Increased immunogenicity is an integral part of the heat shock response following renal ischemia

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Received: 15 October 2010 / Revised: 29 November 2011 / Accepted: 30 November 2011 / Published online: 17 December 2011
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Abstract Renal ischemia increases tubular immunogenicity predisposing to increased risk of kidney allograft rejection. Ischemia–reperfusion not only disrupts cellular homeostasis but also induces the cytoprotective heat shock response that also plays a major role in cellular immune and defense processes. This study therefore tested the hypothesis that upregulation of renal tubular immunogenicity is an integral part of the heat shock response after renal ischemia. Expressions of 70 kDa heat shock protein (Hsp70), major histocompatibility complex (MHC) class II, and intercellular adhesion molecule-1 (ICAM-1) were assessed in normal rat kidney (NRK) cells following ATP depletion (antimycin A for 3 h) and heat (42°C for 24 h). In vitro, transient Hsp70 transfection and heat shock factor-1 (HSF-1) transcription factor decoy treatment were performed. In vivo, ischemic renal cortex was investigated in Sprague–Dawley rats following unilateral renal artery clamping for 45 min and 24 h recovery. Upregulation of Hsp70 was closely and significantly correlated with upregulation of MHC class II and/or ICAM-1 following ATP depletion and heat injury. Bioinformatics analysis searching the TRANSFAC database predicted HSF-1 binding sites in these genes. HSF-1 decoy

significantly reduced the expression of immunogenicity markers in stressed NRK cells. In the in vivo rat model of renal ischemia, concordant upregulation of MHC class II molecules and Hsp70 suggests biological relevance of this link. The results demonstrate that upregulation of renal tubular immunogenicity is an integral part of the heat shock response after renal ischemia. Bioinformatic analysis predicted a molecular link to tubular immunogenicity at the level of the transcription factor HSF-1 that was experimentally verified by HSF-1 decoy treatment. Future studies in HSF-1 knockout mice are needed.

Keywords Renal transplantation · Ischemia · Stress response · Immunogenicity · Heat shock proteins

Introduction

Renal ischemia–reperfusion injury plays an important role in allograft survival after transplantation (de Fijter 2010). Extended cold ischemia time is a risk factor for delayed graft function (Merkus et al. 1991); moreover, renal ischemia increases tubular immunogenicity leading to higher rate of acute and/or chronic rejection (Brockmeyer et al. 1993; Kouwenhoven et al. 2001a, b; Iwaki et al. 1992; Perico et al. 2004).

Ischemia starves cells of oxygen and nutrients, resulting in depletion of ATP, accumulation of waste products, and production of oxygen radicals. Restitution of blood flow with reperfusion sustains renal allograft injury by further generation of free oxygen radicals. Ischemia–reperfusion and the associated oxidative stress not only disrupt cellular homeostasis but also increase the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and major histocompatibility complex (MHC)

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classes I and II in interstitial cells and importantly also in renal tubular cells, thereby increasing immunogenicity and risk for allograft rejection (Mendoza-Carrera et al. 2008; Khazen et al. 2007; Shackleton et al. 1990; Shoskes et al. 1990; Shackleton 1998). However, the direct molecular link between renal ischemia–reperfusion injury and tubular immunogenicity is still unknown.

Renal ischemia–reperfusion induces cellular stress responses such as the heat shock response (HSR) in renal tubular cells (Molitoris 1991; Aufricht et al. 1998a, b; Van Why et al. 1994). We and others have shown activation of heat shock factor-1 (HSF-1) and upregulation of the 70-kDa heat shock protein (Hsp70) in several models of renal ischemia–reperfusion injury and transplantation (Bidmon et al. 2000, 2002; Eickelberg et al. 2002). The HSR fulfills important tasks in cells under normal conditions, such as chaperoning proteins during synthesis, folding, assembly, and degradation. Under stressful conditions, most studies focused on its role of cellular repair and cytoprotection (Riordan et al. 2004; Bidmon et al. 2000, 2004; Aufricht et al. 1998a). However, the HSR also plays a major role in cellular immune and defense processes increasing different markers of immunogenicity, dependent on cell type and stress inducer (Javid et al. 2007; Haug et al. 2007; Young 1990; DeNagel and Pierce 1993; Ito et al. 2001).

