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ORIGINAL ARTICLE

Lipocalin-2 as mediator of chemokine expression and granulocyte infiltration during ischemia and reperfusion

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Summary

Lipocalin-2 (Lcn2) expression contributes to ischemia and reperfusion injury (IRI) by enhancing pro-inflammatory responses. The aim of this work was to elucidate the regulation of Lcn2 during hypoxia and its effects on the expression of key chemokines and adhesion molecules. Lcn2 wt and Lcn2$^{-/-}$ mice were used in a heterotopic heart transplantation model. Quantitative RT-PCR was applied for chemokine gene expression analysis. Reporter gene studies were used to elucidate the regulation of the Lcn2 promoter by hypoxia. HIF-1β expression led to a 2.4-fold induction of the Lcn2 promoter. Apart from an earlier onset of granulocyte infiltration in the Lcn2 wt setting after 2 h of reperfusion compared with the Lcn2$^{-/-}$ setting ($P < 0.013$), exogenous application of recombinant Lcn2 revealed a trend toward increase of granulocyte infiltration. Analyzed chemokines were expressed significantly higher in the Lcn2 wt setting at 2 h of reperfusion ($P \leq 0.05$). The number of apoptotic cells observed in Lcn2$^{-/-}$ grafts was significantly higher than in the Lcn2 wt setting. Our results indicate that Lcn2 affects granulocyte infiltration in the reperfused graft by modulating the expression of chemokines, their receptors and the apoptotic rate.

Introduction

Ischemia and reperfusion injury (IRI) designates a multifactorial, antigen-independent process invariably associated with the transplantation of solid organs. It results from a complex interplay of cellular and molecular events. Although ischemia is unavoidable during transplantation, reperfusion is also a main contributor to IRI as it is linked to the generation of reactive oxygen species (ROS), the perturbation of calcium homeostasis and ultimately cell death [1–5]. ROS have been linked to the enhanced secretion of cytokines and chemokines, resulting in the recruitment of inflammatory cells and further increased tissue damage [1,6]. The inflammatory process is thus a main contributor to the deterioration of organ quality observed under these conditions. On the other hand, only few prophylactic or therapeutic options exist. Thus, a better understanding of the events leading to IRI as well as detection of new biomarkers [7] promises the identification of points for future therapeutic intervention. Lipocalin-2 (Lcn2) is a 24 kDa glycoprotein secreted by neutrophils [7–13] and plays an important role in the innate immune response because of its ability to bind iron siderophores [14–16]. In our own work, we identified for the first time Lcn2 as a potential regulator of inflammation during cardiac IRI [8]. Others suggested Lcn2 gene expression in zero-hour biop-

sies as early candidate biomarker for delayed graft function in renal transplantation [7].

Initial evidence for a possible role of Lcn2 in IRI in the setting of a murine heart transplant model was obtained in a large-scale expression screen [17]. In depth, studies using $Lcn2^{-/-}$ and wt animals confirmed Lcn2 expression at the protein level and provided evidence for an important role in the regulation of granulocyte infiltration [18]. Still there is a controversy about its role in IRI with only few studies addressing administration of recombinant Lcn2 [8,18,19]. Chemokines and their receptors play a crucial role in the pathological process of IRI by regulating the infiltration of neutrophils, monocytes, and lymphocytes to the site of ischemia [20–24]. To obtain a first insight into the regulatory role of Lcn2 in these processes, we aimed to investigate the molecular mechanism underlying Lcn2 expression under hypoxia and additionally analyze possible differences in the expression of chemokines [keratinocyte chemoattractant (KC), Lipopolysaccharide-induced CXC (LIX), macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1)], chemokine receptors, and intercellular adhesion molecule-1 (ICAM-1) between Lcn2 wt and $Lcn2^{-/-}$ hearts and granulocytes.

Material and methods

Cell culture, transient transfections, and luciferase assays

To evaluate whether the Lcn2 promoter is regulated by hypoxia or by the transcription factor HIF-1, COS-7 cells were transfected with the reporter plasmid pGL3-24p3-luc (kindly provided by Prof. S. Gaffen, University of Buffalo, State University of New York, USA) or together with expression plasmids for human HIF-1α or HIF-1β (kindly provided by Dr. D. Richards, Department of Medicine, Université Laval, Quebec, Canada). For induction of hypoxic conditions, the medium was exposed to 1% O_2 at 37 °C for different times. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Samples were normalized to total protein amount.

