

# Suppressed heat shock protein response in the kidney of exercise-trained diabetic rats

J. Lappalainen<sup>1</sup> | N. K. J. Oksala<sup>1,2</sup> | D. E. Laaksonen<sup>1,3</sup> | S. Khanna<sup>4</sup> |  
T. Kokkola<sup>5</sup> | K. Kaarniranta<sup>6</sup> | C. K. Sen<sup>4</sup> | M. Atalay<sup>1</sup>

<sup>1</sup>Institute of Biomedicine, Physiology, University of Eastern Finland, Kuopio, Finland

<sup>2</sup>Faculty of Medicine and Life Sciences, Division of Vascular Surgery, University of Tampere, Tampere University Hospital, Tampere, Finland

<sup>3</sup>Department of Internal Medicine, Kuopio University Hospital, Kuopio, Finland

<sup>4</sup>Department of Surgery, Davis Heart & Lung Research Institute, The Ohio State University Medical Center, Columbus, OH, USA

<sup>5</sup>Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland

<sup>6</sup>Department of Ophthalmology, University of Eastern Finland and Kuopio University Hospital, Kuopio, Finland

## Correspondence

Mustafa Atalay, Institute of Biomedicine, Physiology, University of Eastern Finland, Kuopio, Finland.  
Email: mustafa.atalay@uef.fi

## Funding information

Supported by the grants from the Finnish Ministry of Education; Juho Vainio and Yrjö Jahnsson Foundations; High Technology Foundation of Eastern Finland; COST actions TD1304 and CA16112; Emil Aaltonen Foundation

Impaired expression of heat shock proteins (HSPs) and increased oxidative stress may contribute to the pathophysiology of diabetes by disrupted tissue protection. Acute exercise induces oxidative stress, whereas exercise training up-regulates endogenous antioxidant defenses and HSP expression. Although diabetic nephropathy is a major contributor to diabetic morbidity, information regarding the effect of HSPs on kidney protection is limited. This study evaluated the effects of eight-week exercise training on kidney HSP expression and markers of oxidative stress at rest and after acute exercise in rats with or without streptozotocin-induced diabetes. Induction of diabetes increased DNA-binding activity of heat shock factor-1, but decreased the expression of HSP72, HSP60, and HSP90. The inflammatory markers IL-6 and TNF-alpha were increased in the kidney tissue of diabetic animals. Both exercise training and acute exercise increased HSP72 and HSP90 protein levels only in non-diabetic rats. On the other hand, exercise training appeared to reverse the diabetes-induced histological changes together with decreased expression of TGF-beta as a key inducer of glomerulosclerosis, and decreased levels of IL-6 and TNF-alpha. Notably, HSP72 and TGF-beta were negatively correlated. In conclusion, impaired HSP defense seems to contribute to kidney injury vulnerability in diabetes and exercise training does not up-regulate kidney HSP expression despite the improvements in histopathological and inflammatory markers.

## KEYWORDS

exercise, heat shock proteins, kidney, oxidative stress, training

## 1 | INTRODUCTION

Heat shock proteins (HSPs) are a major component of the cellular stress response to maintain protein homeostasis, to decrease injury, and to accelerate regeneration. HSPs protect against a number of threats and pathological conditions, including diabetes mellitus and its complications.<sup>1</sup> There is a high metabolic energy demand of the kidney,<sup>2</sup> and the diabetic kidney is subjected to metabolic load as a consequence

of alterations in pH, blood glucose, osmolarity, and fatty acid turnover.<sup>3,4</sup> All the above-mentioned challenges will disrupt protein turnover in diabetic kidney and increase the demand for enhanced HSP protection. Moreover, increasing evidence supports a protective role for HSPs during recovery from renal ischemia, in particular in cellular salvage from apoptotic cell death and cytoskeletal restoration.<sup>2,5</sup>

At physiological concentrations, reactive oxygen species (ROS) act as regulatory mediators in signaling processes.<sup>6</sup>

On the other hand, uncontrolled oxidative stress, a state where the increased production of ROS overwhelms endogenous antioxidant protection, disrupts redox signaling and contributes in the pathophysiology of several common diseases.<sup>7</sup> Increased oxidative stress and impaired cellular defenses are also associated with the pathogenesis of diabetes mellitus and its complications including nephropathy.<sup>7,8</sup>

Regular exercise is in many ways beneficial to patients with diabetes, and also recommended for patients with chronic kidney disease.<sup>9</sup> Acute exercise transiently decreases renal blood flow,<sup>5</sup> while chronic exercise normalizes this response.<sup>10</sup> In addition, physical exercise causes a varying extent of oxidant insult to tissues and is expected to result both negative (acute damage) and positive (adaptation) effects.<sup>7</sup> For this extent, exercise training may serve as an approach to offset oxidative stress,<sup>11</sup> to induce HSP responses,<sup>12</sup> and to improve tissue protection.<sup>13</sup>

The impact of HSP expression on whole-body glucose tolerance stresses a role of HSPs in skeletal muscle insulin action.<sup>14</sup> Defects in the function of HSPs could contribute to diabetic complications including nephropathy.<sup>15</sup> Among the many physiological, pharmacological, and genetic approaches that have been postulated to address HSP-targeted therapies in both experimental and clinical setting,<sup>1</sup> physical exercise provides a physiological stimulus of heat shock factor-1 (HSF-1), which translocates to the nucleus upon activation and binds to heat shock elements resulting in increased HSP expression.<sup>16,17</sup> HSPs potentially act in buffering tissues against disease-associated homeostatic disturbance and thereby contribute to the positive biological effects in diabetes.

While diabetes remains a major risk factor to kidney complications,<sup>18</sup> regular exercise training may act as a therapeutic tool to restore tissue HSP-mediated protection.<sup>16</sup> However, information about the protective effect of HSPs on kidney tissue is limited, and to our knowledge, the effects of exercise training on kidney HSP expression in diabetes have not been examined. Thus, this study tested the hypothesis that endurance exercise training can decrease oxidative stress, reduce diabetes-associated kidney injuries as evidenced by histological and biochemical changes, and enhance tissue protection in part through HSP induction. Notably, we utilized acute exhaustive exercise as a physiological inducer of metabolic stress to test acute responses of HSPs and oxidative stress.