Immunogenicity and the HSR may be linked in renal ischemia via specific effector molecules and/or common regulatory mechanisms (Haug et al. 2005, 2007). Antigen processing might represent a special aspect of the chaperoning functions of HSP, and it might be more than coincidence that genes encoding Hsp70 have been mapped in the human MHC next to the loci for complement components and the tumor necrosis factor (Dorak et al. 1993). Wide abundance of HSR inducible genes that are involved in immunologic processes have been previously described in other systems and thus could provide the pathogenic basis for upregulation of tubular immunogenicity following renal ischemia (Moseley 2000; Estruch 2000).

In this study, we tested the hypothesis that the upregulation of renal tubular immunogenicity is an integral part of the HSR after renal ischemia–reperfusion injury. Therefore, we investigated the expression of MHC class II, ICAM-1, and the inducible Hsp70 in renal tubular cells in response to experimental ischemia, heat, and energy deprivation. We aimed to discriminate between the role of Hsp70 versus HSF-1 by applying specific interventions such as transient Hsp70 transfection or HSF-1 transcription factor decoy.

Materials and methods

Standard chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) if not specified otherwise.

Cell culture

Immortalized normal rat kidney (NRK) cells (ATCC CRL-6509, LGC Standards GmbH, Wesel, Germany) were maintained in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, 4.5 g/l glucose, 50 U/ml penicillin, and 50 µg/ml streptomycin as described previously (Madden et al. 2002; Nilakantan et al. 2010). The NRK cell line was established in 1966 by spontaneous immortalization (Best et al. 1999; de Larco and Todaro 1978; Huu et al. 1966). Cultures were kept in 75 cm² plastic dishes (Falcon, BD Bioscience, Franklin Lakes, NJ, USA) or in 12-well plates (Falcon) at 37°C in 5% CO₂ humidified atmosphere and passaged by regular trypsinization. Cells were serum-starved for 12 h before experiments were performed.

Experimental setup

For the cell culture model of ischemia–reperfusion injury, cells were subjected to substrate free media, lacking amino acids and D-glucose, yet containing 0.1 µmol of the mitochondrial inhibitor antimycin A and 100 mg/dl L-glucose to maintain osmolality for 3 h as described previously (van Why et al. 1999). Antimycin A causes ATP depletion and production of the free oxygen radical superoxide through the inhibition of cytochrome c reductase.

For the cell culture model of heat injury, cells were kept at 42°C for 6 or 24 h in a water bath in serum-free growth media as described previously (Aufricht et al. 2001). Cells exposed for 6 h were then allowed to recover in normal growth medium for 18 h. Controls underwent an equivalent number of medium changes and washes.

Transfection experiments

Using total RNA from NRK cells as template, cDNA was generated by reverse transcription PCR. The PCR primers were designed based on a GenBank sequence (BC002453) coding for an inducible human Hsp70. The PCR product was then ligated into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). An appropriate amount of growth medium containing PolyFect transfection reagent (Qiagen, Hilden, Germany) and the DNA transfection complexes (pcDNA3.1-HSP70) or an empty control vector was transferred to the cells and then incubated for 24 h at 37°C and 5% CO₂.

Transcription factor decoy treatment

Inhibition of HSF-1 function was achieved using a transcription factor decoy as previously published (Riordan et al. 2004). The HSF-1 decoy contains the consensus binding

sequence, providing an alternative target for binding. In brief, decoy oligonucleotides were added either to the growth medium at a concentration of 10 µg/500 µl starting 2 h prior to experiment as well as to the injury or the recovery media with a continued presence of decoy oligonucleotides throughout the whole experiments. A decoy oligonucleotide containing a scrambled sequence served as the control. HSF-1 decoy-treated cells as well as Hsp70-transfected cells together with the appropriate controls underwent the *in vitro* experiments described above.

Ischemic injury in an *in vivo* rat model

Animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals and the institutional animal protocol review board approved experimental procedures. All experiments were performed on anesthetized adult male Sprague-Dawley rats. Temperature was monitored and intravenous saline administered. Renal ischemia was accomplished by selective occlusion of the right renal artery and aorta just proximal to the left renal artery. After 45 min, the clamps were removed and reperfusion was visually confirmed. After a reflow interval of 24 h, kidneys were rapidly removed. The left kidney served as non-ischemic control (Aufricht et al. 1998b; Sims et al. 1997). Kidneys were decapsulated immediately after harvest in chilled sterile water. Renal cortex was homogenized in chilled extraction buffer containing 0.1% Triton X-100, 60 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 2 mM trans-1,2-diaminocyclohexane-*N,N,N,N'*-tetraacetic acid, 100 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.75 mg/l leupeptin, and 0.1 mM dithiothreitol, using a Potter-Elvehjem homogenizer (Aufricht et al. 1998b). The homogenate was centrifuged at 680×g and 4°C for 10 min to pellet nuclei and large fragments. The supernatant was used for Western analysis.

