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Original article

High Frequency Electromagnetic Field Induces Lipocalin 2 Expression in Mouse Liver

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Abstract

Objective(s)

Neutrophil gelatinase-associated lipocalin (NGAL/Lcn2), comprise a group of small extracellular proteins with a common β-sheet-dominated 3-dimensional structure. In the past, it was assumed that the predominant role of lipocalin was acting as transport proteins. Recently it has been found that oxidative stress induces Lcn2 expression. It has been also proved that electromagnetic field (EMF) produces reactive oxygen species (ROS) in different tissues. Expression of Lcn2 following exposure to electromagnetic field has been investigated in this study.

Materials and Methods

Balb/c mice (8 weeks old) were exposed to 3 mT, 50 HZ EMF for 2 months, 4 hr/day. Afterwards, the mice were sacrificed by cervical dislocation and livers were removed. The liver specimens were stained with Haematoxylin- Eosin (H&E) and analyzed under an optical microscope. Total RNA was extracted from liver and reverse transcription was performed by SuperScript III reverse transcriptase with 1 µg of total RNA. Assessment of Lcn2 expression was performed by semiquantitative and real time- PCR.

Results

The light microscopic studies revealed that the number of lymphocyte cells was increased compared to control and dilation of sinosoids was observed in the liver. Lcn2 was up-regulated in the mice exposed to EMF both in mRNA and protein levels**.**

Conclusion

To the extent of our knowledge, this is the first report dealing with up-regulation of Lcn2 in liver after exposure to EMF. The up-regulation might be a compensatory response that involves cell defense pathways and protective effects against ROS. However, further and complementary studies are required in this regards.

Keywords: Electromagnetic Field, Liver, Lipocalin 2, Reactive Oxygen Species

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Introduction

Recently, attention is focused on the effects of the electromagnetic field (EMF) due to its widespread use in everyday life. Almost all kinds of household electrical appliances (television sets, personal computers, hair dryers, etc.) emit extremely low frequency magnetic field (ELF-MF) (1, 2).

An ELF-MF can induce a number of changes in biological systems of different living species, like insects, rodents, and humans (3, 4). Epidemiological studies suggest a possible link between ELF-MF exposures and clinically recognized medical disorders in people, such as leukemia, brain cancer, breast cancer, kidney cancer, and other kinds of cancer as well as cardiovascular diseases (5). Rather than chemical processes, physical processes at the atomic level are the bases of reactions between biomolecules in an electromagnetic field, since the field can magnetically affect chemical bonds between adjacent atoms with consequent production of free radicals. There are a number of data on ELF-MF free radical production: such as super oxide anion in different cells and organs, e.g. in macrophages, neutrophils, kidney and liver (5- 9). The liver is the major source of proteins used throughout the body for various functions. Consequently, when the body undergoes severe injury or trauma, the liver is one of the organs to be significantly affected. Upon injury or infection, liver response is characterized by an altered protein synthesis profile (10). The lipocalins constitute a broad but evolutionally conserved family of small proteins; however, the functions of many lipocalins remained unclear to date. Neutrophil gelatinase-associated lipocalin (NGAL; also known as lipocalin 2 or human neutrophil lipocalin) is a 25 kDa glycoprotein that was initially purified from neutrophil granules (11-13). Induction of NGAL/Lcn2 has been reported in various harmful conditions such as infection, cancer, inflammation, kidney injury, heart injury, burn injury, intoxication and β- thalassemia (14- 26). Recently, it was found that oxidative stresses induce Lcn2/NGAL expression. Up-regulation of Lcn2 expression has been reported after exposure to γ-ray in heart, kidney and especially in liver. Up-regulation of Lcn2 expression also has been reported in HepG2 cells after exposure to X-rays or H_2O_2 (15- 16). Present study was designed to clarify whether electromagnetic field (EMF) could induce Lcn2 expression in mouse liver.

Materials and Methods

Mice and irradiation

8 weeks old male Balb/C mice were used in this research. The laboratory was maintained on a 12/12-hr light/dark cycle. Mice were placed inside the EMF exposure cage.10 male mice were irradiated with 3 mT, 50 HZ EMF for 2 months, 6 days/week, 4 hr/day from 8:00 AM to 12:00 PM. Control groups (10 mice) did not received irradiation. After this period, mice were sacrificed by cervical dislocation and their livers were removed and used for purposes of the study. Animal experiments were approved by the ethical committee of Tabriz medical university and performed in accordance with the guidelines.

