DISSERTATIONS IN
HEALTH
SCIENCES

Zekine Pündük

Effects of Exercise and Alpha-Lipoic Acid Supplementation on Brain Tissue Protection in Experimental Diabetes

Publications of the University of Eastern Finland
Dissertations in Health Sciences

UNIVERSITY OF
EASTERN FINLAND

ZEKİNE PÜNDÜK

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To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland for public examination in Mediteknia auditorium, Kuopio, on Saturday, August15th 2015, at 12 noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
Number 291

Institute of Biomedicine, Physiology, School of Medicine,
Faculty of Health Sciences, University of Eastern Finland
Kuopio
2015

Juvenes Print
Tampere, 2015

Distributor:
University of Eastern Finland
Kuopio Campus Library
P.O.Box 1627
FI-70211 Kuopio, Finland
http://www.uef.fi/kirjasto

ISBN (print): 978-952-61-1831-4
ISBN (pdf): 978-952-61-1832-1
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

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Pündük, Zekine
Effects of exercise and alpha-lipoic acid supplementation on brain tissue protection in experimental diabetes
University of Eastern Finland, Faculty of Health Sciences
Publications of the University of Eastern Finland. Dissertations in Health Sciences 291, 2015. 62 p.

ISBN (print): 978-952-61-1831-4
ISBN (pdf): 978-952-61-1832-1
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

ABSTRACT

The present thesis aims to clarify the effects of 8 weeks of exercise training or thiol supplementation (lipoic acid, LA) on exercise-induced oxidative stress and tissue protection, including endogenous antioxidant homeostasis and heat shock proteins (HSPs) in diabetic and non-diabetic rat brain. Protection against oxidative stress, a disruption of redox control of signalling and cellular events, depends on an orchestrated synergism between several exogenous micronutrients and endogenous antioxidants. Exercise-induced oxidative stress stimulates antioxidant protection, which can also be prolonged, and may manifest a sustained response during exercise training. Physical exercise induces HSPs, which have a central role in protein homeostasis and protection in various tissues, predominantly skeletal muscle, while the effect of exercise on brain is limited.

In this study streptozotocin-induced experimental diabetes model in rat brain was used. At baseline, HSP levels, thioredoxin-1 (TRX) protein and activity and levels of thioredoxin-interacting protein (TXNip), an endogenous inhibitor of TRX were not different between SID and non-diabetic animals. Endurance training or diabetes had no effect on protein carbonyl content and other oxidative stress markers, but the proportion of oxidized glutathione (GSSG) to total GSH was increased in diabetic animals, indicating an altered redox status. The levels of elongation factor eEF-1 and eEF-2 kinase were not affected by diabetes or training.

Exercise training increased TRX protein levels in brain, but diabetes down regulated the TRX response to exercise training and induced TXNip mRNA expression. Thus, the beneficial effects of physical exercise on the TRX system were inhibited by diabetes. Similarly, endurance training increased HSP expression in brain tissue, and experimental diabetes impaired the HSP response at the protein level. Acute exhaustive exercise induced mRNA of TRX in the brain. LA supplementation did not prevent diabetes-induced disturbances in GSH and TRX homeostasis; in contrast, LA supplementation increased TXNip transcription. Moreover, LA supplementation increased HSC70 mRNA expression in diabetic animals, but decreased expression in non-diabetic controls. On the other hand, LA supplementation had no effect on the levels of any of the proteins analysed.

Based on this study, brain antioxidant status and redox regulation can be improved in a safe and physiological manner by exercise training, which may provide a means for improving brain health. However, LA supplementation had no beneficial effects on brain protection.

National Library of Medical Classification: WL 348, WK 810, QZ 180, QT 260, QU 55
Medical Subject Headings: Antioxidants; Brain/metabolism; Diabetes Mellitus, Experimental; Heat-Shock Proteins; Homeostasis; Exercise; Muscle, Skeletal; Oxidation-Reduction; Oxidative Stress; Protein Carbonylation; Thiotic Acid; Thioredoxins; Animal Experimentation; Rats

Pündük, Zekine
Liikuntasuorituksen ja alfalipoaatin vaikutus aivojen suojamekanismeihin kokeellisessa diabeteksessa
Itä-Suomen yliopisto, terveystieteiden tiedekunta
Publications of the University of Eastern Finland. Dissertations in Health Sciences 291. 2015. 62 s.