Western analysis

Equal amounts of total protein per sample were size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 7.5% and 12.5% acrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes by semidry transfer, and the membranes were blocked in 5% dry milk (Bio-Rad, Hercules, CA, USA) in TBS Tween buffer (150 mM NaCl, 0.05% Tween 20, 10 mM Tris, pH 7.4). The membranes were then incubated for 1.5 h with the primary antibodies against Hsp70 (SPA810, Stress Gen Biotechnologies Corp., Victoria, BC, Canada), ICAM-1 (1A29ab, Abcam, Cambridge, UK), MHC class II (mouse anti-rat MHC II OX6 from Serotec, Oxford, UK), and actin (Sigma-Aldrich). After incubation with secondary horseradish peroxidase-coupled antibodies

(Sigma-Aldrich), detection was accomplished using enhanced chemiluminescence solution (Western Lightning reagent, Perkin Elmer, Boston, MA, USA).

Immunofluorescence staining

Twenty-four hours after energy depletion or heat injury experiments, cells were washed two times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilized for 20 min with 0.1% saponin in PBS. Nonspecific binding sites were blocked for 45 min in 5% FCS and 1% bovine serum albumin in PBS. Hsp70, MHC class II, and ICAM-1 were immunodetected by sequential incubation with the same primary antibodies as described above and Alexa Fluor®680-coupled secondary antibodies (goat anti-rat IgG, A21096, Invitrogen, Carlsbad, CA, USA). Images were acquired with an Olympus AX-70 fluorescent microscope (Olympus Inc, Center Valley, PA, USA).

Flow cytometric analysis

For flow cytometric analysis, NRK cells were detached using Accutase for 20 min at 37°C. Single-cell suspensions were prepared and washed twice in PBS. Aliquots of 250,000 cells were stained with 5 µl fluorescein isothiocyanate-conjugated MHC class II antibody (clone RT1B, AbDserotec MCA46FT) for 30 min on ice. Unstained control samples were left on ice for the same time. After incubation, cells were washed twice with PBS and resuspended in 300 µl PBS prior to the analyses on the BD LSRFortessa (Core Facility Flow Cytometry, Medical University of Vienna, Andreas Spittler). Data were analyzed with BD FACSDiva software. In each run, at least 10,000 gated events were analyzed.

Data analysis

Protein abundance was quantified from the specific immunodensitometric signal determined in the linear range of the protein concentration/ signal intensity relationship and compared to an internal standard. Absolute values of densitometry measurements were normalized to actin. Analysis of variance was used where appropriate. Values for treatment groups were compared to the respective control and considered significantly different if the *p* value was lower than 0.05. Changes are expressed as mean and 95% confidence intervals. MHC class II and ICAM-1 antigen expression was compared to the expression of Hsp70 by bivariate correlation (Pearson correlation).

Promoter analysis

For bioinformatic analysis of the promoter regions of the genes investigated in the in vitro model, the according genes were searched using the ENSEMBL database with the protein specificity of the antibodies described above as the starting point. Nucleotide sequences were downloaded for Hsp70 (HSPA1A, ENSRNOG00000033526), MHC class II (RT1-Bb, ENSRNOG00000032708), and ICAM-1 (ENSRNOG00000020679) as well as for the gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an example for an independent housekeeping gene (ENSRNOG00000018630). Of each of the mentioned genes, a 5'-flanking region, 3,000 bp in length (2,500 bp upstream and 500 bp downstream of the transcriptional starting site), was subjected to TFSearch (<http://mbs.cbrc.jp/research/db/TFSEARCH.html>) searching the TRANSFAC database (Heinemeyer et al. 1998) (threshold score 85.0), and the results were filtered for HSF-1 binding sites.