Haematoxilin and Eosin staining

After 2 months of irradiation, the mice were sacrificed with cervical dislocation and their livers were removed. The specimens were stored in 10% formalin solution for 24 hr and, after that, they were submitted to the routine process of slide preparation with 5 mm sections, stained with Haematoxylin- Eosin (H&E), to be analyzed under an optical microscope.

Immunohistochemistry

Liver tissues were fixed in 10% formalin and cryoprotected by sinking in 10% and then in 30% sucrose (in 0.1 M phosphate buffer) at 4°C. 20 micrometer thick sections were prepared with cryostat. The primary antibody incubation, Lcn2, was carried out at 4 °C for overnight with dilution of 1:100 of polyclonal goat NGAL (M-12) (sc-18695, Santa Cruz, USA). Further incubation was carried out with 1/100 dilution of horse radish peroxidase-coupled secondary anti-goat IgG-HRP (sc-3851, Santa Cruz, USA) antibody for 2 hr at room temperature (RT). For color

development DAB solution (Sigma, USA) was used in this experiment.

RNA Extraction

Total RNA from liver tissue was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The quantity and quality of RNA were determined by spectrophotometry (ND-1000; Nanodrop, Wilmington, DE) and electrophoresis, respectively.

cDNA Synthesis

Reverse transcription was performed by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with 1 μg of total RNA followed by DNaseI (Invitrogen, Carlsbad, CA) treatment and heat inactivation.

Assessment of Lcn2 Expression

Semi-quantitative PCR was performed using Taq DNA polymerase (Roche, Germany) in a GeneAmp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Wellesley, MA). After initial denaturation (5 min at 94 °C), cDNA was subjected to 30 cycles of PCR. Primer set for the mouse Lcn2 included forward 5′-CCA GTT CGC CAT GGT ATT TTTC-3′ and reverse 5′-CAC ACT CAC CAC CCA TTC AGTT-3′. For the normalization, expression of β-actin was examined and the primer set was forward 5′-TTC TAC AAT GAG CTG CGT GTG G -3′ and reverse 5′-GTG TTG AAG GTC TCA AAC ATG AT-3′. PCR annealing temperature was 60 °C for mouse Lcn2 and 59 °C for β -actin. PCR products were evaluated in a 2% agarose gel. Intensity of the bands was assessed by UVIdoc software version 12.5 Real-time PCR analysis was performed in a Rotor-Gene RG 3000 (Corbett Research, Sydney, Australia). Amplification was conducted using AB solute Syber green mix (ABgene, Surrey, UK) according to the manufacturer's instructions. PCR condition included an initial denaturation at 94 °C for 15 min followed by 40 cycles consisting of denaturation at 94 °C for 30 sec, annealing at suitable temperature for 30 sec and extension at 72 °C for 30 sec. Threshold cycle values were normalized by β -actin expression.

Statistical analysis

The results are expressed as mean±SD of three independent experiments. Differences were compared using student t- test.

Results

Light microscopic findings in liver

Infiltration of lymphocytes was observed in portal space. Pericentral lymphocytes infiltration in central venule of liver was also observed. Dilation of sinusoids occurred in the liver exposed to EMF compared to the control (Figure 1).

Immunohistochemistry findings

Immunohistochemistry findings showed that Lcn2 was upregulated in pericentral region and sinusoids of liver lobules (Figure 2).

Induction of Lcn2 in mouse liver after exposure to EMF

To determine whether EMF induces Lcn2 expression, mice were exposed to EMF and gene expression was assessed by RT-PCR. First, semiquantitative RT-PCR was carried out. Expression of Lcn2 was observed in the liver of control samples which indicates that expression of Lcn2 in liver is necessary for normal physiology of the cells but Lcn2 was up-regulated in the exposed samples compared to normal (Figure 3a and b). Then, we quantified Lcn2 expression in exposed samples by real-time RT-PCR. Lcn2 expression increased about 8 fold (8±1.84, $P< 0.001$) compared to control samples indicating that EMF induces Lcn2 expression.

Discussion

Expression of Lcn2 has been reported in harmful conditions (14-26). Recently it has been shown that oxidative stress induces Lcn2 expression (15). This study was performed to clarify whether EMF could induce Lcn2 expression. Our results revealed that Lcn2 was up-regulated in mRNA level after EMF exposure. In present study, pathological