The heterotopic murine heart transplantation model

Hearts were transplanted using a modified cervical cuff technique for revascularization after 6 h of cold ischemic time (CIT) described elsewhere [25]. $Lcn2^{-/-}$ animals were obtained from Thorsten Berger, The Campbell Family Institute for Cancer Research, University of Toronto, Princess Margaret Hospital, Toronto, Canada. Mice were housed in a specific pathogen-free environment, and experiments were conducted following approval by the Institutional Animal use under Austrian Federal Law Gazette No. 162/2005, GZ 66.011/64-BrGT/2004. For our experiments, $Lcn2^{-/-}$ mice were backcrossed into the C57BL/6 background for at least 10 generations. Offsprings were genotyped by PCR of genomic DNA derived from tail clippings. Wild-type C57BL/6 animals (Lcn2 wt) were purchased from Harlan-Winkelmann (Borchen, Germany) and all animals were kept under special pathogen-free environment with unlimited access to water and standard laboratory chow according to the Austrian Animal Care Law. Male inbred C57BL/6 or $Lcn2^{-/-}$ mice weighing 24–29 g were used as size-matched donor and recipient pairs ($n = 6$ in each group). Anesthesia for transplant procedures was induced by intramuscular injection of ketamine and xylazine. At different time points (0, 2, 12, 24 and 48 h), the graft was recovered and dissected, snap-frozen in liquid nitrogen, and stored at −80 °C until further analysis. All experiments were approved by the National Animal Welfare Committee.

Isolation of primary murine granulocytes

Primary granulocytes were isolated from the peritoneal lavage following casein stimulation of Lcn2 wt and $Lcn2^{-/-}$ mice by percoll gradient centrifugation.

Determination of granulocyte infiltration

For histological examination, grafts were fixed in 4% formaldehyde for 24 h and embedded in paraffin wax. Sections of 5 μm were mounted on conventional glass slides and stained with hematoxylin and eosin using standard histological methods. Granulocytes were counted in a blinded fashion and granulocyte numbers per high-power field (40× magnification) were reported in categories of 1 (<5 cells), 2 (5–10 cells), 3 (11–20 cells), 4 (21–50 cells), or 5 (>50 cells).

Apoptosis detection

Apoptosis in the heart was determined by TdT-mediated dUTP-biotin nick end labelin (TUNEL) staining of paraffin-embedded thin sections using a commercial kit (Roche Diagnostics, Vienna, Austria). Apoptotic cells were quantified by calculating the mean of TUNEL-positive nuclei in at least three high-power fields (40× magnification) for each section.

RNA isolation and cDNA synthesis

Heart tissue was immediately placed in AllProtect Tissue Reagent (Qiagen, Hilden, Germany) and stored at −20 °C. RNA and protein were extracted from the same sample

using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Quantitative real time polymerase chain reaction (qRT-PCR)

Primers for the murine chemokines and cytokines MIP-2, LIX, CCL6, IL-6, and the murine chemokine receptors CXCR2 and CCR2 were designed using FastPCR. Primer sequences for KC, MCP-1, and ICAM-1 were used as described by Natarajan *et al.* [26]. Quantitative PCR were performed on a BioRad iQ5 Cycler (Bio-Rad Laboratories GmbH, Munich, Germany). Data were normalized to the housekeeping gene β-actin and calculated relative to the untreated controls using the in-built software (normalized fold expression).

Purification and FITC-labeling of recombinant Lcn2

Recombinant human Lcn2 (rLcn2) was expressed as a glutathione-S-transferase (GST)-fusion protein in *E. coli* BL21 and, after proteolytic removal of GST with thrombin (GE Healthcare, Vienna, Austria), purified to homogeneity by cation exchange chromatography on CIM-SO3 (BIA Separations, Ljubljana, Slovenia). Lcn2 was labeled with fluorescein isothiocyanate (FITC) using standard procedures as described by Fluckinger *et al.* [27].

Internalization of rLcn2 by various target cells

Cultures of COS-7, human endothelial cells [Human Umbilical Vein Endothelial Cells (HUVEC)], Madin-Darby canine kidney (MDCK) and HL-1 cells were seeded at a density of 50 000 cells per well on eight-well chamber slides and incubated overnight at 37 °C. The cells were washed twice with serum-free medium, and recombinant FITC-labeled Lcn2 (3 μg) was added in serum-free medium and incubated for 3 h at 37 °C. To remove cell surface-bound Lcn2, cells were washed twice with phosphate-buffered saline (PBS) and treated with 50 mM glycine, 150 mM NaCl, pH 3.0, for 2 min. The cells were washed with Dulbecco's PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells without protein served as control. Uptake of FITC-labeled Lcn2 was analyzed using an inverse microscope (IX70, Olympus, Vienna, Austria) mounted to the UltraVIEW RS 3-line confocal System (Perkin Elmer, Wellesley, USA). Images were acquired with a 60× PlanApo oil immersion objective (numerical aperture 1.4). Image acquisition and processing was carried out with the UltraVIEW™ software (Perkin Elmer). Confocal images captured as a stack of images at different focal points (at 0.2 μm steps) were processed with Volocity™3D/4D visualization software (Perkin Elmer, Wellesley, MA, USA).

