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Oxidative Stress Induced Lipocalin 2 Gene Expression: Addressing its Expression under the Harmful Conditions

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Lipocalin 2/H_2O_2/oxidative stress/radiation/liver.

Lipocalin 2 (Lcn2, NGAL) is a member of the lipocalin superfamily with diverse functions such as the transport of fatty acids and the induction of apoptosis. Previous reports indicated that expression of *Lcn2* is induced under harmful conditions. However, the mechanisms of the induction of *Lcn2* expression remain to be elucidated. In this report, we intended to identify the factor or factors that induce *Lcn2* expression. Up-regulation of *Lcn2* expression after X-ray exposure was detected in the heart, the kidney and especially in the liver. Primary culture of liver component cells revealed that this up-regulation in the liver was induced in hepatocytes. Up-regulation of *Lcn2* expression was also detected in HepG2 cells after the administration of X-rays or H_2O_2. Interestingly, up-regulation of *Lcn2* expression after H_2O_2 treatment was canceled by the addition of the anti-oxidants, dimethylsulfoxide or cysteamine. These results strongly suggest that *Lcn2* expression is induced by reactive oxygen species. Therefore, Lcn2 could be a useful biomarker to identify oxidative stress both *in vitro* and *in vivo*.

INTRODUCTION

The lipocalins constitute a broad but evolutionally conserved family of small proteins. Although the primary function of the lipocalins is thought to be involved in the transport of small ligands such as fatty acids and pheromones, they have also been implicated in a variety of different functions such as retinol transport, cryptic coloration, olfaction, prostaglandin synthesis, regulation of the immune response and cell homeostatic mediation.[1)] Neutrophil gelatinase-associated lipocalin (NGAL, lipocalin 2, Lcn2) is a 25 kDa glycoprotein that was initially purified from neutrophil granules.[2)] The Lcn2 protein exists as a 25 kDa monomer, as a 46 kDa homodimer, and in a covalent complex with neutrophil gelatinase that is known as matrix metalloproteinase 9.[2)] A variety of functions of the Lcn2 protein have been reported, such as the transport of fatty acids and iron,[3–4)] the induction of apoptosis,[5)] the suppression of bacterial growth,[6)] and the modulation of inflammatory responses.[7)] The expression of the *Lcn2* gene is detected in mouse fibroblasts stimulated by a number of growth factors such as serum, basic fibroblast growth factor and phorbol esters. *Lcn2* expression is also observed in the mouse liver after treatment with some carcinogens or reactive oxygen species (ROS) producing agents such as diethylnitrosamine.[8)] These findings suggest that the Lcn2 protein may play a role in regulating cellular growth. This hypothesis is further supported by the expression of the Lcn2 protein in various malignant tumors.[9–11)] Contrary to previous reports, most functions of the Lcn2 protein, with the exception of its role in innate immunity, are not verified in *Lcn2* deficient mice.[12)] Another member of the lipocalin family, human tear lipocalin (Lcn1), acts as an oxidative-stress induced scavenger of potentially harmful products.[13)] In spite of the fact that there is little similarity between the Lcn1 and the Lcn2 proteins, they commonly exhibit antimicrobial activity.[6,14)] Several reports indicated that *Lcn2* expression is induced in various cells under harmful conditions such as cancer, intoxication, infection, inflammation, kidney injury, heart injury and burn injury where production of free radicals has been reported.[9–11,15–19)] Our previous study revealed that *Lcn2* expression was induced in the mouse liver when exposed to alpha particles (Roudkenar *et al*., manuscript submitted). It has also been

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reported that *Lcn2* expression was up-regulated in some radio-resistant cell lines established by continuous fractionated exposure to X-rays.[20] From the above results we hypothesized that *Lcn2* expression is induced by ROS. In this study we examined *Lcn2* expression in mouse tissues and HepG2 cells after irradiation. We also examined *Lcn2* expression after the administration of H_2O_2 and its scavengers.

MATERIALS AND METHODS

Mice

Seven-week old male C3H/Hex mice were used. Animal protocols were approved by the ethical committee of the Institute of Development, Aging and Cancer (IDAC), Tohoku University, and were performed according to the institutional guidelines.

Cell culture

HepG2 cells derived from human hepatoblastoma were obtained from the Cell Resource Center for Biomedical Research in IDAC, Tohoku University. Cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Cell cultures were raised at 37°C in an atmosphere of 5% CO_2.