Results

We found a strong and significant upregulation of Hsp70 in NRK cells following antimycin A treatment (4.50 ± 1.20 -fold, $p < 0.05$) as well as after heat treatment (6.79 ± 1.99 -fold, $p < 0.05$), compared to control conditions (Fig. 1a). Markers of immunogenicity like MHC class II and ICAM-1 were also significantly and strongly upregulated. MHC class II demonstrated a 3.4-fold (3.39 ± 0.76 , $p < 0.05$) increase following antimycin A treatment and a 4.6-fold (4.58 ± 1.40 , $p < 0.05$) increase after heat treatment; ICAM-1 demonstrated a 3.6-fold (3.58 ± 0.88 , $p < 0.05$) increase after antimycin A treatment and a 5.8-fold (5.77 ± 1.21 , $p < 0.05$) increase after heat treatment.

In both in vitro stress models, a strong correlation between Hsp70 and immunogenicity markers can be observed (Fig. 1b). Expression of MHC class II as well as ICAM-1 molecules can be expressed as functions of Hsp70 24 h after antimycin A treatment or heat treatment (MHC class II: $R = 0.941$; $y = 0.599x + 0.554$; $n = 21$; $p < 0.003$) (ICAM-1: $R = 0.943$; $y = 0.731x + 0.469$; $n = 21$; $p < 0.05$).

Hsp70 was upregulated in a perinuclear pattern after heat treatment and in a mainly cytosolic pattern after antimycin A treatment (Fig. 1c). Following strong upregulation after heat treatment as well as after antimycin A treatment, the staining pattern of ICAM-1 revealed a Golgi pattern, while MHC class II showed a more diffuse pattern.

As shown in Fig. 2, flow cytometric analysis further assessed the effect of heat treatment on NRK cell-surface MHC class II expression. Stress-induced MHC class II expression was markedly upregulated from 0.1% basal positivity to 4.3% and finally 58.7% MHC class II-positive cells with more extended heat treatment.

Fig. 1 Markers of the heat shock response and of immunogenicity (MHC class II and ICAM-1) in in vitro stress experiments in normal rat kidney NRK cells following 24 h recovery after 3 h of antimycin A treatment (ATP depletion) as well as after 24 h heat treatment at 42°C, compared to control conditions. The data are obtained from seven cell cultures under each experimental condition. **a** Densitometry and representative Western blots of Hsp70 (upper), MHC class II (middle), and ICAM-1 (lower) demonstrated an upregulation after antimycin A treatment (ATP depletion) and heat treatment. The asterisk indicates a $p < 0.05$. **b** Immunogenicity markers were significantly and closely correlated with the expression of Hsp70, as shown for MHC class II (upper) and for ICAM-1 molecules (lower) after antimycin A treatment (ATP depletion) and heat treatment. The optical densitometry data (OD) were normalized to actin. **c** Immunofluorescence staining of NRK cells shows that Hsp70 (upper) is constitutively expressed (1) and shows marked upregulation under stress conditions, with a mainly perinuclear pattern after heat treatment (2) and a cytosolic pattern after antimycin A treatment (ATP depletion) (3). MHC class II (4) and ICAM-1 (7) have low basal expression rates but demonstrate strong upregulation after heat treatment (5, 8) and after antimycin A treatment (ATP depletion) (6, 9) with a diffuse staining pattern for MHC class II and a Golgi pattern for ICAM-1

Fig. 1 (continued)

As shown in Fig. 3, transient transfection of Hsp70 resulted in a significant 6.9-fold (6.97 ± 2.05 , $p < 0.03$)

upregulation of Hsp70 compared to bare vector treatment. Following transfection, minor changes of immunogenicity

Fig. 2 Cell surface expression of MHC class II in heat-treated NRK cells. Confluent NRK cells were exposed to heat for 6 or 24 h. Cells treated for 6 h were then allowed to recover for 18 h. Control cells were

kept at 37°C in 5% CO₂ humidified atmosphere. Harvested NRK cells were then analyzed by flow cytometry using an anti-MHC II antibody. Presented findings are representative of three separate experiments

markers compared to bare vector-treated cells were found. While MHC class II showed a significant upregulation after transfection with Hsp70 (1.89 ± 0.44 -fold; $p < 0.05$), ICAM-1 showed no significant changes (0.79 ± 0.18 -fold) compared to bare vector treatment.