In vivo administration of recombinant Lcn2

Preliminary data were obtained from a randomized-controlled, double-blinded trial regarding administration of different concentrations of iron-free and iron-bound rLcn2 (group 1 = control, injection of PBS; group 2 = 50 μg rLcn2; group 3 = 250 μg rLcn2; group 4 = 50 μg rLcn2 + Enterobactin + Fe^{3+}; group 5 = 250 μg rLcn2 + Enterobactin + Fe^{3+}) in a syngeneic murine heterotopic heart transplantation model (see above) with 6 h of CIT. The time points of intraperitoneal Lcn2-administration were 1 h before, during and 1 h after reperfusion and enrichment of the preservation solution as well. $Lcn2^{-/-}$ mice were used for this study and organ retrieval was scheduled 24 h after reperfusion.

Statistical analysis

Statistical analyses were performed using SPSS statistics 19 software for Windows (SPSS Inc., Chicago, IL, USA). Students *t*-test was used to analyze differences in reporter gene expression in transfected cells under normoxic or hypoxic conditions. The Kruskal–Wallis test and the Mann–Whitney *U*-test were employed to analyze global and specific differences, respectively, in gene expression between Lcn2 wt and $Lcn2^{-/-}$ grafts and group differences in apoptosis. Differences in granulocyte infiltration were analyzed using Pearson's chi-square granulocyte infiltration with reperfusion time and with the number of apoptotic cells were analyzed using Spearman's correlation statistics. Values were expressed as means and standard deviations.

Results

Regulation of the Lcn2 promoter under hypoxic conditions

After transient transfection of the Lcn2 reporter plasmid pGL3-24p3-luc we observed only a mild induction of luciferase gene activity of 1.43±0.299 at 21 h of hypoxia in COS-7 cells (Fig. 1a). However, overexpression of HIF-1β, but not HIF-1α, stimulated Lcn2 promoter activity 2.41-fold under normoxic conditions (Fig. 1b). Co-transfection of both subunits further enhanced the reporter gene activity as compared with HIF-1α alone (2.64±0.28-fold; $P < 0.01$).

To confirm stabilization of HIF-1α in our experimental system, Western blot analysis using crude nuclear extracts from our transfected COS-7 cells was performed. The HIF-1α immunoblots shown in Figure 1c demonstrates that HIF-1α is stabilized following hypoxic treatment of the cells. To confirm switch to hypoxic conditions in our cells under the experimental conditions