## 2 | METHODS

### 2.1 | Animals

The animals were twelve-week-old male outbred Wistar rats that were maintained at  $22 \pm 2^\circ\text{C}$  with 12:12-h light–dark cycles with free access to standard rat chow and water. The experimental procedure was in accordance with the Guide for

the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985), and was approved by the Ethics Committee for the laboratory animal research of University of Eastern Finland. One-half of the rats were randomly assigned to the diabetic group, which was induced by streptozotocin as described below. The other half was kept as the control group. Rats with sustained diabetes and the non-diabetic control rats were further divided into training and non-training groups. Half of the rats from all groups were sacrificed by decapitation at rest or approximately 72 h after the last exercise training session, and the other half immediately after acute exhaustive exercise. After exclusions (no induction of diabetes, infection, and injury due to treadmill running), the groups consisted of 7–10 animals.

#### 2.1.1 | Preparation of diabetic rats

Streptozotocin destroys pancreatic beta cells and is used in experimental models of type 1 diabetes.<sup>19</sup> In this study, a single intraperitoneal streptozotocin injection (60 mg/kg, prepared in 0.1 mol/L citrate buffer, pH 4.5) was used as described.<sup>16</sup> The state of diabetes was confirmed by glucosuria using dipstick urine test (Combur, Roche Diagnostics, France) 1 week after the injection. Urine test was repeated once a week during the study for detection of glucosuria. Proteinuria was detected using dipstick test (Roche). In addition, blood glucose levels were measured at the end of the study in truncal blood collected immediately after decapitation using a commercial kit (Gluco-quant Glucose/HK, Roche Diagnostics). Rats without sustained diabetes indicated by glucosuria at least 20 mmol/L 14 days after the injection till the end of the study were excluded from the study.

#### 2.1.2 | Exercise training and acute exercise protocol

The rats subjected to exercise training ran on a treadmill for 8 weeks, 5 days a week. Briefly, after 1 week of familiarizing the rats to the treadmill system, the non-diabetic animals started their training from 1.08 km/h, 30 min/d, with gradual increases in training speed and duration until reaching 1.08 km/h for 1.5 h/d by the end of the first week and 1.8 km/h 1.5 h/d until week 4, and continued training 5 days a week 1.8 km/h 1.5 h/d for 4 weeks. In diabetic rats, similar training duration was used with slightly lower intensity due to observed slightly lower body weight (Table 1) and endurance capacity, which was determined according to the continuous monitoring of the optimal running intensities that all the diabetic rats were able to tolerate. More specifically, the exercise training for the diabetic animals started from 1.08 km/h for 30 min/d for week 1 and then gradually increased until 1.45 km/h, 1.5 h/d was reached by week 4, and continued at this intensity (1.45 km/h, 1.5 h/d) for 4 weeks.

Experimental group	Body weight at week 8 (g)	Blood glucose (mmol/L)	Citrate synthase activity	
			VL (nmol/min/mg protein)	RG (nmol/min/mg protein)
Control rest	418 (18)	8.79 (0.65)	125 (10)	328 (44)
Control Tr rest	370 <sup>††</sup> (27)	8.17 (0.25)	149 <sup>†</sup> (18)	426 <sup>†††</sup> (16)
Diabetes rest	296 <sup>***</sup> (40)	18.19 <sup>***</sup> (4.22)	92 <sup>**</sup> (10)	233 <sup>*</sup> (15)
Diabetes Tr rest	281 <sup>***,††</sup> (53)	14.76 <sup>***,†</sup> (4.69)	110 <sup>**</sup> (9)	406 <sup>*,†††</sup> (35)

Data for citrate synthase activity were previously presented.<sup>15</sup>

Data are presented as mean (SD).

VL; vastus lateralis, RG: red gastrocnemius muscles.

Difference due to diabetes (\* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ).

Effect of training (<sup>†</sup> $P < .05$ , <sup>††</sup> $P < .01$ , <sup>†††</sup> $P < .001$ ).

The animals appeared to respond to the training well and were able to increase their running distance and intensity according to the training protocol. The efficiency of the exercise protocol was confirmed by citrate synthase (CS) activity as marker of oxidative metabolism in the exercised skeletal muscle (vastus lateralis and red gastrocnemius muscles) (Table 1) and measured as previously described.<sup>16</sup> Total protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL).

At the end of the exercise training period, the animals randomly selected to the acute exercise protocol ran until exhaustion in a single exercise session in order to induce metabolic stress. For this experiment, the running speed was kept 1 km/h (10% uphill gradient) for the first 10 minutes, and then gradually increased to 2.1 km/h until exhaustion. A detailed protocol is provided in the Data S1.

### 2.1.3 | Sample collection

Following decapitation, the kidneys of the animals were quickly removed, rinsed in ice-cold saline and blotted, cut into small pieces and placed in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  for later homogenization and biochemical determinations.

## 2.2 | Histology

Kidney samples were fixed overnight with 4% formalin solution buffered with sodium phosphate at  $5^{\circ}\text{C}$ . Routine paraffin-embedding and tissue processing were performed. Sections of 3  $\mu\text{m}$  thickness were mounted on glass slides and stained with hematoxylin–eosin. Histological samples from six randomly selected rats from each study group were analyzed and scored according to Renal Pathology Society (RPS) classification of diabetic nephropathy<sup>20</sup> as follows: no injury = score 0, class I: glomerular basement membrane thickening = score 1; class IIa: mild mesangial expansion = score 2; class IIb: severe mesangial expansion = score

**TABLE 1** Effect of streptozotocin-induced diabetes and 8 wk of exercise training (Tr) on body weight, blood glucose, and citrate synthase activity

3; class III: nodular sclerosis = score 4; and class IV: global glomerulosclerosis in  $>50\%$  of glomeruli = score 5.

### 2.3 | Preparation of samples for electrophoresis and subsequent Western blot analysis

The samples were pulverized under liquid nitrogen and sonicated in a buffer containing 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L  $\text{MgCl}_2$ , 0.2 mmol/L EDTA, 20 mmol/L HEPES, 5  $\mu\text{mol/L}$  DTT, and 5  $\mu\text{mol/L}$  PMSF at  $4^{\circ}\text{C}$ .

Next, protein extracts (30  $\mu\text{g}$  protein per lane) were electrophoresed together with molecular weight markers on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, MA). Equal loading and transfer of the Western blot samples were further verified and compared with beta-actin and by reversible total protein staining of the nitrocellulose membrane using Ponceau S.