H_2O_2 treatment

Before H_2O_2 treatment, HepG2 cells were cultured in serum-free medium for 16 hrs. After washing with phosphate buffered saline (PBS), cells were cultured in serum-free medium containing 0.1 mM H_2O_2 for 45 min. For scavenger treatments we also added 1 mM cysteamin or 5% dimethyl sulfoxide (DMSO; Sigma) to the H_2O_2 containing medium.

Irradiation

Mice were exposed to 2, 5 or 8.5 Gy of ^{60}Co γ-rays (0.34 Gy/min) at the Research Reactor Institute, Kyoto University. Liver, lungs, heart, spleen, testis and kidneys were dissected out from mice after sacrificed by cervical dislocation. All tissues were immediately frozen and kept at –80°C until use. HepG2 cells and mice for the isolation of parenchymal cells (hepatocytes, PC) and non-parenchymal cells (NPC) were exposed to 8.5 Gy X-rays (1.0 Gy/min.) using a 150-KVp X-rays generator (Model MBR-1520R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminum plus 0.1 mm copper filter. Long-term irradiated HepG2 cells were exposed to 0.5 Gy X-rays every 12 hrs for more than 4 years, and the total exposure dose was over 1,600 Gy.

Assessment of Lcn2 *gene expression*

Total RNA from mouse tissues and cells were extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription (RT) was performed by SuperScript III reverse transcriptase (Invitrogen) with 500 ng of *DNaseI* (Invitrogen) treated total RNA. Polymerase chain reaction (PCR) was performed using Animal *Taq* DNA polymerase (ABgene, Surrey, UK) in a GeneAmp PCR system 9600 (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA, USA). After initial denaturation (5 min at 94°C), complementary DNA was subjected to 30 cycles of PCR. Primers for the amplification of mouse *Lcn2* were; forward 5'-CCA GTT CGC CAT GGT ATT TTT C-

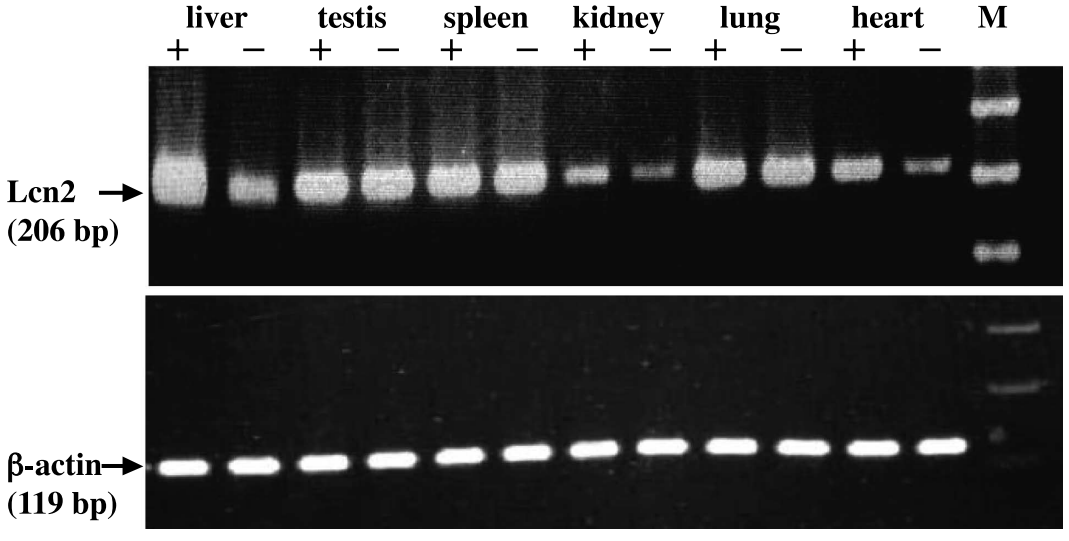

Fig. 1. Expression of *Lcn2* in mouse organs with or without exposure to γ-rays. Twenty-four hrs after exposure to 8.5 Gy of γ-rays *Lcn2* expression was determined by semi-quantitative RT- PCR. *Lcn2* expression was detected in all the organs examined before irradiation. After γ-ray irradiation *Lcn2* expression was up-regulated in the liver, the kidney and the heart. M; 100 bp marker. +; with γ-rays. –; without γ-rays.