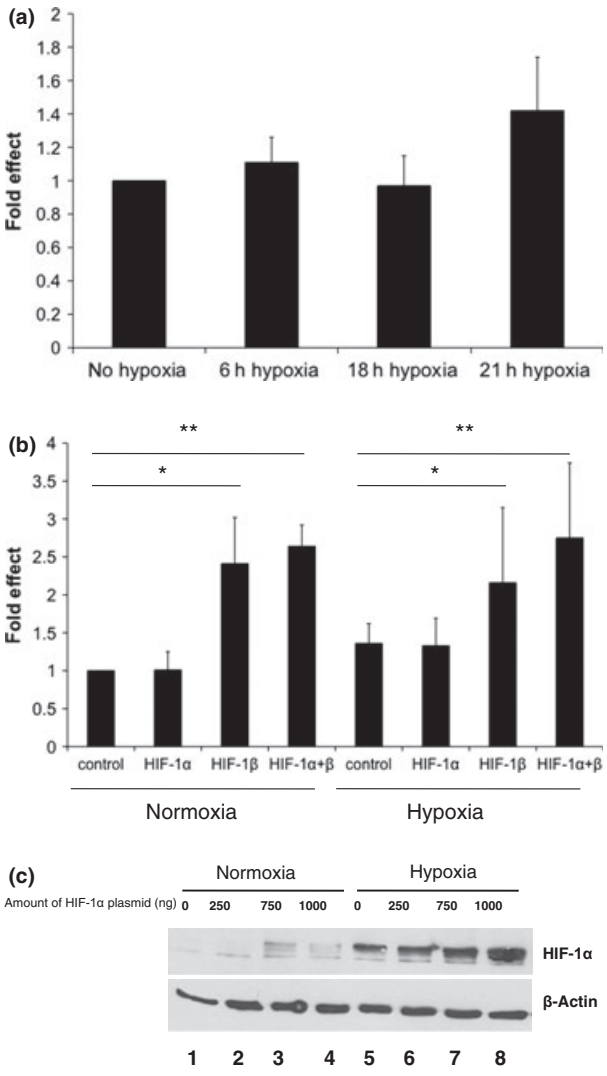

Figure 1 Regulation of the Lipocalin-2 (Lcn2) promoter by hypoxia and HIF-1. (a) Regulation of the Lcn2 promoter by hypoxia. COS-7 cells were transiently transfected in triplicates with the reporter plasmid pGL3-24p3-luc and subjected to hypoxia (1% O_2) for the indicated times. Values are means ± SD fold induction of reporter gene activity of four independent transfections. (b) The Lcn2 promoter activity is slightly increased by HIF-1β, but not by HIF-1α. COS-7 cells were transiently co-transfected in triplicate with the reporter plasmid pGL3-24p3-luc and the indicated expression plasmids for either HIF-1α or HIF-1β or both. Transfected cells were either subjected to hypoxia (1% O_2) for 18 h or kept under normoxic conditions. Values are expressed as means ± SD fold induction of reporter gene activity of 3–4 independent transfections (*$P < 0.05$, ** $P < 0.01$). (c) COS-7 cells were transiently transfected either with empty pcDNA3 vector (lanes 1 and 5) or with increasing amounts of HIF-1α expression plasmid (lanes 2–4 and 6–8). Following transfection, the cells underwent hypoxic treatment (21 h, 1% O_2) (lanes 5–8) or were left untreated (lanes 1–4). Crude nuclear extracts were prepared according to the protocol for the monoclonal HIF-1α antibody (Abcam, Cambridge, UK); loading control: β-actin. The HIF-1α blot clearly shows stabilization/induction of HIF-1α protein following hypoxic treatment (lanes 5–8).

studied, we transfected the cells with the plasmid hypoxia response element (HRE)-luc (kindly provided by Prof. Paul Schumacher, University of Chicago, USA), containing three hypoxia response elements (HIF-1 binding sites) in tandem fused to the luciferase reporter gene. Hypoxia alone led to a 50-fold, overexpression of HIF-1α led to 82-fold, and the combination of hypoxia and HIF-1α overexpression to 177-fold stimulation of the reporter gene activity. Thus, we confirmed that in our experimental system HIF-1α protein was not only stabilized, but also functionally active (data not shown).

Expression of chemokines, chemokine receptors and adhesion molecules in Lcn2 wt and Lcn2$^{-/-}$ hearts during IRI following murine syngeneic heart transplantation

Six pairs of male inbred C57BL/6 Lcn2$^{-/-}$ and Lcn2 wt mice were transplanted in groups with different reperfusion periods (0, 2, 12, 24 and 48 h). To look for possible direct or indirect effects of Lcn2 on the recruitment and infiltration of neutrophils in the reperfused graft, we performed quantitative qPCR assays for chemokines (CCL6, KC, MIP-2, LIX, MCP-1) and chemokine receptors (CXCR2 and CCR2) known to be critical for neutrophil migration [28] as well as for the cytokine IL-6, and the adhesion molecule ICAM-1 in the Lcn2$^{-/-}$ and the Lcn2 wt heart transplantation setting. Significant differences in the gene expression were observed between Lcn2 wt and Lcn2$^{-/-}$ grafts at the various time points analyzed ($P \leq 0.05$; Fig. 2). In the ischemic, nonreperfused groups, expression levels for CCL6, CCR2, MIP-2, and MCP-1 were already significantly higher in the Lcn2 wt than in the Lcn2$^{-/-}$ grafts, indicating that Lcn2 is critical for the expression of chemokines in response to prolonged CIT alone (Fig. 2 a, b, h, i). Accordingly, the expression patterns of the chemokine receptors, CCR2 (MCP-1) and CXCR2 (MIP-2) were strikingly different between the Lcn2$^{-/-}$ and the Lcn2 wt models, but showed a significantly higher expression only for MCP-1 in the wt setting. Most of the analyzed chemokines were expressed significantly higher in the Lcn2 wt setting at the early phase of reperfusion (2 h) ($P \leq 0.05$; Fig. 2a, c, d, f, h).