The above-mentioned pulverized samples were then analyzed for HSP expressions by Western blotting.<sup>16,21</sup> Briefly, after blocking with a 5% fat-free milk solution at  $37^{\circ}\text{C}$  for 60 minutes, the membranes were treated with 1:1000 diluted monoclonal antibodies (all from StressGen, Victoria, Canada) recognizing heme oxygenase-1 (HO-1), the 60 kDa HSP (HSP60), the 72 kDa inducible form of HSP (HSP72), the 90 kDa HSP (HSP90), and glucose-regulated protein 75 (GRP75). Horseradish peroxidase (HRP)-conjugated anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rat immunoglobulins (Zymed Laboratories, San Francisco, CA) secondary antibodies were used in 1:10 000 dilution. For data normalization, mouse monoclonal antibody to beta-actin (Sigma, St. Louis, MO) was used as an endogenous control.

The membranes were developed with the enhanced chemiluminescence method (NEN Life Sciences, Boston, MA), and the signals were detected by X-ray film (Kodak) and quantified using image-analysis software (Scion Corp, Frederick, MD). An external reference sample was included

in each membrane to calibrate variations in film exposure times. The normalized data was expressed relative to the untrained non-diabetic group.

## 2.4 | Analysis of protein carbonyls and 4-HNE as markers of oxidative damage

For protein carbonyl measurements, the pulverized kidneys samples described above were first derivatized with 2,4-dinitrophenylhydrazine, subjected to electrophoretic separation (30 µg protein per lane), and detected by Western blotting.<sup>16,21</sup> Rat monoclonal antibodies against 2,4-dinitrophenyl and anti-rat immunoglobulins (Zymed Laboratories) were used as primary and secondary antibodies, respectively. Measurement of 4-hydroxy-2-nonenal (4-HNE) protein adducts was taken as described<sup>16,21</sup> using rabbit 4-HNE anti-serum (Alpha Diagnostics, San Antonio, TX) and HRP-conjugated anti-rabbit immunoglobulins (Santa Cruz Biotechnology) as secondary antibody. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

## 2.5 | Analysis of inflammatory markers TGF-β, IL-6 and TNF-α

TGF-β was used as a marker of glomerulosclerosis<sup>22</sup> and IL-6 and TNF-α served as general inflammatory markers. Their levels were measured from the supernatants of kidney homogenates by commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The results were normalized to the total protein concentration of tissue homogenates.

## 2.6 | Analysis of gene expression

The mRNA expression of HSP60, HSP72, HSP90, GRP75, HO-1, and beta actin (ACTB) in kidney tissue was analyzed by quantitative real-time RT-PCR. Briefly, 100 mg of tissue was first homogenized with Ultra-Turrax and total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). RNA concentrations were determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the integrity of the RNA was checked with gel electrophoresis. One microgram of total RNA from each sample was then converted to cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT) primers (Promega, Madison, WI). For PCR primer design, the annotated nucleotide sequences were retrieved from GenBank database (National Center for Biotechnology Information, Bethesda, MD) and designed not to amplify genomic DNA. The primer sequences were as follows: HSP60 forward primer (-F) 5'-AAAGCTGAACGAGCGACTTG-3' and reverse primer

(-R) 5'-ATCACTTGTCCTCCAACCTTC-3'; HSP72-F 5'-GGACAAGTCGGAGAACG-3' and HSP72-R 5'-CCGA GTAGGTGGTGAAG-3'; HSP90-F 5'-GTACGAAACA GCACTCCTGTCTTC-3' and HSP90-R 5'-ATCCTCATCA ATACCTAGACCAAGC-3'; GRP75-F 5'-ACGAGGATG CCAAGGTTC-3' and GRP75-R 5'-TGAATGGCAGCTC CAATGG-3'; HO-1-F 5'-GGAAGGCTTTAAGCTGGTG ATG-3' and HO-1-R 5'-GGTTCTGCTTGTTCGCTCT ATC-3'; ACTB-F 5'-AGCCTTCCTTCCTGGGTATG -3' and ACTB-R 5'-CGGATGTCAACGTCACTTC -3'.

The samples were amplified in duplicate using Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA) with 200 nM of each gene-specific primer, and run on Mx3000P System (Stratagene) with the following program: a 10 minutes pre-incubation at 95°C, followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 59°C and 25 seconds at 72°C. The data were normalized relative to expression of ACTB using the previously introduced algorithm.<sup>23</sup> Unique amplification products and the absence of primer-dimers were evaluated by melt curve analysis.

## 2.7 | Analysis of HSF-1 DNA-binding activity

For the measurement of HSF-1 DNA-binding activity, an electrophoretic mobility shift assay (EMSA) was performed as previously described.<sup>16</sup> The protein extracts were prepared similar to Western blot analysis and mixed with <sup>32</sup>P-labeled probes corresponding to the two overlapping heat shock elements. The protein-DNA complexes were resolved on a non-denaturing polyacrylamide gel, the EMSA gels were dried, and the radioactivity was detected by autoradiography.

## 2.8 | Statistics

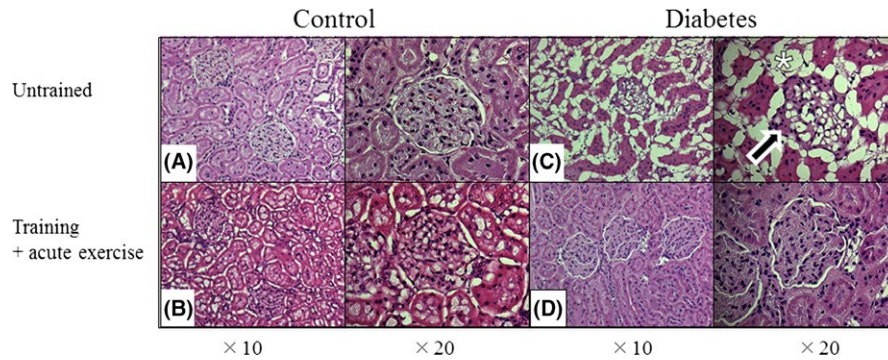
The effect of diabetes, acute exercise and exercise training on measured variables was tested by two- or three-way ANOVA with Bonferroni's correction, when appropriate. Pearson's and Spearman's correlation coefficients were used for the detection of associations between HSPs, TGF-β and the inflammation-related cytokines. Statistical significance was set at  $P < .05$ . Data are shown as means ± SEM, or means ± SD when appropriate.

