad Sci USA 99:3505–3510.

Frei MR, Ryan KL, Berger RE, Jauchem JR. 1995. Sustained 35-GHz radiofrequency irradiation induces circulatory failure. Shock 4:289–293.

Fultz CD, Witzmann FA. 1997. Locating Western blotted and immunostained proteins within complex two-dimensional patterns. Anal Biochem 251:288–291.

Gadgil HS, Pabst KM, Giorgianni F, Umstot ES, Desiderio DM, Beranova-Giorgianni S, Gerling IC, Pabst MJ. 2003. Proteome of monocytes primed with lipopolysaccharide: Analysis of the abundant proteins. Proteomics 3:1767–1780.

Ghesquiere B, Colaert N, Helsens K, Dejager L, Vanhaute C, Verleysen K, Kas K, Timmerman E, Goethals M, Libert C, Vandekerckhove J, Gevaert K. 2009. In vitro and in vivo protein-bound tyrosine nitration characterized by diagonal chromatography. Mol Cell Proteomics 8:2642–2652.

Ghosh S, Janocha AJ, Aronica MA, Swaidani S, Comhair SA, Xu W, Zheng L, Kaveti S, Kinter M, Hazen SL, Erzurum SC. 2006. Nitrotyrosine proteome survey in asthma identifies oxidative mechanism of catalase inactivation. J Immunol 176:5587–5597.

Helmke RJ, German VF, Mangos JA. 1989. A continuous alveolar macrophage cell line: Comparisons with freshly derived alveolar macrophages. In Vitro Cell Dev Biol 25:44–48.

IEEE Committee on Man and Radiation (COMAR). 2002. Medical aspects of radiofrequency radiation overexposure. Health Phys 82:387–391.

IEEE International Committee on Electromagnetic Safety (SCC39). 2006. IEEE standard for safety levels with respect to human exposure to radio frequency electromagnetic fields, 3 kHz to 300 GHz, IEEE Standard C95. 1-2005. New York: The Institute of Electrical and Electronics Engineers.

Kalns J, Ryan KL, Mason PA, Bruno JG, Gooden R, Kiel JL. 2000. Oxidative stress precedes circulatory failure induced by 35-GHz microwave heating. Shock 13:52–59.

Leon LR. 2007. Heat stroke and cytokines. Prog Brain Res 162:481–524.

Leon LR, Blaha MD, DuBose DA. 2006. Time course of cytokine, corticosterone, and tissue injury responses in mice during heat strain recovery. J Appl Physiol 100:1400–1409.

Makar V, Logani M, Szabo I, Ziskin M. 2003. Effect of millimeter waves on cyclophosphamide induced suppression of T cell functions. Bioelectromagnetics 24:356–365.

Makar VR, Logani MK, Bhanushali A, Kataoka M, Ziskin MC. 2005. Effect of millimeter waves on natural killer cell activation. Bioelectromagnetics 26:10–19.

Makar VR, Logani MK, Bhanushali A, Alekseev SI, Ziskin MC. 2006. Effect of cyclophosphamide and 61.22 GHz millimeter waves on T-cell, B-cell, and macrophage functions. Bioelectromagnetics 27:458–466.

Millenbaugh NJ, Kiel JL, Ryan KL, Blystone RV, Kalns JE, Brott BJ, Cerna CZ, Lawrence WS, Soza LL, Mason PA. 2006. Comparison of blood pressure and thermal responses in rats exposed to millimeter wave energy or environmental heat. Shock 25:625–632.

Millenbaugh NJ, Roth C, Sypniewska R, Chan V, Eggers JS, Kiel JL, Blystone RV, Mason PA. 2008. Gene expression changes in the skin of rats induced by prolonged 35 GHz millimeter-wave exposure. Radiat Res 169:288–300.

Miyagi M, Sakaguchi H, Darrow RM, Yan L, West KA, Aulak KS, Stuehr DJ, Hollyfield JG, Organisciak DT, Crabb JW. 2002. Evidence that light modulates protein nitration in rat retina. Mol Cell Proteomics 1:293–303.

National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press. NIH Publication No. 86-23.

Reeves GI. 2000. Review of extensive workups of 34 patients overexposed to radiofrequency radiation. Aviat Space Environ Med 71:206–215.

Rojavin MA, Tsygankov AY, Ziskin MC. 1997. In vivo effects of millimeter waves on cellular immunity of cyclophosphamide-treated mice. Electromagn Biol Med 16:281–292.

Ryan KL, D'Andrea JA, Jauchem JR, Mason PA. 2000. Radio frequency radiation of millimeter wavelength: Potential occupational safety issues relating to surface heating. Health Phys 78:170–181.

Walters TJ, Ryan KL, Belcher JC, Doyle JM, Tehrany MR, Mason PA. 1998a. Regional brain heating during microwave exposure (2.06 GHz), warm-water immersion, environmental heating and exercise. Bioelectromagnetics 19:341–353.

Walters TJ, Ryan KL, Tehrany MR, Jones MB, Paulus LA, Mason PA. 1998b. HSP70 expression in the CNS in response to exercise and heat stress in rats. J Appl Physiol 84:1269–1277.

Wehling JH. 2005. Multifunctional millimeter-wave systems for armored vehicle application. IEEE Trans Microw Theory Tech 53:1021–1025.

Williams RA, Webb TS. 1980. Exposure to radio-frequency radiation from an aircraft radar unit. Aviat Space Environ Med 51:1243–1244.

Witzmann FA, Clack JW, Geiss K, Hussain S, Juhl MJ, Rice CM, Wang C. 2002. Proteomic evaluation of cell preparation methods in primary hepatocyte cell culture. Electrophoresis 23:2223–2232.

Witzmann FA, Arnold RJ, Bai F, Hrncirova P, Kimpel MW, Mechref YS, McBride WJ, Novotny MV, Pedrick NM, Ringham HN, Simon JR. 2005. A proteomic survey of rat cerebral cortical synaptosomes. Proteomics 5:2177–2201.

Xiao GG, Nel AE, Loo JA. 2005. Nitrotyrosine-modified proteins and oxidative stress induced by diesel exhaust particles. Electrophoresis 26:280–292.