ISBN (print): 978-952-61-1831-4
ISBN (pdf): 978-952-61-1832-1
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

TIIVISTELMÄ

Tutkimuksen tarkoituksena oli selvittää liikuntasuorituksen ja tioliantioksidanttilisän (lipoaatti, LA) vaikutus hapetusstressiin ja toisaalta näiden tekijöiden vaikutus aivojen suojamekanismeihin, erityisesti antioksidanttitasapainoon ja ns. lämpösokkiproteiineihin (HSP).

Suoja hapetusstressiä vastaan ja hapetus-pelkistystasapainon välittämä solunsisäinen signalointi on tarkasti säädelty prosessi johon vaikuttavat ulkopuolelta saadut ravintoaineet ja toisaalta sisäsyntyiset antioksidantit. Liikuntasuorituksen aiheuttama hapetusstressi johtaa pitkittyessään pitkäkestoiseen antioksidantti- ja HSP-välitteiseen suojavaikutukseen. HSP suojaa valkuaisaineita erityisesti luurankolihaksessa vaikkakin sen vaikutus aivoihin on rajallinen.

Mallina käytettiin kokeellista rotan diabetesmallia Altistamattomilla eläimillä HSP- ja tioredoksiini-1 (TRX-1)-proteiinitasot, aktiivisuus ja tioredoksiinin kanssa vuorovaikuttava proteiini (TXNip) joka on TRX:n sisäsyntyinen estäjä, eivät eronneet verrattaessa rottia joilla oli kokeellinen diabetes (SID) terveisiin rottiin. Liikuntaharjoittelulla eikä diabeteksellä ollut vaikutusta proteiinikarbonyylien ja hapetusstressin merkkiaineisiin. Sitä vastoin, hapettuneen glutationin (GSSG) suhde kokonaisglutationiin (GSH) kasvoi diabeetikkorotilla heijastaen lisääntynyttä hapetusstressiä. Diabeteksellä tai kestävyysharjoittelulla ei ollut kuitenkaan vaikutusta elongaatiotekijä-1- (eEF-1) tai eEF-2-kinaasiin. Liikuntaharjoittelu lisäsi TRX-1:n pitoisuutta aivoissa kun taas diabetes vähensi TRX-vastetta liikuntaharjoitteluun ja lisäsi TXNip:n pitoisuutta. Tämän perusteella diabetes esti liikuntaharjoittelun hyödyllistä vaikutusta TRX-järjestelmään. Vastaavasti kestävyysharjoittelu lisäsi HSP pitoisuutta aivoissa ja kokeellinen diabetes heikensi HSP-vastetta proteiinitasolla. Äkillinen liikuntaharjoittelu lisäsi TRX-1:n mRNA-pitoisuutta aivoissa. LA-lisällä ei kyetty estämään diabeteksen aiheuttamia muutoksia GSH- ja TRX-järjestelmissä, sitä vastoin, LA-lisä lisäsi TXNip:n transkriptiota. Lisäksi LA-lisä lisäsi HSC70 mRNA-pitoisuutta diabeetikkorotilla mutta vähensi sitä ei-diabeetikkorotilla. LA-lisällä ei ollut kuitenkaan vaikutusta tutkittuihin tekijöihin proteiinitasolla.

Tämän tutkimuksen perusteella aivojen antioksidanttitasapainoa ja hapetus-pelkistys-välitteistä solun toimintojen säätelyä voidaan parantaa turvallisesti ja fysiologisesti käyttäen liikuntaharjoittelua. Tämä voi mahdollistaa uusia keinoja edistää aivojen terveyttä. LA-lisällä ei kuitenkaan havaittu hyödyllisiä aivoja suojaavia vaikutuksia.

Luokitus: WL 348, WK 810, QZ 180, QT 260, QU 55
Yleinen suomalainen asiasanasto: antioksidantit; aivot; diabetes; eläinkokeet; kestävyysharjoittelu; liikunta; oksidatiivinen stressi; rotta

To gezi parkı,

Acknowledgements

This work was carried out in the Institute of Biomedicine, Physiology of University of Eastern Finland, Kuopio. I owe warmest thanks to all people who have contributed to this work. Especially, I would like to express my special thank you to my supervisors.

The deepest and the most sincere thanks of all I owe to my principle supervisor Docent Mustafa Atalay, for his invaluable support and guidance that have motivated and carried me over the hard times of this project. I could not have finished this work without your help!

I am thankful to Docent David Laaksonen, my second supervisor, for his excellent contribution to planning of the studies and preparing the manuscripts, and revising the English language of this thesis.

I am grateful to Associate Professor Niku Oksala, my third supervisor, for planning the studies and revising this thesis. I would also like to thank Professor Chandan K. Sen for his professional expertise and support to this work.