# 3 | RESULTS

## 3.1 | Effects of experimental diabetes

Induction of experimental diabetes resulted in elevated blood glucose (Table 1) and characteristic kidney injury, particularly glomerulosclerosis (see Figure 1 and Table 2). Kidney homogenates from diabetic animals contained increased





**FIGURE 1** Representative images of kidney histology in non-diabetic rat (A); non-diabetic exercise-trained rat after acute exercise (B); streptozotocin-induced diabetes (Diabetes) (C); exercise-trained diabetic rat after acute exercise (D). Stained with hematoxylin–eosin. Magnifications are 10× and 20×. Asterisks indicate tubular dilatation and arrows indicate mesangial expansion

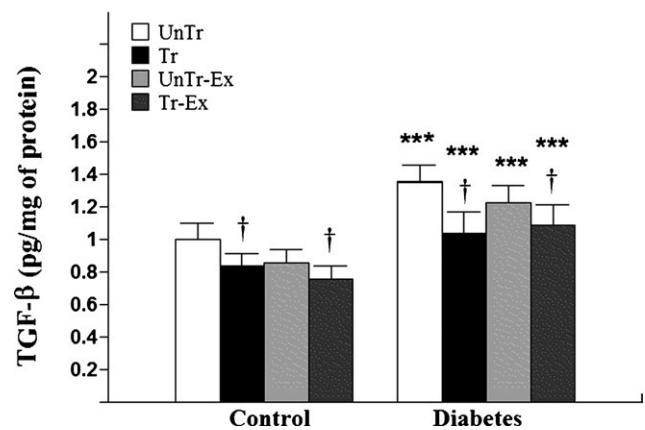
**TABLE 2** Effect of streptozotocin-induced diabetes, 8 wk of exercise training (Tr), and acute exercise on glomerular histology according to classification of diabetic nephropathy

Experimental group	Histological injury score
Control rest	0.0 (0.0)
Control Tr rest	0.3 (0.2)
Control + exercise	0.0 (0.0)
Control Tr + exercise	0.2 (0.2)
Diabetes rest	3.8 (0.2)
Diabetes Tr rest	1.7 <sup>†</sup> (0.2)
Diabetes + exercise	3.8 (0.4)
Diabetes Tr + exercise	2.0 <sup>†</sup> (0.3)

Data are presented as mean (SEM).  
Effect of exercise training (<sup>†</sup> $P < .05$ ).

levels of TGF- $\beta$  ( $P < .001$ , Figure 2) and IL-6 and TNF- $\alpha$  ( $P < .01$  and  $P < .05$ , respectively, Table 3). Moreover, a significant correlation between TGF- $\beta$  and TNF- $\alpha$  was found in diabetic animals ( $R = .892$ ,  $P = .017$ ). Using a graded dipstick test (scaled as follows: +, ++, +++, +++++), all diabetic animals were tested positive for proteinuria as an early marker of glomerular filtration membrane pathology. More specifically, 50% of the animals were grade ++ and 50% were grade +++. Exercise training had no effect on proteinuria results as the proportion of the aforementioned grade remained identical in untrained and exercise-trained animals (50%/50% for grade ++/+). Dipstick test for glucosuria was positive (+++++) only in diabetic animals.

Despite the increased HSF-1 DNA-binding activity ( $P < .001$ , Figure 3), induction of diabetes decreased HSP72 protein levels ( $P < .01$ , Figure 4). Diabetes also decreased HSP90, but increased GRP75 and HSP60 protein levels although HSP60 mRNA expression did not reflect the protein levels (Figure 4). Moreover, diabetes increased HO-1 mRNA expression ( $P < .001$ ) although the respective protein levels



**FIGURE 2** Effect of streptozotocin-induced diabetes, acute exercise (Ex), and 8 wk of exercise training (Tr) on kidney levels of transforming growth factor beta (TGF- $\beta$ ). Open bars represent untrained rats (UnTr); closed bars represent exercise-trained (Tr) rats; light gray bars represent untrained rats after acute exercise (UnTr-Ex); dark gray bars are exercise-trained rats after acute exercise (Tr-Ex). Each experimental group consisted of 7–10 animals. Values are per mg of tissue and expressed as mean  $\pm$  SEM. Difference due to diabetes: \*\*\* $P < .001$ ; difference due to exercise training: <sup>†</sup> $P < .05$

were decreased ( $P < .05$ , Figure 4). Overall our findings may point out a compromised tissue protection and altered HSP response in diabetic kidney.

### 3.2 | Effects of acute exercise

The running time until exhaustion in the acute exercise model was longer in diabetic animals compared to non-diabetic rats ( $168.5 \pm 31.75$  vs  $137.5 \pm 9.40$  minutes,  $P < .05$ ). Due to the different physical capacities of the experimental groups of animals, varying increments in exercise intensity were applied till running exhaustion was achieved for non-diabetic and diabetic animals, as well as in trained and untrained animals (see Data S1 for exercise protocol).

Acute exercise increased HSP72 mRNA and protein ( $P < .001$  and  $P < .05$ , respectively, Figure 4), HSP60 mRNA ( $P < .05$ ), and HSP90 mRNA and protein ( $P < .001$  and  $P < .05$ , respectively, Figure 4). Acute exercise induced oxidative stress indicated by increased levels of HO-1 mRNA and protein (both  $P < .001$ , Figure 4), 4-HNE protein adducts, and protein carbonyls (both  $P < .01$ , Table 4). There were significant interactions of diabetes with acute exercise for HSP72 mRNA ( $P < .01$ ), for HO-1 protein ( $P < .001$ ), for HSP60 mRNA and protein (both  $P < .05$ ), for GRP75 protein ( $P < .05$ ), for HSP90 mRNA and protein ( $P < .001$  and  $P < .05$ , respectively), also for protein carbonyls and 4-HNE protein adducts (both  $P < .05$ ). Together these data indicate that diabetes attenuates the induction of HSPs and HO-1 and results in decreased peroxidation of cellular lipids and proteins in response to acute exercise.