Correlation between chemokine expression and neutrophil infiltration

A time-dependent increase in infiltrating polymorphonuclear cells (mostly neutrophilic granulocytes) was observed in Lcn2 wt and Lcn2$^{-/-}$ animals ($P \leq 0.001$), with early onset of granulocyte infiltration in the Lcn2 wt setting after 2 h of reperfusion and diffuse infiltration already after 24 h

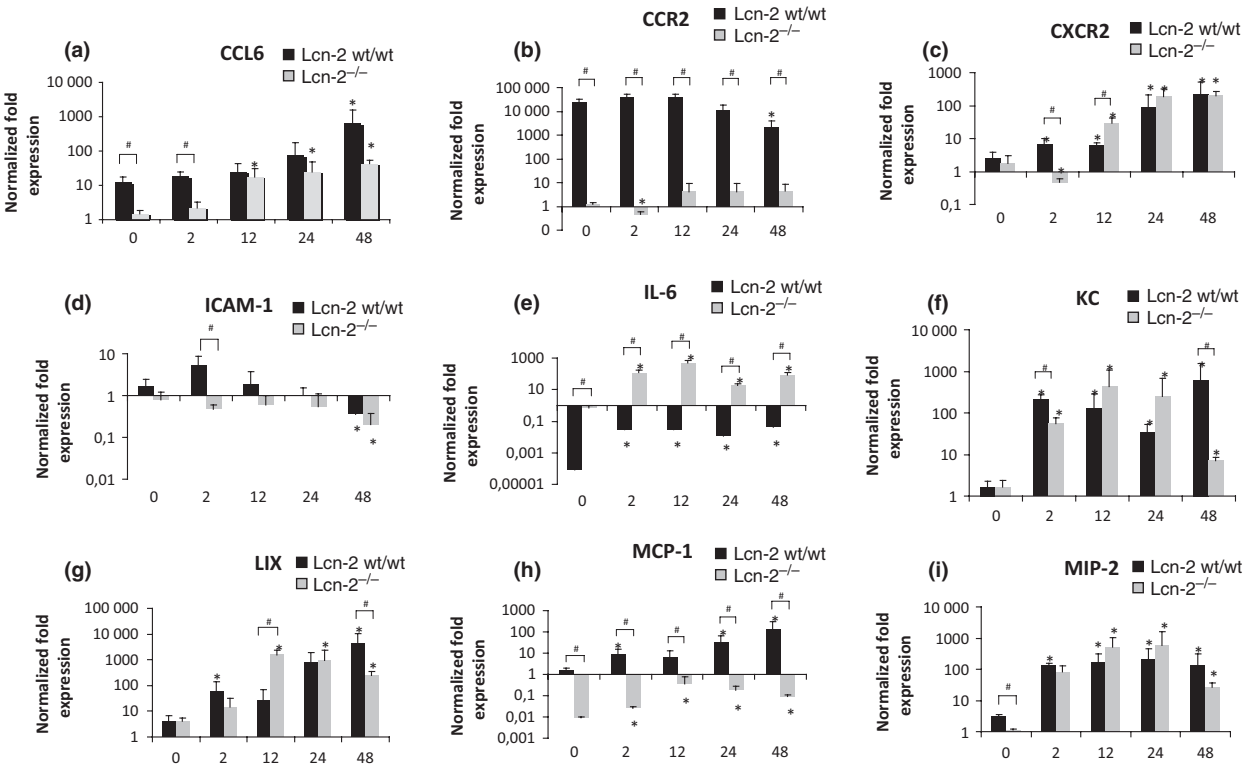

Figure 2 Expression of chemokines, chemokine receptors, and intercellular adhesion molecule in reperfused murine hearts. Quantitative real-time PCR (qRT-PCR) analysis of CCL6 (a), CCR2 (b), CXCR2 (c), ICAM-1 (d), IL-6 (e), KC (f), LIX (g), MCP-1 (h), and MIP-2 (i) in murine Lcn2 wt or Lcn2$^{-/-}$ hearts following ischemia/reperfusion. In each setting, five different groups were included for each reperfusion period (0 h, 2 h, 12 h, 24 h and 48 h). Each group consisted of six mice with a constant cold ischemia time (CIT) of 6 h. In the control group, the animals were sacrificed after 6 h of CIT without reperfusion (t = 0 h). Data were normalized to the housekeeping gene β-actin and are presented as the relative change in each mRNA as compared with the nonreperfused hearts (t = 0 h). Asterisks (*) indicate significant differences ($P \leq 0.05$) between the Lcn2 wt and the Lcn2$^{-/-}$ setting for each time point of reperfusion as compared with the nonreperfused control (t = 0). Hash marks (#) denote significant differences ($P \leq 0.05$) in gene expression between Lcn2 wt and Lcn2$^{-/-}$ hearts for each individual time point.

as compared with the Lcn2$^{-/-}$ setting (Fig. 3). The number of infiltrating neutrophil granulocytes was significantly lower in the absence of Lcn2 ($P < 0.013$) as demonstrated in a previous study [8].

Preliminary data from our pilot study of exogenous administration of rLcn2 in the Lcn2$^{-/-}$ setting revealed a trend toward increase in granulocyte infiltration in the treatment groups although not significantly different (13.5 vs 27.3, 38.5, 15.6 and 10.5 neutrophils/high-power field in the control and groups 1–5, respectively; $P = 0.13$).