As shown in Fig. 4, bioinformatic analysis of encoding genes and their promoter regions revealed three transcription factor binding sites (TFBS) for HSF-1 (= the highly specific consensus sequence of the heat shock element) in the coding and regulatory nucleotide sequences of Hsp70 (HSPA1A) as the gold standard for an HSF-1-regulated gene. These consensus sequence patterns were also identified by the search algorithm for MHC class II (two TFBSs found) and ICAM-1 (one TFBS found). No binding site for HSF-1 could be identified for the negative control gene GAPDH in the investigated region, 2,500 bp upstream and 500 bp downstream of the starting point of transcription.

As shown in Fig. 5, HSF decoy treatment resulted in a significant 73% reduction of Hsp70 upregulation after heat treatment (from 6.79- to 1.81-fold control, $p < 0.05$) and a significant 49% reduction after antimycin A treatment (from 4.50- to 2.29-fold control, $p < 0.05$). Similar effects, but to a minor extent, could also be observed for MHC class II expression with an 18% reduction of upregulation after heat treatment (from 4.58- to 3.76-fold control, $p < 0.05$) and a 23% reduction after antimycin A treatment (from 3.39- to 2.61-fold control, $p < 0.05$). The addition of HSF decoy

resulted in a significant 53% reduction of ICAM-1 upregulation after heat treatment (from 5.77- to 2.71-fold control, $p < 0.05$) and a 46% fold reduction after antimycin A treatment (from 3.58- to 1.93-fold control, $p < 0.05$).

In the rat model of renal ischemia, control kidneys showed only low levels of Hsp70 and MHC class II. As shown in Fig. 6a, ischemia resulted in a significant 5.7-fold increase of Hsp70 expression (5.71 ± 4.19 ; $p < 0.03$) in the ischemic kidneys with 24 h of reflow. Expression of MHC class II also showed a significant 3-fold increase in the ischemic kidneys (3.03 ± 1.11 ; $p < 0.03$). In this in vivo ischemic model, a strong correlation between Hsp70 and the immunogenicity marker can be observed (Fig. 6b). Expression of MHC class II molecules can be expressed as functions of Hsp70 24 h after ischemic induction ($R = 0.883$; $y = 0.174x + 0.715$, $n = 18$, $p < 0.003$).

Discussion

Renal tubular ischemia-reperfusion injury has a major impact on acute- and long-term allograft survival in kidney transplantation. In addition to the direct detrimental effects of energy depletion and oxidative stress, ischemia-reperfusion also leads to triggered immunogenicity and thus predisposes to allograft rejection (Brockmeyer et al. 1993; Iwaki et al. 1992; Kouwenhoven et al. 2001a, b; Perico et

Fig. 3 Densitometric analysis of Western blots of markers of the heat shock response and of immunogenicity in normal rat kidney NRK cells following transient transfection. Transfection of *hsp70* (right bars) resulted in a strong upregulation of Hsp70 (upper), mild upregulation of MHC class II (middle), and unchanged expression of ICAM-1 (lower), all compared to bare vector treatment (left bars). The asterisk indicates a $p < 0.05$, and the data are obtained each with 18 experiments

al. 2004). This study clearly demonstrates that increased immunogenicity after ischemia–reperfusion stress is an integral part of renal HSR. Based on studies of MHC class II and ICAM-1 as key markers of renal immunogenicity (Khazen et al. 2007; Mendoza-Carrera et al. 2008; Shackleton 1998; Shoskes and Halloran 1991) and Hsp70 as the key marker and HSF-1 as the key regulator of the renal HSR (Shamovsky and Nudler 2008; Pirkkala et al. 2001), our data show that HSF-1 plays a role in the concordant upregulation

of tubular immunogenicity and HSR, representing a direct molecular link between ischemia–reperfusion injury and renal immunogenicity.

MHC class II is an integral transmembrane protein encoded by polymorphic genes whose role is to present extracellular pathogenic material, degraded in endocytic vesicles, to the cell surface for recognition by lymphocytes (Banu et al. 2002; Haas et al. 1995). The intercellular adhesion molecule ICAM-1 is part of the immunoglobulin superfamily and is crucial for co-stimulation and adhesion of neutrophils, lymphocytes, and monocytes, inducing intracellular signaling followed by secretion of cytokines (Schmal et al. 1998). The tubule cell expression of ICAM-1 is significantly increased after ischemia–reperfusion under such conditions, and targeting ICAM-1 has been shown to ameliorate signs of tissue injury (Phull et al. 2008; Rusai et al. 2008; Kelly et al. 1994; Rabb et al. 1995). Both MHC class II and ICAM-1 are known to be transcriptionally regulated by cytokines, and their tissue distribution is specific and depends on the presence of cytokine receptors, signal transduction pathways, and transcription factors. In this study, we decided to focus on the regulation of these molecules at the level of the renal tubule cell. Since induction of renal tubular HSR is a major response mechanism upon ischemia–reperfusion, we hypothesized that this pathway is a particular attractive candidate to also regulate stress-induced renal tubular immunogenicity at the cellular level.