**TABLE 3** Effect of streptozotocin-induced diabetes, 8 wk of exercise training (Tr), and acute exercise on the inflammatory markers IL-6 and TNF- $\alpha$  in kidney homogenates

Experimental group	IL-6 (pg/mg protein)	TNF- $\alpha$ (pg/mg protein)
Control rest	4.2 (1.49)	23.99 (4.79)
Control Tr rest	4.2 (1.51)	25.47 (2.25)
Control + exercise	3.88 (1.47)	20.96 (8.94)
Control Tr + exercise	4.22 (1.63)	22.50 (10.38)
Diabetes rest	7.92** (3.29)	32.93* (8.06)
Diabetes Tr rest	4.77 <sup>†</sup> (0.97)	24.53 <sup>†</sup> (5.09)
Diabetes + exercise	7.05 (3.76)	28.27 (4.13)
Diabetes Tr + exercise	4.58 (1.15)	22.77 (6.49)

Data are presented as mean (SD).

Difference due to diabetes (\* $P < .05$ , \*\* $P < .01$ ).

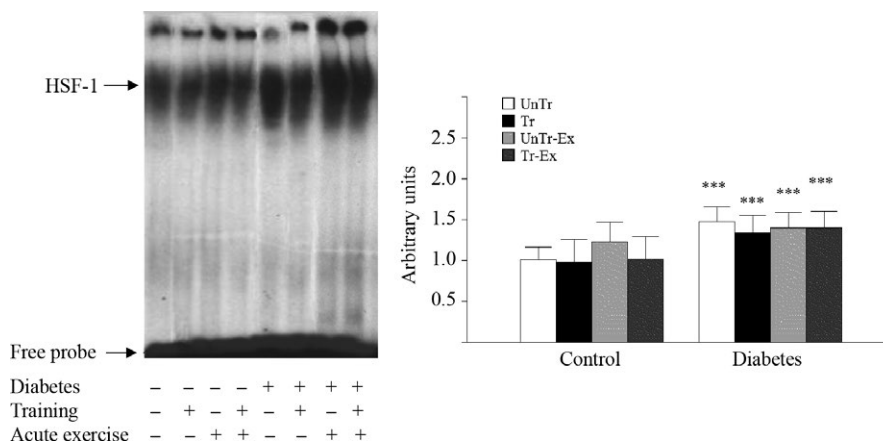
Effect of exercise training (<sup>†</sup> $P < .05$ ).

Nevertheless, a lower induction of HSPs and HO-1, and less oxidative damage to macromolecules in response to acute exercise could also be attributed to the differences in the intensities of acute exercise protocol based on the observed running time to exhaustion.

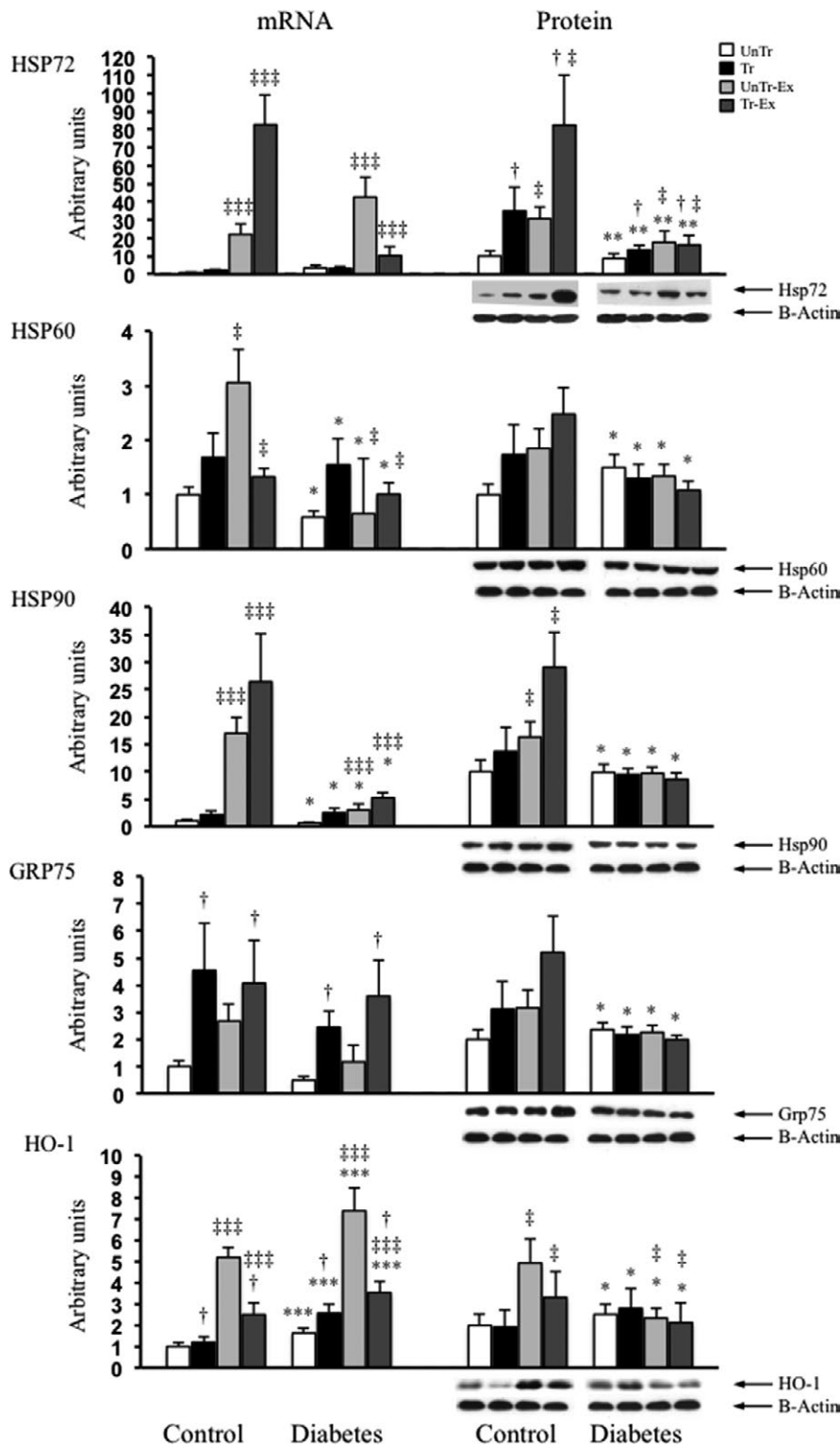
### 3.3 | Effects of exercise training

The efficiency of the exercise training was evident by increased running time till exhaustion, and also by enhanced vastus lateralis muscle CS activity in both diabetic and non-diabetic rats (Table 1). The time until exhaustion was even longer in diabetic rats compared to non-diabetic control rats ( $220.57 \pm 48.39$  minutes vs  $168.5 \pm 31.75$  minutes,  $P < .05$ ). Although no significant interaction between diabetes and exercise training was observed, the training effect on vastus lateralis and red grastocnemius CS activities was likely to be more pronounced in diabetic rats compared to the non-diabetic rats (74% vs 30% induction, respectively). On the other hand, overall CS activities in skeletal muscle were lower in diabetic rats, as described previously.<sup>16</sup>