In the Lcn2$^{-/-}$ setting, granulocyte infiltration correlated positively with CCL6 ($r = 0.645$, $P = 0.009$) and negatively with ICAM-1 expression ($r = -0.596$, $P = 0.024$). In the Lcn2 wt grafts, granulocyte infiltration correlated positively with expression of CCL6 ($r = 0.661$, $P = 0.007$), CXCR2 ($r = 0.806$, $P = 0.000$), LIX ($r = 0.691$, $P = 0.004$), and MCP1 ($r = 0.744$, $P = 0.004$) and negatively with CCR2 ($r = -0.583$, $P = 0.023$) and ICAM-1 ($r = -0.652$, $P = 0.008$).

Expression of chemokines and chemokine receptors in primary granulocytes from Lcn2 wt and Lcn2$^{-/-}$ mice

We also analyzed the expression of the chemokines and chemokine receptors in primary neutrophil granulocytes isolated from Lcn2 wt and Lcn2$^{-/-}$ mice. As shown in Fig. 4, KC and MCP-1 chemokine mRNAs are abundantly expressed in isolated primary granulocytes from both Lcn2 wt and Lcn2$^{-/-}$ mice, whereas MIP-2 mRNA is expressed to a lesser extent. The mRNA for LIX is weakly expressed but more than 5-fold upregulated in Lcn2$^{-/-}$ granulocytes, whereas expression of the chemokine receptors CXCR2 and CCR2 is significantly lower in Lcn2$^{-/-}$ granulocytes as compared with cells from Lcn2 wt animals.

Apoptosis during IRI following murine syngeneic heart transplantation

Apoptotic cells were detected in transplanted hearts by TUNEL staining in all groups. The mean number of

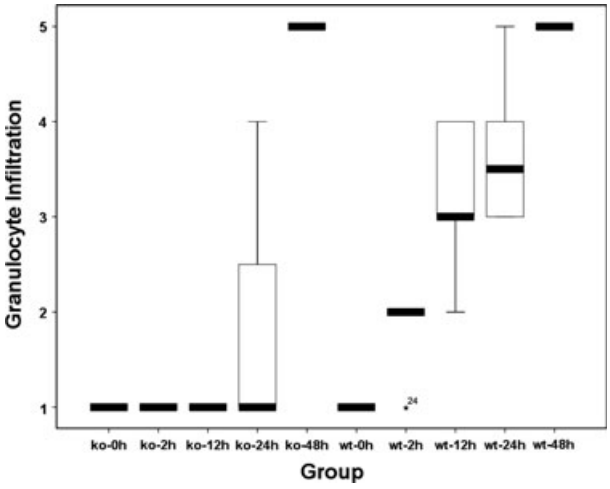

Figure 3 Time-dependent increase in granulocyte infiltration. Box plot of granulocyte infiltration in Lcn2$^{-/-}$ and Lcn2 wt animals at different reperfusion times. Granulocyte infiltration is categorized as 1 (<5 cells), 2 (5–10 cells), 3 (11–20 cells), 4 (21–50 cells), or 5 (>50 cells) and is significantly lower in Lcn2$^{-/-}$ (ko) than in Lcn2 wt animals ($P < 0.013$; Pearson's Chi-Square Test).

apoptotic cells per high-power field was significantly higher ($P = 0.002$) in the Lcn2$^{-/-}$ setting (9.91 ± 1.89) compared with the Lcn2 wt setting (3.55 ± 0.99). At 0 h, 2 h, 12 h, 24 h and 48 h of reperfusion, the mean number of TUNEL-positive nuclei was 0.73 ± 0.15, 16.47 ± 2.35, 16.63 ± 4.92, 9.00 ± 2.01, and 6.73 ± 0.15, respectively, in the Lcn2$^{-/-}$ group and 0.15 ± 0.05, 9.50 ± 4.21, 2.82 ± 0.48, 3.25 ± 0.82, and 2.03 ± 0.36, respectively, in the Lcn2 wt group. The differences between both groups were statistically significant ($P < 0.05$) at 0 h, 12 h, and 48 h. The number of apoptotic cells did not correlate significantly with neutrophil infiltration ($P = 0.847$) in either the Lcn2$^{-/-}$ or the Lcn2 wt setting, nor in the treatment group of rLcn2 administration in the Lcn2$^{-/-}$ setting ($P = 0.663$, data not shown).