3' and reverse 5'- CAC ACT CAC CAC CCA TTC AGT T-3'. Primers for human *Lcn2* were; forward 5'-TCA CCT CCG TCC TGT TTA GG-3' and reverse 5'-CGA AGT CAG CTC CTT GGT TC-3'. Gene expressions were normalized by *β-actin* expression and primers were; 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 human and mouse *Lcn2* and 59°C for *β-actin*. PCR products were separated in a 2% agarose gel. In order to determine gene expression, real-time PCR was performed in a BIO-RAD icycler iQ, SA-THK Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Amplification was conducted using AB solute SYBR green ROX mix (ABgene) according to the manufacture's instructions. The PCR conditions were; initial denaturation at 96°C for 15 min followed by 40 amplification cycles consisting of denaturation at 96°C for 30 sec, annealing at a suitable temperature for 30 sec and extension at 72°C for 30 sec. Threshold cycle values were normalized by *β-actin* expression.

Isolation of parenchymal cells (PC) and non-parenchymal cells (NPC)

PC and NPC were isolated from mouse liver tissue as described elsewhere.[21] Briefly, 24 hrs after exposure to 8.5 Gy of X-rays, mice were anaesthetized with pentobarbital (Dainippon Sumitomo Pharma, Co., Ltd., Osaka, Japan) followed by cannulation of the portal vein with a 24-gauge needle. Then, the liver was perfused with prewarmed calcium-free Hank's Balanced Salt Solution (HBSS, Sigma), at a flow rate of 3 ml/min. Subsequently, the liver was perfused with collagenase solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan; 0.5 mg/ml HBSS containing 5 mM calcium) for 7 min. Liver component cells were gently isolated and were maintained in HBSS. Differential centrifugations were used to separate PC from NPC. The PC fraction was separated from total cells by centrifugation for 2 min at 50*g* and the pellet was washed three times with HBSS. Viability of isolated cells was assessed by the trypan blue dye exclusion test immediately after isolation and was found to be more than 70%. For separation of the NPC fraction, the cell suspension was centrifuged twice for 5 min at 50*g* to eliminate PC. Subsequently, the NPC fraction was sedimented by centrifugation of supernatant for 10 min at 150*g*.

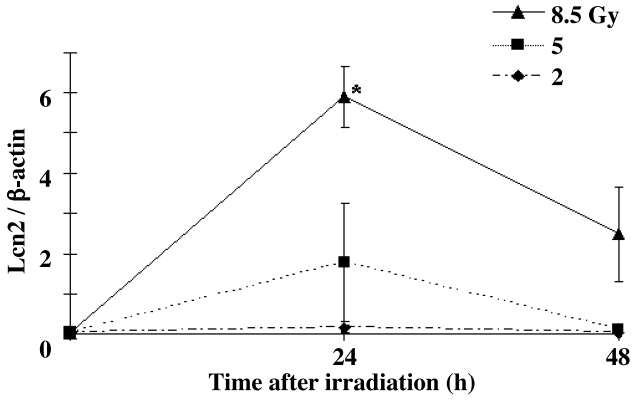

Fig. 2. Real-time PCR analysis of *Lcn2* expression in the mouse liver after exposure to γ-rays. Dose dependent up-regulation of *Lcn2* expression was observed 24 hrs after irradiation. (Mean ± SD, *; $p < 0.001$)

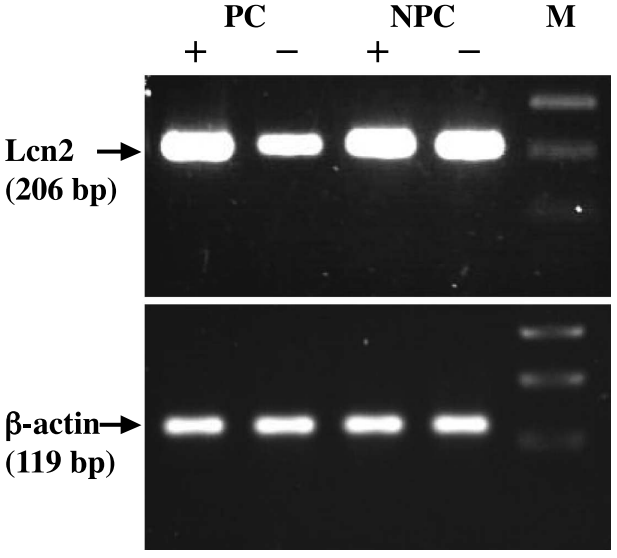

Fig. 3. Up-regulation of *Lcn2* expression in PC after exposure to 8.5 Gy of X-rays. PC and NPC were separately obtained from the irradiated mouse liver by perfusion. PC; parenchymal cells. NPC; non-parenchymal cells. M; 100 bp marker. +; with X-rays. –; without X-rays.