First, we investigated expression of these markers following exposure of NRK cell cultures to antimycin A treatment or to heat. Whereas antimycin A treatment is a well-accepted in vitro model for the ischemia–reperfusion-associated insults from energy deprivation and oxidative stress, heat exposure represents the gold standard for stress research. As expected from previous studies, proximal tubular epithelial cells increased their expression of MHC class II (Hagerty and Allen 1992; Muller et al. 1989; Wuthrich et al. 1989) and ICAM-1 (Jevnikar et al. 1990; Chow et al. 1992) upon experimental ischemia/oxidative stress. The extent of upregulation of tubular MHC class II molecule was—albeit smaller—shown to be comparable to that on interstitial cells following in vivo ischemia–reperfusion injury (Shoskes et al. 1990). As a new finding, our results showed a significant correlation between the investigated immunogenicity markers ICAM-1 and MHC class II and Hsp70 expression in tubule cells. The reproducibility of these results following heat injury further supports the hypothesis that alterations of renal immunogenicity might be regarded as an integral part of the HSR at the tubular cellular level. For the first time, our study also demonstrated increased surface expression of MHC class II by flow cytometry analysis.

Fig. 4 Promoter analysis of the regulatory regions of the rat Hsp70, ICAM-1, and MHC class II genes. The 5'-flanking regions, 3,000 bp in length (2,500 bp upstream and 500 bp downstream of the transcriptional starting site), were searched for transcription factor binding sites (TFBSs) specific for HSF-1 using the web tool TFSearch. Three TFBSs were found in the Hsp70 (HSPA1A) sequence as the gold standard for an HSF-1 regulated gene, one TFBS was found in ICAM-1, and two TFBSs were found in the MHC class II sequence. No TFBS could be identified in the according region of GAPDH as a possible negative control (sequence not shown). Nucleotide sequences are given with their position relative to the transcriptional starting site. Identified consensus sequences for HSF-1 are *underlined*

Hsp70 (HSPA1A)		3 TFBSs found	
-3500	TAACCAAAAC CACAGAATCT TCACAATCAA AATAGCAAGA TCCCTGATAA	-3450	
	HSF-1		
-600	AAAATGGAGG GAGTGGGGGG CAGACACAGA CAGGCGAGCA TTCCACAGGC	-550	
	HSF-1		
-200	ATTACTCATG GGAGGCGGAG AAGCTCTAAC AGACCCGAAA CTGCTGGAAG	-150	
-150	ATTCTTGCC CCAAGGCCTC CTCCCGCTCG CTGATTGGCC CATGGGAGGG	-100	
	HSF-1		
ICAM-1		1TFBS found	
-1350	GCCGGGTATA TGAATATATT TAAACACTGG GAAAATTCTA TCACTGTCTC	-1300	
	HSF-1		
MHC class II (RT1-Bb)		2 TFBSs found	
-2500	GCTTCAAGCC ACTGGCCTGC AGCCTGCATT TCTTCTGTAA ATGAACITGA	-2450	
-2450	ATCTTCTGAA GTTCAAATGG CGCGTCTGTG CTCGCTCTGC GGCTGCACG	-2400	
	HSF-1		
-200	GGAACAGATA CCTCCAATGT TCATCCAGTG AGGCTCCGTG AAGAAGCAGA	-150	
-150	ACGTGCCCTG CTTAGAGAGC CTTTGTAAC CAAGTCTGCC CAGAGACAGA	-100	
	HSF-1		

In previous *in vivo* studies, the induction of inflammatory mediators and markers by renal ischemia was associated with recruitment of allogeneic-mediated immune cell infiltration (Akca et al. 2009; Kouwenhoven et al. 2001a; Shackleton et al. 1990). Our results add to these data as they prove the “intrinsic” molecular pathogenesis for tubular immunogenicity, without intercellular interactions with immune cells that might have been induced by local injury in the *in vivo* setting.