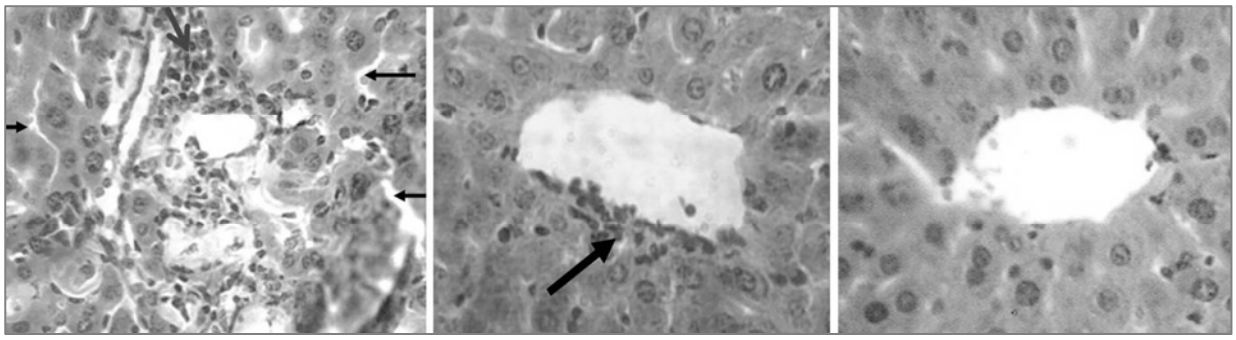

Figure 1. Histological findings in mouse liver tissue after exposure to EMF. Lymphocytes infiltration in portal space in the group exposed to EMF has been shown with brown arrow and black arrows point to sinusoidal dilation (a), × 400. Figure 1 (B) shows the pericentral venule lymphocytes infiltration in experimental group, × 400. Figure (C); Unexposed group (Control), × 400.

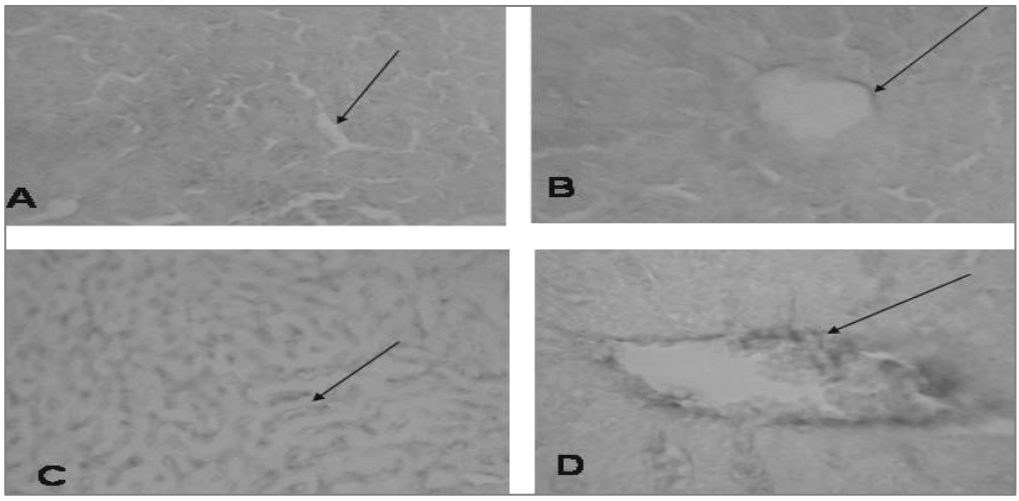

Figure 2. Immunohistochemistry findings in mouse liver after exposure to EMF. Controls show low staining in sinusoids (A) and pericental vein region (B). Lcn2 was expressed in liver sinusoids (C) and around the central vein (pericentral) (D).