I am privileged to have Professor Shuzo Kumagai as my opponent and Professor Haydar Demirel and Professor Khalid Rahman as reviewers of my thesis. Their constructive and valuable critiques helped me to improve thesis.

Without our research group I would not be able to accomplish this work. I am grateful to Dr. Jani Lappalainen, for planning the study and improving the thesis papers for this project. I, also thank Dr. Savita Khanna for her collaboration and efforts during the initial phase of these studies. I would like to thank, laboratory technicians Ms. Taina Vihavainen, Ms Satu Marttila and Ms. Taija Hukkanen for their skilful technical assistance.

This study has been financially supported by Finnish Ministry of Education, Centre for International Mobility (CIMO), Foundation of Pajulahti College of Sports, Juho Vainio and Yrjö Jahnsson Foundations, Helsinki, Finland, high Technology Foundation of Eastern Finland, and by COST actions B35, BM0602, CM1001, TD1304 and National Doctoral Programme of Musculoskeletal Disorders and Biomaterials.

Balıkesir, May 2015

Zekine Pündük

List of the original publications

This dissertation is based on the following original publications, which will be referred to by their Roman numerals (I-IV) in the text:

I Lappalainen Z, Lappalainen J, Oksala N.K.J., Laaksonen D.E., Khanna S, Sen C.K., Atalay M. Diabetes impairs exercise training-associated thioredoxin response and glutathione status in rat brain. Journal of Applied Physiology 106:461-467, 2009. Doi:10.1152/japplphysiol.91252.2008.

II Lappalainen Z, Lappalainen J, Oksala N.K.J., Laaksonen D.E., Khanna S, Sen C.K, Atalay M. Exercise training and experimental diabetes modulate heat shock protein response in brain. Scandinavian Journal of Medicine & Science in Sports 20:83-89, 2010. Doi: 10.1111/j.1600-0838.2008.00872.x.

III Lappalainen Z, Lappalainen J, Laaksonen D.E., Oksala N.K.J., Khanna S, Sen C.K., Atalay M. Acute exercise and thioredoxin-1 in rat brain, and alpha-lipoic acid and thioredoxin-interacting protein response, in diabetes. International Journal of Sport Nutrition and Exercise Metabolism 20:206-215, 2010.

IV Lappalainen J, Lappalainen Z, Oksala N.K.J, Laaksonen D.E, Khanna S, Sen C.K., Atalay M. Alpha-lipoic acid does not alter stress protein response to acute exercise in diabetic brain. Cell Biochemistry and Function 28:644-650, 2010. Doi: 10.10002/cbf.1702.

Contents

Abbreviations

ALA	alpha-lipoic acid
AP-1	activator protein 1
CAT	catalase
Cu,Zn-SOD	copper-zinc superoxide dismutase
DBD	DNA-binding domain
DHLA	dihydrolipoic acid
DM	diabetes mellitus
eEF-1	elongation factor-1
eEF-2	elongation factor-2
eHSP	extracellular heat shock protein 72
eNOS	endothelial nitric oxide synthase
ERK	extracellular regulated kinase
GLUT4	glucose transporter protein
GPx	glutathione peroxidise
GRD	glutathione reductase
GRP75	glucose-regulated protein 75
GRP78	glucose-regulated protein 78
GRX	glutaredoxin
GSH	glutathione
GSH/GSSG	glutathione (reduced/oxidised)
GSK-3	glycogen synthase kinase-3
GSSG	glutathione disulphide
HO-1	hem oxygenase 1
HSC73	heat shock cognate protein 73
HSF	heat shock factor
HSP60	heat shock protein 60

HSP70	heat shock protein 70
HSP72	heat shock protein 72
HSP90	heat shock protein 90
HSPA2	heat shock 70kDa protein 2
HSPs	heat shock proteins
JNK	c-Jun N-terminal kinase
kDA	kilo Dalton
LA	lipoic acid
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MnSOD	manganese superoxide dismutase
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NF-kappaB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	nuclear factor erythroid 2-related factor
P53	tumor protein
Prxs	peroxiredoxins
RNS	reactive nitrogen species
ROS	reactive oxygen species
SID	streptozotocin-induced diabetes
SOD	superoxide dismutase
TRX-1	thioredoxin-1
TRxRd	thioredoxin reductase
TXNip	thioredoxin-interacting protein
VEGF	vascular endothelial growth factor