As next step, we therefore searched for a link between HSR and immunogenicity in the NRK cell culture model. HSR was manipulated via transient transfection to evaluate effects of Hsp70 overexpression on markers of tubular immunogenicity (Bolhassani and Rafati 2008). HSP facilitate uptake, processing, and presentation of HSP-peptide complexes in class I and class II MHC complexes. Furthermore, HSP have immunomodulatory effects contributing to cross-presentation and direct activation of dendritic cells. Membrane-bound HSP on tumor cells interacts with and activates NK cells (Radons and Multhoff 2005). As a result, HSP emerge as potential vaccines in different infectious diseases and cancer (Bolhassani and Rafati 2008). At increased Hsp70 abundances that were comparable to those found with antimycin A treatment or heat treatment, there were only mild increases of MHC class II and no effects on ICAM-1 expression. Effects on MHC class II are likely non-specific, as Hsp70 directly interacts with MHC gene products during their biogenesis to become functional antigen-presenting molecules (Hochstenbach et al. 1992; Bonnerot et al. 1994). Hsp70 is known to guide and assist the proper folding and translocation of complex protein

molecules such as MHC class II within and between subcellular compartments by their chaperoning function. Thus, in our study, the overall weak effect of Hsp70 overexpression on markers of immunogenicity suggested that the regulatory link via HSR might rather be upstream of the effector HSP, such as a common regulatory cellular mechanism.

The HSR is known to be mainly regulated at the transcriptional level with HSF-1 as major transcription factor to control stress induced expression of heat shock genes (Shamovsky and Nudler 2008; Pirkkala et al. 2001). During ischemia-reperfusion, HSF-1 is activated by multiple factors, such as increased levels of unfolded proteins, acidosis, decreased levels of high-energy phosphate compounds caused by ischemia (Benjamin et al. 1992), or alterations in the redox state resulting from oxidative stress (Paroo et al. 2002). Activation of HSF-1 then results in DNA binding to the so-called heat shock elements in the promoter region of heat shock genes as pre-requisite for transcriptional activity, whereas artificial oversupply of non-functional oligonucleotides containing the heat shock element base sequence (HSF decoy) effectively blocks expression of these genes (Riordan et al. 2004). In addition to the classical HSP genes, several other key mediators in inflammation and host defense, such as immunophilins, ABC transporter, inducible nitric oxide synthase, and cytokines like IL-7 and tumor necrosis factor (TNF)- α , have also been shown to be stress inducible (Perrot-Applanat et al. 1995; Fruman et al. 1994; Srivastava 1993; Young 1990; Goldring et al. 2000) and to contain heat shock elements in their promoter region. To our knowledge,

Fig. 5 Densitometric analysis of Western blots of markers of the heat shock response and of immunogenicity in *in vitro* stress experiments in NRK cells. Treatment with HSF decoy resulted in no effects under control conditions, but in consistent reduction of upregulation of Hsp70 (*upper*), MHC class II (*middle*), and ICAM-1 (*lower*) following 24 h recovery after 3 h of antimycin A treatment (ATP depletion) as well as after 24 h heat treatment at 42°C. The *asterisk* indicates a $p < 0.05$ compared to unstressed controls; the data are obtained each with 18 experiments

neither MHC class II nor ICAM-1 has previously been reported to be regulated by HSF-1.

To elucidate the molecular mechanism of the common joint regulation of the investigated marker proteins, we performed a bioinformatic analysis of the encoding genes and their promoter regions. Coding and regulatory nucleotide sequences of immunogenicity markers were analyzed in comparison to Hsp70 as the gold standard for an HSF-1-regulated gene and to GAPDH as negative control. This *in silico* analysis demonstrated that MHC class II and ICAM-1 contain highly specific consensus sequence patterns as putative transcription factor binding sites for HSF-1,

suggesting a regulatory link at the transcription factor level. Indeed, experimental blockade of HSF-1 binding by addition of HSF decoy containing the proposed binding site not only reduced Hsp70 expression in stressed renal tubular cells but also reduced expression of MHC class II and ICAM-1 following antimycin A or heat treatment. Interestingly, effects of decoy were more pronounced after heat than after antimycin A treatment and affected expression of ICAM-1 stronger than that of MHC class II, likely reflecting the respective impact of HSR on protein expression. These results suggest that HSF-1 represents a direct molecular mechanistic link between the HSR and tubular markers of