Eight weeks of exercise training did not alter kidney histology in non-diabetic rats, but partially reversed the mesangial expansion in diabetic rats (Figure 1, Table 2), and moderately decreased levels of TGF- $\beta$  ( $P < .05$ , Figure 2) as well as IL-6 and TNF- $\alpha$  ( $P < .05$ , Table 3). Exercise training overall up-regulated HSP72 protein levels ( $P < .05$ , Figure 4), as well as HO-1 and GRP75 mRNA expression ( $P < .05$  and  $P < .01$ , respectively, Figure 4). On the other hand, the observed interactions of diabetes with exercise training for HSP72, HSP60, GRP75, and HSP90 proteins (each  $P < .05$ ), and for HSP72 and HSP60 mRNA ( $P < .001$  and  $P < .05$ ) together indicate that diabetes affects the positive effects of exercise training on kidney HSP expression.



**FIGURE 3** Gel mobility shift image and densitometric values of heat shock factor-1 (HSF-1) DNA-binding activity in kidney tissue in response to streptozotocin-induced diabetes, acute exercise, and eight-week exercise training. Groups are as in Figure 2. Values are mean  $\pm$  SEM. Difference due to diabetes: \*\*\* $P < .001$



**FIGURE 4** Kidney levels of heat shock protein 72 (HSP72), HSP60, HSP90, glucose-regulated protein 75 (GRP75), and heme oxygenase-1 (HO-1) in response to streptozotocin-induced diabetes, acute exercise, and 8 wk of exercise training. Groups are as in Figure 1. Densitometric values are mean  $\pm$  SEM. Difference due to diabetes: \* $P$  < .05; \*\* $P$  < .01; \*\*\* $P$  < .001. Difference due to exercise training: † $P$  < .05. Effect of acute exercise: ‡ $P$  < .05; †‡‡ $P$  < .001. Representative Western blots for the protein expression are also provided. Groups are as in Figure 2. Beta-actin was used as a loading control

In correlation analysis, a significant negative correlation between HSP72 and TGF- $\beta$  ( $R = -.775$ ,  $P < .01$ ) was observed when both diabetic and non-diabetic animals were included. Furthermore, 4-HNE as the marker of lipid peroxidation was strongly correlated with HSP72 in diabetic animals ( $R = .975$ ,  $P < .001$ ) and with HSF-1 in all animals ( $R = .821$ ,  $P < .001$ ).

## 4 | DISCUSSION

The present study demonstrated an enhanced HSP response in the kidney of non-diabetic rats in response to both acute exercise and 8 weeks of endurance exercise training. In diabetic rats, kidney HSP response was impaired especially in the major inducible stress protein HSP72 levels, despite

**TABLE 4** Effect of streptozotocin-induced diabetes, 8 wk of exercise training (Tr), and acute exercise on kidney levels of protein carbonyls and 4-HNE adducts

Experimental group	Protein carbonyls	4-HNE adducts
	(arbitrary units)	(arbitrary units)
Control rest	1.00 (0.15)	1.00 (0.06)
Control Tr rest	0.90 (0.12)	1.23 (0.23)
Control + exercise	1.56* (0.23)	2.00* (0.21)
Control Tr + exercise	1.21* (0.21)	1.37* (0.14)
Diabetes rest	0.93 (1.08)	1.34 (0.11)
Diabetes Tr rest	1.08 (0.16)	1.58 (0.18)
Diabetes + exercise	1.16* (0.14)	1.48* (0.13)
Diabetes Tr + exercise	1.36* (0.15)	1.66* (0.19)

Data are presented as mean (SEM).  
Effect of acute exercise (\* $P < .01$ ).

increased HSF-1 DNA-binding activity. The diabetic rats also exhibited glomerulosclerosis indicated by increased synthesis of TGF- $\beta$  and glomerular injury characteristic of diabetic nephropathy.<sup>22</sup> Despite an overall impaired HSP response in diabetes, exercise training appeared to attenuate the development of glomerulosclerosis, as indicated by lower TGF- $\beta$  levels and partially reversed mesangial expansion. Moreover, the protective role of exercise training in diabetic kidney may also be mediated by its anti-inflammatory effects. Compelling evidence has proved an interplay between the inflammatory response and the fibroproliferative process, including TGF- $\beta$  signaling.<sup>24</sup> Consistently, in the present study, we found that TGF- $\beta$  and the two key inflammation-related cytokines IL-6 and TNF- $\alpha$  were increased and also positively correlated in diabetic rats, while the eight-week exercise training appeared to offset these changes in diabetes.

Although the reported effects of various experimental models of diabetes are diverse,<sup>25,26</sup> an impaired HSP defense seems to predispose the kidney to nephropathy, consistent with the present results. On the other hand, there is increasing evidence for a protective role for HSPs during recovery from renal ischemia.<sup>2,5,27</sup> The present study reported a significant negative correlation between the most inducible and abundant HSP, namely HSP72 and TGF- $\beta$  emphasizing the protective role of HSPs against renal injury and kidney fibrosis. Induction of HSP72 expression may contribute to overall adaptation to exercise<sup>12</sup> and also provide protection against diabetic complications.<sup>25,26</sup> HSP72 has also been shown to improve oxidative metabolism and insulin sensitivity,<sup>28</sup> which is a key mechanism in the treatment of type 2 diabetes. Evidently, the present study explored for the first time HSP responses by exercise training in diabetic kidney. The eight-week exercise-training program used in the present study markedly increased endurance capacity in diabetic animals.<sup>29</sup> However, despite

the improved exercise tolerance, a lower HSP72 protein response was observed compared to non-diabetic animals (55% vs 250% relative increase in the present study). The ability of exercise training to increase kidney HSP72 levels is in line with reports in other tissues.<sup>11,16,30,31</sup> It should be noted that that the exercise-induced HSP responses are dependent on exercise intensity and duration,<sup>32</sup> reflecting a physiological response to the heat shock and oxidative stress of exercise. Our finding that acute exercise-induced kidney HSP72 levels is consistent with a previous study in non-diabetic rats.<sup>33</sup> We hypothesize that exposure to exercise-induced metabolic and oxidative stress, and the accompanying decrease in blood flow and pH together induce kidney HSP response with most prominent effects seen on HSP72. In line with this hypothesis, we observed that 4-HNE protein adducts as a marker of oxidative stress were strongly correlated with HSP72 levels in diabetic animals, and with HSF-1 levels in all animals.