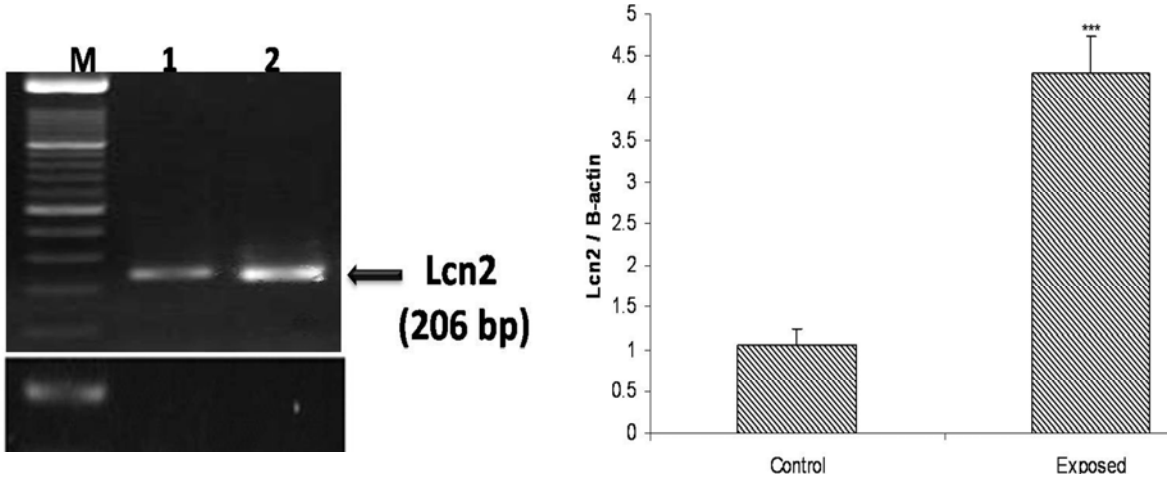

Figure 3. Expression of Lcn2 in mouse liver after exposure to EMF. Two months after exposure to 3mT and 50 Hz of EMF expression of Lcn2 was determined by semiquantitative RT-PCR. (a); After irradiation expression of Lcn2 was upregulated (lane 2) compared to the control, without exposure, (Lane 1). M, 100-bp marker. Lower figure indicates the expression of β- actin in both mice i.e. irradiated and control. M, 100-bp marker (b); Densitometric quantitation of the bands was performed by using UVIdoc software version 12.5. (Mean±SD; ****P* <0.001; number of replicates, 3.

findings such as increased lymphocyte infiltration in central venule and portal space and dilation of sinusoids were observed in liver tissue after exposure to electromagnetic filed. In another research reported in 2008, slight increase in the number and size of Kupffer cells and dilation of sinusoids has been detected in mice liver exposed to α particle radiation (16). Similarly, induction of Lcn2 expression was observed in mice liver exposed to α particle radiation. Up regulation has been also reported in the liver of mice exposed to γ- ray (16). Induction of Lcn2 expression has been also reported in acute lung injury induced by lipopolysaccharide (LPS) and diesel exhaust particles (DEP) (27). Taken together, these indicate that inflammatory response plays an important role for induction of Lcn2. During inflammation macrophages and endothelial cells secret the so-called pro-inflammatory cytokins such as tumor necrosis factor-α (TNF-α), Interleukin 1-β and Interleukin 6. Lipocalin 2 is also an acute-phase protein (APP) involved in a mammalian defense mechanism against bacterial infection and works by adding itself to the iron group within bacterial iron-containing siderophores (16).

It has been known that reactive oxygen species (ROS) induce Lcn2 expression (15). ROS are constantly generated in small amounts during metabolic processes and in several biochemical events in living organisms. It has been proved that reactive oxygen species are produced after EMF exposure in different cells (6-9, 28). In various pathophysiological conditions such as infection, cancer, inflammation, kidney injury, heart injury, burning and intoxication; expression of Lcn2/NGAL is induced (14-26). The pathophysiologic functions of 24p3/Lcn2/NGAL are unclear, but it has been suggested that they may act as an immunomodulator by binding to or inactivation of bacterial products, or through direct actions on the inflammatory cells (18). Similar to our findings, induction of Lcn2 expression has been reported in mice exposed to light. Photo-oxidative stress has been implicated in light damage pathogenesis (29). We previously showed that X-ray and H_2O_2 induce Lcn2 expression *in vitro* (30). Interestingly, induction of Lcn2 was abolished by administration of antioxidants. More recently, we found that Lcn2/NGAL acts as a protective factor against cisplatin and H_2O_2 toxicities (31, 32). Taken together, induction of Lcn2 in mice exposed to EMF would be attributed to ROS production in liver tissue and the up-regulation might be a compensatory response that involves cell defense pathways and protective effects against ROS.

Expression of heat shock proteins after EMFexposure has been proved. Exposure of HL60 cells by a 60Hz magnetic field at normal growth temperatures results in heat shock factor 1 activation and heat shock element binding, a sequence of events that mediates the stress-induced transcription of the stress gene HSP70 and increases synthesis of the stress response protein hsp70kD. Thus, the events mediating the electromagnetic field-stimulated stress response appear to be similar to those reported for other physiological stresses (e.g., heavy metals, oxidative stress, hyperthermia, oxidative stress) and could well be the general mechanism of interaction of electromagnetic fields with cells (33).

According to our results up-regulation of Lcn2 in liver after EMF exposure indicates its protective effects against ROS produced in the cell, however further and complementary studies are required in this regard.

Conclusion

Our data suggest that induction of Lcn2 is an adaptive response to ameliorate the injuries induced by EMF, and in other words, reestablishment of homeostasis. However, further and comprehensive studies are required to clarify the precise role of Lcn2 in EMF stress.

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