1 Introduction

Diabetes mellitus (DM) is a metabolic disorder defined by relative or absolute deficiency of insulin secretion and/or insulin resistance that causes chronic hyperglycaemia and impaired carbohydrate, lipid and protein metabolism (Maritim et al. 2003). In general, diabetes can be classified into four clinical categories: type 1 diabetes (due to beta-cell destruction, usually leading to absolute insulin deficiency); type 2 diabetes (due to a progressive insulin secretory defect in the background of insulin resistance); other specific types of diabetes due to genetic defects or different diseases and gestational diabetes mellitus (ADA 2014). Moreover, diabetes is associated with oxidative stress (Rahimi et al. 2005), with an imbalance between free radical formation and the ability of the organism's natural antioxidants (Chaturvedi 2007). It further leads to oxidative abnormalities of cell components such as protein, lipid and nucleic acid and may play a role in the development and progression of DM and its complications, both in type 1 and type 2 DM (Maritim et al. 2003; Rahimi et al. 2005; Song et al. 2007; Chaturverdi 2007).

The benefits of physical activity in health promotion and prevention of diseases are well established, and it can be used as a therapeutic tool for disease prevention (Warburton et al. 2006). Moreover, physical exercise has a protective effect on the brain and its cognitive functions (Radak et al. 2001a). This is especially important in disease conditions such as diabetes mellitus (DM), which is a risk factor for decline in cognitive function and ischemic stroke (Ma et al. 2014, Passler et al. 2014). However, despite the positive effects on health, physical exercise is also a major source of toxic oxygen metabolites (Radak et al. 2013). During exercise, oxygen consumption increases by 8-10 fold, and oxygen flux through the working muscles may increase up to 100-fold (Sen et al. 1994). This may increase free radical production and overwhelm body antioxidant defences, resulting in oxidative damage (Sen and Packer 2000). In the last decade oxidative stress has been re-defined as the perturbation of redox control of signalling and cellular events, especially disruption of thiol redox circuits (Jones 2006), and may contribute to the development of a wide range of diseases, including diabetes which may make tissues more susceptible to oxidative stress (Atalay et al. 2004).

Protection against oxidative stress depends primarily on an orchestrated synergism between exogenous micronutrients and endogenous antioxidants (Atalay et al. 2006). Thiols are important players in protein-protein interactions and have multifaceted functions in cellular functions, including a pivotal role in antioxidant defence. Glutathione (GSH) is the most abundant intracellular thiol with antioxidant properties and plays a key role in many physiological functions, whereas the TRX system is the major protein disulphide reductase in cells and comprises TRX and its reductase (TrxR), and NADPH. TRX, a small multi-functional thiol protein, acts as a central antioxidant and is also one of the key regulators of signalling in the cellular responses against various stresses, including oxidative stress. TRX translocates from the cytosol into the nucleus to regulate the expression of various genes (Holmgren et al. 2010). Importantly, the expression of thioredoxin-interacting protein (TXNip), an endogenous inhibitor of TRX (Nishiyama et al. 1999) was markedly increased in animals with diabetes (Schulze et al. 2004, Shalev 2014). Responses to oxidative stress during physical exercise are believed to be regulated by signalling pathways involving the TRX system. Indeed, TRX may act as a key regulator of intracellular signalling in response to stressful conditions since TRX enhances the binding of *Jun*/*Fos* complex to the AP-1 site by interacting with a redox-sensitive nuclear factor (Tanito et al.2004).In addition, TRX is

involved in redox signalling, regulating the activities of several redox sensitive transcription factors like NF-kappaB, Nrf2, P53(Lu and Holmgren 2014a).Exercise-induced responses to oxidative stress have an effect on TRX induction, and this delayed and prolonged over-expression of TRX may contribute to the post-exercise recovery processes and training response (Sumitani et al. 2002,Fisher-Wellman et al. 2013). To date, only a few studies are available on the protective and regulatory role of TRX against exercise-induced oxidative stress.

Lipoic acid (LA) is a natural thiol redox modulator that can enhance the endogenous antioxidant defence system and is used to treat complications associated with diabetes (Sen and Packer 2000). Furthermore, endogenous LA is a cofactor of mitochondrial oxidative enzymes and may increase glucose uptake in skeletal muscle via its insulin-like effects (Khanna et al. 1999a). Our group has shown that LA protects against exercise-induced oxidative stress and up-regulates endogenous protection mechanisms, including antioxidant and heat shock protein (HSP; stress protein) defences (Khanna et al. 1999a, Oksala et al. 2006, Kinnunen et al. 2009a, Kinnunen et al. 2009b).