Internalization of recombinant Lcn2 by various target cells

Our *in vitro* findings pointed to a modulatory role of Lcn2 on chemokine LIX, adhesion molecule ICAM-1 and chemokine receptor expression in granulocytes. To analyze the susceptibility of possible target cell types, which are also present in the heart transplantation model, COS-7, human endothelial cells HUVEC, MDCK and HL-1 cells were incubated for 3 h with FITC-labeled recombinant human Lcn2. After fixation and washing of the cells, we analyzed the internalization of Lcn2 by confocal fluorescence microscopy. As shown in Fig. 5, Lcn2 is strongly internalized by HUVEC cells and by HL-1 cardiomyocytes, whereas uptake of Lcn2 into COS-7 and MDCK cells was not significant.

Discussion

The results of our reporter gene assays showed that hypoxia had only a mild effect on the Lcn2 promoter, whereas HIF-1β induced it more than 2-fold independent of hypoxia. Induction of Lcn2 expression in mouse liver by anemia and mild hypoxia (10% O_2) has been reported by Jiang *et al.* [28]. It is conceivable that in the cellular system used in our reporter gene assays essential cofactor(s) necessary for efficient induction of the Lcn2 promoter that are present in the liver, such as CBP/p300 and HNF-4, [29,30] are lacking. Altogether, these results suggest a cell/tissue-specific regulation of Lcn2 expression by hypoxia. Consequently, reperfusion causes Lcn2 expression by infiltrating polymorphonuclear cells as already demonstrated in a previous study [8]. Simultaneously, the expression profiles of various chemokines, chemokine receptors, and the adhesion

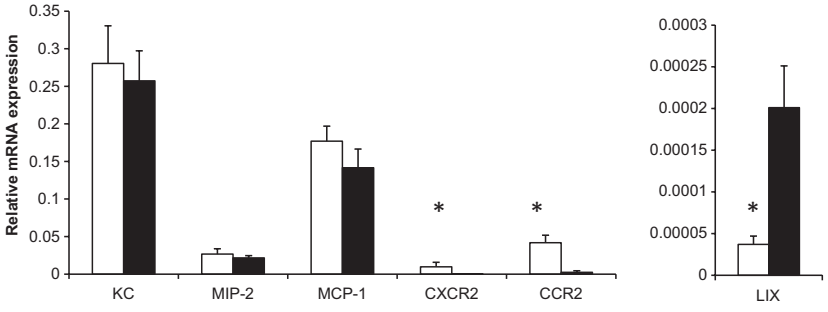

Figure 4 Chemokine and chemokine receptor mRNA expression in primary murine granulocytes. Constitutive expression of the indicated chemokine and chemokine receptor mRNAs in Lcn2 wt and Lcn2$^{-/-}$ granulocytes without hypoxia. Expression levels were normalized to β-actin. White bars indicate Lcn2 wt and black bars Lcn2$^{-/-}$ granulocytes. Asterisks (*) indicate significant differences ($P \leq 0.05$) between the Lcn2 wt and the Lcn2$^{-/-}$ setting.

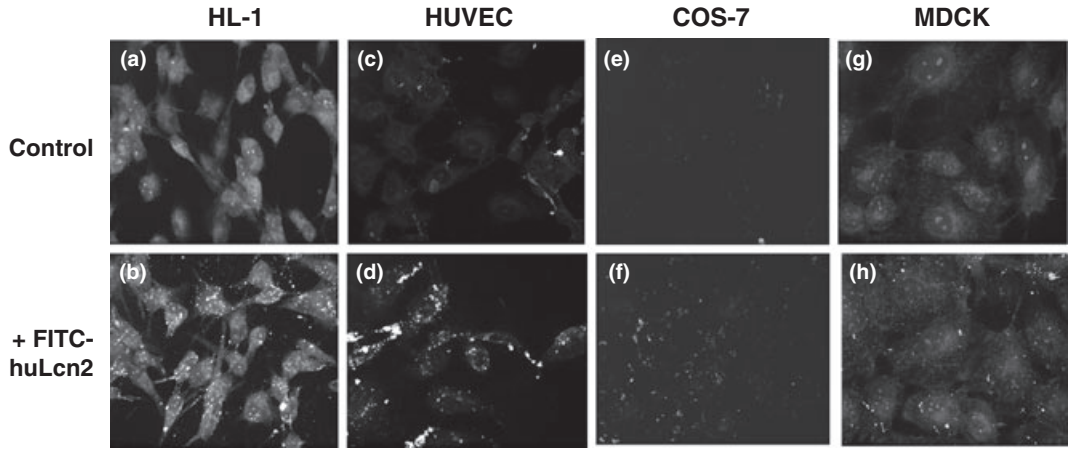

Figure 5 Internalization of recombinant Lcn2 by various cells. FITC-labeled recombinant human Lcn2 (+FITC-Lcn2) or PBS (control) was added to HL-1 cells, human endothelial cells HUVEC, COS-7 and MDCK uptake of FITC-labeled Lcn2 was analyzed by confocal fluorescence microscopy (Olympus IX-70 inverse microscope).