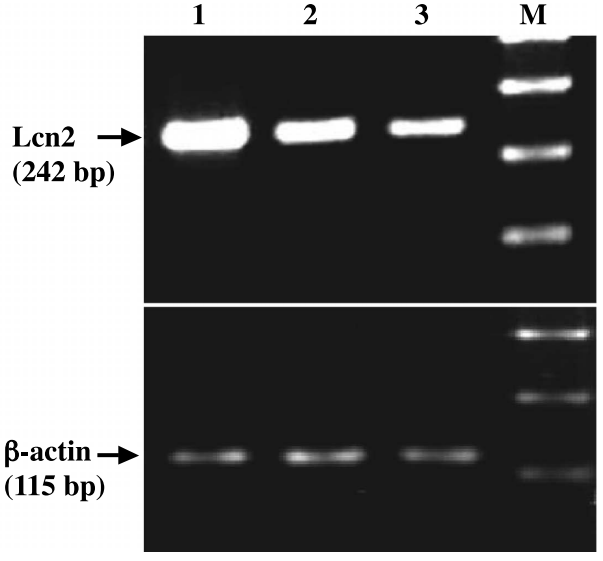

Fig. 4. Up-regulation of *Lcn2* expression after acute or long-term X-ray irradiation. Total RNA was extracted from HepG2 cells and *Lcn2* expression was examined by semi-quantitative RT-PCR. Lane 1; Long-term X-rays. Lane 2; 8.5 Gy of X-rays. Lane 3; without X-rays. M; 100 bp marker.

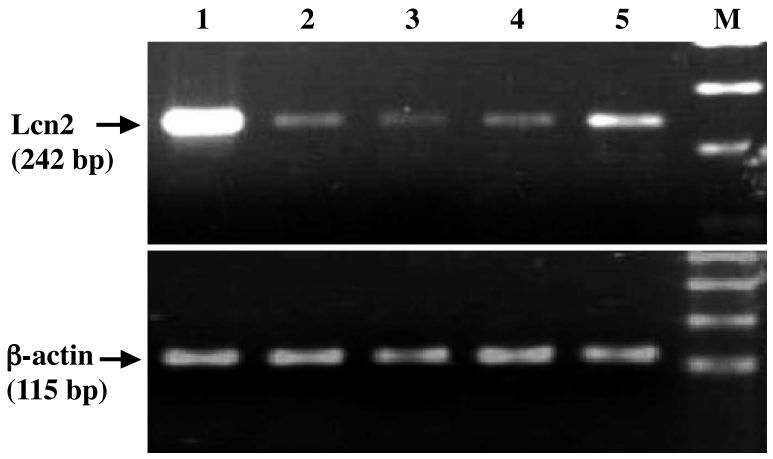

Fig. 5. Up-regulation of *Lcn2* expression after treatment with H_2O_2. Up-regulation of *Lcn2* expression was induced by the administration of H_2O_2. This up-regulation was suppressed by the addition of 5% DMSO or 1 mM cysteamine in the medium containing H_2O_2. Lane 1; 0.1 mM H_2O_2. Lane 2; 0.1 mM H_2O_2 and 1 mM cysteamine. Lane 3; 0.1 mM H_2O_2 and 5% DMSO. Lane 4; serum-free medium. Lane 5; No treatment. M; 100 bp marker.

Statistical analysis

The results were expressed as the mean ± SD of three independent experiments. Differences between groups were compared using the Student's *t*-test.

RESULTS

Lcn2 *expressions in mouse tissues after exposure to γ-rays*

We examined the effect of γ-rays on *Lcn2* expression by semi-quantitative RT-PCR. Before irradiation, *Lcn2* expression was detected in all the organs examined (Fig. 1). Twenty-four hrs after exposure to 8.5 Gy of γ-rays, *Lcn2* expression was up-regulated in the kidney, the heart and especially in the liver (Fig. 1). We then quantified *Lcn2* expression in the irradiated liver by real-time PCR (Fig. 2). Up-regulation of *Lcn2* expression was dose dependent and statistical significance was detected in the 8.5 Gy exposed group compared to non-irradiated mice ($p < 0.001$). In order to identify which compartment of the liver expressed *Lcn2*, we separated mouse liver cells into PC and NPC by the perfusion sedimentation technique. PC and NPC fraction mainly contained hepatocytes and Kupffer cells, respectively. Basal level of *Lcn2* expression in NPC was higher than that in PC (Fig. 3). After 8.5 Gy X-rays administration, up-regulation of *Lcn2* expression was detected in PC but not in NPC (Fig. 3).