Fig. 6 Markers of the heat shock response and of immunogenicity in rat renal cortex homogenates from nine kidneys obtained at 24 h of reflow after 45 min of unilateral renal ischemia and from nine contralateral control kidneys in the in vivo model of renal ischemia. **a** Densitometry and representative Western blots of Hsp70 (*upper*) and MHC class II (*lower*) demonstrated upregulation. The optical densitometry data (*OD*) were normalized to actin. The *asterisk* represents a significant difference with a $p < 0.05$. **b** The immunogenicity marker MHC class II was significantly and closely correlated with the expression of Hsp70

immunogenicity and that HSF-1 is not the only regulator of MHC class II and ICAM-1 expressions under these

conditions. Indeed at least four additional regulating factors are known for MHC class II (Reith et al. 2005) and at least

two more factors for ICAM-1 (Niessen et al. 2002; Chen 2006). Therefore, blocking the HSR via HSF-1 decoy treatment caused a significant decrease, but did not completely abolish the expression of MHC class II and ICAM-1.

To test the biological relevance of these findings, the final part of our study investigated whether HSR was also related to upregulation of renal immunogenicity in the rat model of renal ischemia–reperfusion injury. In this model, independent studies have previously demonstrated activation of HSF-1 (Eickelberg et al. 2002; Van Why et al. 1994), upregulation of Hsp70 (Kelly et al. 2001; Bidmon et al. 2000), Hsp25 (Smoyer et al. 2000; Aufricht et al. 1998b), and of MHC class II molecules (Shackleton 1998; Shackleton et al. 1990). Our study describes a strong correlation between the expression of Hsp70 and MHC class II following renal ischemia. These results support that the HSR represents the link by which ischemic injury increases tubular immunogenicity in vivo and might thus predispose the allograft to the development of rejection (Iwaki et al. 1992; Kouwenhoven et al. 2001a, b; Brockmeyer et al. 1993). Furthermore, Hsp70, as biomarker of the ischemic HSR, could be tested for its predictive value for acute rejection of the renal graft (Vargha et al. 2005).

In this study, we primarily focused on regulation of immunogenicity marker protein expression at the (isolated) tubule cell level. Therefore, effects of HSR/HSF-1 on highly relevant upstream mechanisms such as the interplay of other organs, additional cellular populations (endothelial cells, immune cells), and cytokines were not examined. The effects of HSF-1 on these mediators of postischemic inflammation, however, are not trivial to predict: For example, IL-1 β (Cahill et al. 1996) and TNF- α (Fashena et al. 1990) have been shown to contain heat shock elements in their promoter regions in macrophages. IL-1 β and TNF- α were repressed by HSF-1 in macrophages, and plasma levels of TNF- α were higher in HSF-/- mice after LPS treatment (Xiao et al. 1999). Interestingly, expression of MHC class II and ICAM-1 is known to be upregulated by IL-1 β and/or TNF- α (Kittur et al. 2002). Therefore, these reports suggest that activation of HSF-1 may differentially influence immunogenicity dependent on the investigated/involved target cell and/or organ. It is likely that the current findings have application beyond the kidney. Interestingly, for example, organ-specific renal overexpression of HSP has been recently shown to be cytoprotective in ischemia–reperfusion injury—but global overexpression of the same HSP in a transgenic animal was associated with increased inflammatory responses and organ damage (Kim et al. 2010). Thus, our observations on the renal tubule cell level clearly need additional studies to be transferable to the whole kidney, then to other organs, and finally to the complex interplay with the immune system.

Taken together, our study demonstrates that upregulation of renal tubular immunogenicity is an integral part of the HSR, in particular after renal ischemia–reperfusion. Changes of the expression of inducible Hsp70 were correlated with the expression of MHC class II and ICAM-1 following insults such as heat or antimycin A treatment. Manipulation of the HSR by means of HSP transfection and HSF-1 decoy elucidated a molecular link to tubular immunogenicity at the level of the transcription factor HSF-1 that was predicted by bioinformatics analysis. In the in vivo rat model of renal ischemia–reperfusion, concordant upregulation of MHC class II molecules and Hsp70 further suggests biological relevance. Future studies in knockout animal models for HSF-1 are needed to prove the functional interdependence in the in vivo setting.

Acknowledgments The authors would like to thank Michael Riordan for valuable comments and by courtesy of HSF-1 transcription factor decoy. We would like to thank Andreas Spittler for the possibility to let us perform our analyses on the LSR Fortessa in his FACS Facility.

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