molecule ICAM-1 showed significant differences in Lcn2 wt and Lcn2$^{-/-}$ grafts. Most notably, the correlations between granulocyte infiltration and gene expression profiles differed significantly between the Lcn2$^{-/-}$ and the Lcn2 wt setting. This observation supports our previously proposed model that Lcn2 plays a crucial role in chemoattraction during IRI following murine heart transplantation [8]. Our results presented here support an indirect function of Lcn2 in this process, because the expression of certain chemokines and chemokine receptors was deregulated during ischemia/reperfusion in our Lcn2$^{-/-}$ heart transplant model.

In the early phase of reperfusion (2 h) all genes except LIX and MIP-2 showed significant differences in their expression levels between Lcn2 wt and Lcn2$^{-/-}$ grafts (Fig. 2). Expression of CCR2, IL-6 and MCP-1 differed significantly between Lcn2 wt and Lcn2$^{-/-}$ grafts at all time points with most dramatic differences in the CCR2 expression profile (several 1000-fold higher expression in the Lcn2 wt setting). This expression largely depends on Lcn2, possibly explaining its effect on the recruitment of monocytes and macrophages [31,32].

Our observation of IL-6 upregulation in the Lcn2$^{-/-}$ setting agrees with findings reported earlier [33,34]. Previous studies using experimental models of inflammation demonstrated that IL-6 can suppress neutrophil recruitment *in vivo* [35,36]. As shown by McLoughlin *et al.* [33], IL-6 deficiency was associated with increased neutrophil infiltration following inflammatory stimulation.

Our experiments support a scenario where neutrophils infiltrate the graft during IRI, synthesizing and perhaps releasing Lcn2. Lcn2 binds to and is internalized by cardiomyocytes and endothelial cells, which is supported by our uptake studies (Fig. 5), and inhibits IL-6 expression. Thus, in the Lcn2$^{-/-}$ setting IL-6 expression was increased, thus inhibiting neutrophil recruitment.

The increased number of TUNEL-positive cells observed in Lcn2$^{-/-}$ grafts as compared with that in the Lcn2 wt setting indicates an anti-apoptotic impact of Lcn2 on polymorphonuclear cells. On the other hand, it may be explained by elevated intracellular iron and consecutively increased oxidative stress in Lcn2 deficiency as described recently [37]. This observation is in contrast to a potential pro-apoptotic function of Lcn2 published by others [38–40]. However, also pro-survival activity of Lcn2 has been reported [41–43]. The rate of post-transplant apoptosis in the graft might be influenced by Lcn2 indirectly, the impact of CIT (6 h) was more obvious as basically no change between wt and Lcn2 deficiency was observed in our previous study [8].

According to our uptake studies shown in Fig. 5 and our preliminary results from the administration study, we propose that blocking Lcn2 could be a potential therapeutic approach in terms of regulation of IRI following organ transplantation. Endothelial cells and cardiomyocytes as the predominant donor-specific target cells, showed a remarkable uptake of recombinant Lcn2 (Fig. 5). Our chemokine expression experiments (Fig. 2) indicate that Lcn2 should exert a maximum effect on target cells, predominantly neutrophils, during the early phase of reperfusion. Experiments are in progress to elucidate the optimal application strategy *in vivo*.

Within this study, we examined the effect of Lcn2 on neutrophil recruitment and chemokine secretion. Data from preliminary experiments indicate that application of recombinant Lcn2 increases neutrophil infiltration in the transplanted heart dependent on its iron-binding status. This is also in agreement with published data showing, that

Lcn2 function is closely related iron-binding [44,45]. Whether intra or extracellular iron chelation is relevant to this proposed mode of action needs to be proven in future experiments.

Recent publications on endogenous availability of constitutively produced siderophores demonstrate complex formations with a stress-activated protein, Lcn2, which traffics and clears iron. Features of intracellular iron homeostasis may link the disparate roles of Lcn2 in different diseases [45].

Conclusion

A well-orchestrated regulatory network of gene expression holds the balance between pro- and anti-inflammatory pathways during ischemia and reperfusion. Lcn2 has chemoattractant properties, especially during the early phase of reperfusion.

Authorship

SS, FA: designed research, performed research, collected data, analyzed data, wrote the article. HM: performed research, collected data, analyzed data, wrote the paper. SK, NV, MK, PS, MH, PO: performed research. HS: performed research, analyzed data, revised article, contributed important reagents. SS: designed research, revised article. RM, JT, JP: revised article.

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