Induction of Lcn2 expression in HepG2 cells after acute and long-term X-rays exposure

Since the liver was the most sensitive organ for the induction of *Lcn2* after irradiation (Fig. 1), we examined *Lcn2* expression *in vitro* using HepG2 cells. Up-regulation of *Lcn2* expression was detected 12 hrs after exposure to 8.5 Gy of X-rays (Fig. 4). *Lcn2* expression level in cells with long-term fractionated irradiation was higher than in cells after an acute exposure (Fig. 4).

Induction of Lcn2 *expression after H_2O_2 treatment*

The effect of ROS on *Lcn2* expression was examined by treating HepG2 cells with H_2O_2. Before H_2O_2 treatment, HepG2 cells were cultured in serum-free RPMI-1640 medium to avoid any influence of external free radicals such as lipid peroxidation products or antioxidants present in the serum. Without serum, *Lcn2* expression decreased, however, the administration of H_2O_2 drastically up-regulated *Lcn2* expression (Fig. 5). With the presence of 5% DMSO or 0.1 mM cysteamine in the H_2O_2 containing medium, up-regulation of *Lcn2* expression was canceled (Fig. 5).

DISCUSSION

Several studies indicate that *Lcn2* expression is induced under harmful conditions such as intoxication, infection, inflammation or other forms of cellular stresses,[8–11,15–19] however, the reason for the induction remains to be elucidated. Previous reports indicated that expression of *Lcn1*, a member of the lipocalin superfamily, was induced by harmful products such as ROS under conditions such as kidney injury, heart failure and burn injury.[15–19] Therefore, we hypothesized that harmful products such as ROS also induce *Lcn2* expression. To confirm our assumption, we examined *Lcn2* expression after the administration of H_2O_2 and also after Ionizing Radiation (IR), that is also a potent inducer of ROS.[22–23]

Without irradiation, *Lcn2* expression was detected in all the mouse organs examined. The Lcn2 protein in the testis

is known to be involved in protection of spermatogenic cells from genotoxic stresses such as ROS, and high expression of the *Lcn2* protein in the lung might be necessary to cope with the high level of ROS produced in this organ.[24] In this study, drastic up-regulation of *Lcn2* expression induced by IR was detected in the liver. Previous reports indicated that hepatocyte growth factor (HGF) induced by IR subsequently induces *Lcn2* expression.[25] HGF expression is reportedly induced under the condition of renal tubular injury, heart and liver diseases[26–28] accompanied with the production of ROS and induction of the *Lcn2* gene expression.[15–19] Therefore, high level of *Lcn2* expression in the irradiated liver might be due to the HGF induced by ROS. However, there is no report which indicates that ROS induces HGF expression. The up-regulation of *Lcn2* expression in the irradiated mouse liver may be attributed to both HGF and ROS. A previous report indicated that HGF is produced in Kupffer cells and endothelial cells.[29] Therefore, we further studied *Lcn2* expression after separating liver cells into PC and NPC. Basal level of *Lcn2* expression in NPC was higher than that in PC presumably because ROS are endogenously produced in Kupffer cells for the mediators in antigen presentation.[30] In this study, IR induced *Lcn2* upregulation in PC but not in NPC. It is noted that ROS are prominently produced in hepatocytes, but not in Kupffer cells after the administration of toxic injury.[31] These suggest that *Lcn2* expression in the irradiated liver is attribute to ROS produced in PC.

After acute and long-term exposure to X-rays, *Lcn2* expression was found to be upregulated in HepG2 cells. The level of up-regulation after long-term exposure to X-rays was higher than that after single acute exposure. This is consistent with the previous result using esophageal cancer cell lines.[20] In the present study, H_2O_2 induced up-regulated *Lcn2* expression in HepG2 cells and this up-regulation was canceled by the administration of ROS scavengers. Thus, we confirmed that *Lcn2* up-regulation in the liver is induced via ROS produced by IR. It is suggested that the Lcn2 protein might have a function as a scavenger and protect cells from ROS. The study to confirm these assumptions is now underway in our laboratory. This study also suggested that Lcn2 could be a useful biomarker for the detection of oxidative stress *in vitro* and *in vivo*.

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