HSP60 levels were increased in diabetic animals, consistent with previous report by us<sup>25</sup> and others.<sup>34</sup> Autoimmunity against HSP60 and the consequent drop in circulating HSP60 levels may be important in the pathogenesis of diabetes.<sup>35</sup> However, appearance of antibodies to the HSP60-derived peptide p277 has been reported to arrest autoimmune diabetes induced by streptozotocin;<sup>36</sup> thus, the exercise-induced decrease in HSP60 levels in diabetic animals could also be regarded cytoprotective.

In the present study, diabetic animals showed discrepancies between HSF-1 DNA-binding activity and both HSP mRNA and protein responses to exercise, indicating an impaired protein turnover. In addition to increased DNA-binding activity and oligomerization, HSF-1 undergoes many post-translational stages which may all have affect its transcriptional activity.<sup>37</sup> As HSF-1 is a master stress-induced transcription factor to regulate HSP expression through heat shock elements, these complex regulators could potentially alter either HSP transcription or translation in diabetes. Moreover, HSF-1 regulates many other genes that may be modulated in our experimental diabetes model.<sup>38</sup>

Exercise training increased HO-1, which is an inducible HSP mainly involved in the protection against the adverse effects of oxidative stress.<sup>39</sup> Previously, exercise training has been shown favorable effects on kidney oxidative stress in diabetes,<sup>29,40</sup> but the HO-1 responses have been very little studied. Adaptations of endogenous antioxidants to oxidative stress and the beneficial effects of exercise on the markers of oxidative stress are well demonstrated.<sup>41,42</sup> We specifically explored the HSP response; hence, the antioxidant network was left aside in the present study. Although a persistent and uncontrolled oxidative stress may impair signal transduction giving rise to pathological conditions (reviewed in Radak et al<sup>42</sup>), our data provide evidence for a compensatory regulation of cellular functions during transient oxidative stress, which may actually be beneficial for adaptive responses such



as increased HSP expression. On the other hand, the absence of significant acute exercise-induced oxidative stress in diabetic animals must be regarded as a contributing factor to the low HSP response in the present study.

In conclusion, we report here that HSP72 levels were negatively correlated with TGF- $\beta$ , a key inducer of glomerulosclerosis and the induction of diabetes resulted in alterations of kidney HSP levels, which could not be counteracted by exercise training, contrary to other tissues. On the other hand, exercise training increased kidney HSP levels and decreased acute exercise-induced oxidative stress in non-diabetic animals, and notably in diabetic kidney reversed the histopathological findings of glomerulosclerosis, and decreased the levels of TGF- $\beta$  and the inflammation-related cytokines IL-6 and TNF- $\alpha$ . An impaired HSP response likely is a contributing factor in diabetic nephropathy, although all aspects of their role in this process remain unknown.

#### 4.1 | Perspectives

The various roles of HSPs help to maintain the physiology of multicellular organisms. Activation of the HSP response by exercise might well account for many of the health benefits associated with increased physical activity. Diabetes is the leading cause of kidney disease. Diabetic organs represent altered physiological states predisposing to many complications where an impaired HSP response is also involved. Understanding better the mechanisms of cytoprotective HSP induction and tissue-specific effects of exercise training, and developing pharmacological modulation to augment HSP synthesis for the successful therapeutic interventions are potential strategies to ameliorate kidney injury as a major cause of morbidity in diabetes.

#### ACKNOWLEDGEMENTS

We thank Taina Vihavainen for skillful technical assistance and Prof. Mustafa Gül for providing blood glucose data.

#### CONFLICT OF INTEREST

The authors have no disclosures. There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

#### ORCID

M. Atalay  <http://orcid.org/0000-0001-8999-1426>

#### REFERENCES

- Atalay M, Oksala N, Lappalainen J, Laaksonen DE, Sen CK, Roy S. Heat shock proteins in diabetes and wound healing. *Curr Protein Pept Sci*. 2009;10:85-95.
- Li F, Mao HP, Ruchalski KL, et al. Heat stress prevents mitochondrial injury in ATP-depleted renal epithelial cells. *Am J Physiol Cell Physiol*. 2002;283:C917-C926.
- Danda RS, Habiba NM, Rincon-Choles H, et al. Kidney involvement in a nongenetic rat model of type 2 diabetes. *Kidney Int*. 2005;68:2562-2571.
- Sieber J, Jehle AW. Free Fatty acids and their metabolism affect function and survival of podocytes. *Front Endocrinol (Lausanne)*. 2014;5:186.
- Suzuki S, Maruyama S, Sato W, et al. Geranylgeranylacetone ameliorates ischemic acute renal failure via induction of Hsp70. *Kidney Int*. 2005;67:2210-2220.
- Fittipaldi S, Dimauro I, Mercatelli N, Caporossi D. Role of exercise-induced reactive oxygen species in the modulation of heat shock protein response. *Free Radic Res*. 2014;48:52-70.
- Atalay M, Laaksonen DE. Diabetes, oxidative stress and physical exercise. *J Sports Sci Med*. 2002;1:1-14.
- Kashihara N, Haruna Y, Kondeti VK, Kanwar YS. Oxidative stress in diabetic nephropathy. *Curr Med Chem*. 2010;17:4256-4269.
- Heiwe S, Jacobson SH. Exercise training for adults with chronic kidney disease. *Cochrane Database Syst Rev* 2011;10:CD003236.
- Kanazawa M, Kawamura T, Li L, et al. Combination of exercise and enalapril enhances renoprotective and peripheral effects in rats with renal ablation. *Am J Hypertens*. 2006;19:80-86.
- Smolka MB, Zoppi CC, Alves AA, et al. HSP72 as a complementary protection against oxidative stress induced by exercise in the soleus muscle of rats. *Am J Physiol Regul Integr Comp Physiol*. 2000;279:1539-1545.
- Henstridge DC, Febbraio MA, Hargreaves M. Heat shock proteins and exercise adaptations. Our knowledge thus far and the road still ahead. *J Appl Physiol (1985)*. 2016;120:683-691.
- Powers SK, Locke M, Demirel HA. Exercise, heat shock proteins, and myocardial protection from I-R injury. *Med Sci Sports Exerc* 2001;33:386-392.
- Geiger PC, Gupte AA. Heat shock proteins are important mediators of skeletal muscle insulin sensitivity. *Exerc Sport Sci Rev*. 2011;39:34-42.
- Chebotareva N, Bobkova I, Shilov E. Heat shock proteins and kidney disease: perspectives of HSP therapy. *Cell Stress Chaperones*. 2017;22:319-343.
- Atalay M, Oksala NK, Laaksonen DE, et al. Exercise training modulates heat shock protein response in diabetic rats. *J Appl Physiol*. 2004;97:605-611.
- Walsh RC, Koukoulas I, Garnham A, Moseley PL, Hargreaves M, Febbraio MA. Exercise increases serum Hsp72 in humans. *Cell Stress Chaperones*. 2001;6:386-393.
- Jha V, Garcia-Garcia G, Iseki K, et al. Chronic kidney disease: global dimension and perspectives. *Lancet*. 2013;382:260-272.
- Wang RN, Bouwens L, Kloppel G. Beta-cell proliferation in normal and streptozotocin-treated newborn rats: site, dynamics and capacity. *Diabetologia*. 1994;37:1088-1096.
- Tervaert TW, Mooyaart AL, Amann K, et al. ; Renal Pathology Society. Pathologic classification of diabetic nephropathy. *J Am Soc Nephrol*. 2010;21:556-563.
- Oksala NK, Laaksonen DE, Lappalainen J, et al. Heat shock protein 60 response to exercise in diabetes: effects of alpha-lipoic acid supplementation. *J Diabetes Complications*. 2006;20:257-261.
- Loeffler I, Wolf G. Transforming growth factor-beta and the progression of renal disease. *Nephrol Dial Transplant*. 2014;29(Suppl 1):i37-i45.

23. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001;29:2002-2007.
24. Richter K, Konzack A, Pihlajaniemi T, Heljasvaara R, Kietzmann T. Redox-fibrosis: impact of TGFbeta1 on ROS generators, mediators and functional consequences. *Redox Biol.* 2015;6:344-352.
25. Oksala NK, Lappalainen J, Laaksonen DE, et al. Alpha-lipoic Acid modulates heat shock factor-1 expression in streptozotocin-induced diabetic rat kidney. *Antioxid Redox Signal.* 2007;9:497-506.
26. Sharma AK, Bharti S, Ojha S, et al. Up-regulation of PPARgamma, heat shock protein-27 and -72 by naringin attenuates insulin resistance, beta-cell dysfunction, hepatic steatosis and kidney damage in a rat model of type 2 diabetes. *Br J Nutr.* 2011;106:1713-1723.
27. Lee J, Park S, Kim WK. Exercise preconditioning reduces acute ischemic renal injury in Hsp70.1 knockout mouse. *Histol Histopathol.* 2013;28:1223-1233.
28. Henstridge DC, Bruce CR, Drew BG, et al. Activating HSP72 in rodent skeletal muscle increases mitochondrial number and oxidative capacity and decreases insulin resistance. *Diabetes.* 2014;63:1881-1894.
29. Gul M, Laaksonen DE, Atalay M, Vider L, Hänninen O. Effects of endurance training on tissue glutathione homeostasis and lipid peroxidation in streptozotocin-induced diabetic rats. *Scand J Med Sci Sports.* 2002;12:163-170.
30. Naito H, Powers SK, Demirel HA, Aoki J. Exercise training increases heat shock protein in skeletal muscles of old rats. *Med Sci Sports Exerc.* 2001;33:729-734.
31. Lappalainen Z, Lappalainen J, Oksala NK, et al. Exercise training and experimental diabetes modulate heat shock protein response in brain. *Scand J Med Sci Sports.* 2010;20:83-89.
32. Liu Y, Lormes W, Baur C, et al. Human skeletal muscle HSP70 response to physical training depends on exercise intensity. *Int J Sports Med.* 2000;21:351-355.
33. Lollo PC, Moura CS, Morato PN, Amaya-Farfan J. Differential response of heat shock proteins to uphill and downhill exercise in heart, skeletal muscle, lung and kidney tissues. *J Sports Sci Med.* 2013;12:461-466.
34. Dogukan A, Tuzcu M, Juturu V, et al. Effects of chromium histidinate on renal function, oxidative stress, and heat-shock proteins in fat-fed and streptozotocin-treated rats. *J Ren Nutr.* 2010;20:112-120.
35. Birk OS, Douek DC, Elias D, et al. A role of Hsp60 in autoimmune diabetes: analysis in a transgenic model. *Proc Natl Acad Sci U S A.* 1996;93:1032-1037.
36. Elias D, Cohen IR. The hsp60 peptide p277 arrests the autoimmune diabetes induced by the toxin streptozotocin. *Diabetes.* 1996;45:1168-1172.
37. Anckar J, Sistonen L. Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annu Rev Biochem.* 2011;80:1089-1115.
38. Hayashida N, Fujimoto M, Tan K, et al. Heat shock factor 1 ameliorates proteotoxicity in cooperation with the transcription factor NFAT. *EMBO J.* 2010;29:3459-3469.
39. Negi G, Nakkina V, Kamble P, Sharma SS. Heme oxygenase-1, a novel target for the treatment of diabetic complications: focus on diabetic peripheral neuropathy. *Pharmacol Res.* 2015;102:158-167.
40. Ishikawa Y, Gohda T, Tanimoto M, et al. Effect of exercise on kidney function, oxidative stress, and inflammation in type 2 diabetic KK-A(y) mice. *Exp Diabetes Res.* 2012;2012:702948.
41. Done AJ, Traustadottir T. Nrf2 mediates redox adaptations to exercise. *Redox Biol.* 2016;10:191-199.
42. Radak Z, Zhao Z, Koltai E, Ohno H, Atalay M. Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. *Antioxid Redox Signal.* 2013;18:1208-1246.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Lappalainen J, Oksala NKJ, Laaksonen DE, et al. Suppressed heat shock protein response in the kidney of exercise-trained diabetic rats. *Scand J Med Sci Sports.* 2018;28:1808–1817. <https://doi.org/10.1111/sms.13079>