Skeletal muscle is subjected to a high level of oxidative stress during exercise due to increased production of reactive oxygen species (ROS), thus requiring greater antioxidant protection during or after physical exercise. Nevertheless, exercise training appears to upregulate antioxidant protection against oxidative stress also in skeletal muscle (Khanna et al. 1999b, Henriksen and Saengsirisuwan 2003, Atalay et al. 2004, Radak et al. 2013). However, there is little information on how the beneficial effects of physical exercise are mediated in brain compared to other tissues, such as skeletal muscle.

The series of studies presented in this thesis explore the effects of physical exercise and LA supplementation on brain HSP expression, endogenous antioxidant protection, oxidative stress-induced damage and inflammatory processes by using both acute exercise and exercise training models, in a set of experiments performed in rats with or without diabetes. Moreover, in this thesis, the effects of LA supplementation on TRX synthesis and activity in the brain are evaluated for the first time.

2 Review of the Literature

Oxidative stress was defined in 1985 by Helmut Sies as a "disturbance in the pro-oxidant-antioxidant balance in favour of the former" (Sies 1993). According to this definition oxidative stress is manifested by diminished antioxidant levels, increased production of reactive species or increased oxidative macromolecule damage including DNA, lipid and protein oxidation. In this regard, oxidative stress was classically defined as a disturbance in the balance between the production of ROS and antioxidant defences, which can lead to tissue damage (Radak et al. 2013). An accumulation of research data on redox signalling pathways, antioxidant intervention trials, and oxidative stress markers indicated that a more useful definition is "a disruption of redox signalling and control" (Jones 2006, Sies 2007).

Study results indicated that ROS-mediated signalling and redox-sensitive thiols play an important role in the redox state, including regulation of cellular processes in response to intracellular changes (Sen 2000). The thiol GSH and TRX systems play a main role in the antioxidant defences against oxidative stress by regulating cellular events (Sen 2000). In addition, TRX and GSH defences systems have various biological functions such as regulation of enzyme activity, receptors, transcription factors, redox-sensitive signal transduction, short-term storage of cysteine, protein structure, cell growth, proliferation, and programmed cell death (Lu and Holmgren 2012, Sen and Packer 2000). Therefore, at physiological conditions, low levels ROS are crucial for redox regulation of cellular functions and may induce protective responses at temporarily increased higher levels, but uncontrolled oxidative stress can damage DNA, trigger death by apoptosis, necrosis, or cell death mechanisms (Juránek et al. 2013).

An early study using electron spin resonance spectroscopy showed directly that exhaustive exercise resulted in a two- to three-fold increase in free radical species in muscle and liver tissues of rats (Davies et al. 1982). It has been well demonstrated that exercise-induced oxidative insults are closely linked to muscle damage and decreased muscle performance (Powers et al. 2010). However, ROS generated during muscle contraction have a key physiological role in the adaptation to exercise. In response to free radical assault, the cell has developed a number of endogenous defences composed of enzymatic and non-enzymatic components, in which thiol antioxidants play major roles (Radak et al. 2013). Low concentrations of reactive oxygen species can induce the expression of antioxidant enzymes or other defence mechanisms. Previous studies have well demonstrated that acute intensive exercise causes less oxidative stress in trained than untrained animals or humans, which could be attributed to upregulation of primary antioxidant defences, secondary antioxidant defences such as HSPs which maintains protein homeostasis and decreased inflammation (Atalay et al. 1996, Khanna et al. 1999b, Sen 1999, Atalay et al. 2004, Jackson 2013, Radak et al. 2013). However, there is little information available on how the beneficial effects of physical exercise are mediated in brain compared to the information regarding other tissues such as skeletal muscle.

Increased oxidative stress as measured by indices of lipid peroxidation and protein oxidation has been shown to be increased in type 1 DM, type 2 DM and experimental DM (Atalay and Laaksonen 2002). Despite experimental evidence indicating that oxidative stress may contribute to the onset and progression of diabetic complications (Atalay and Laaksonen 2002, Laaksonen et al. 2000), controversy exists about whether the increased oxidative stress is merely associative rather than causal in DM (Laaksonen et al. 1996).

Hyperglycaemia has been found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Atalay and Laaksonen 2002). Hyperglycaemia also increased oxidative stress through TXNip (Chen et al. 2008). Previous studies have suggested that oxidative stress contributes to hyperglycaemia-induced neuronal apoptosis and cognitive decline in experimental diabetes (Kamboj et al. 2008b, Alvarez et al. 2009, Kapitulnik et al. 2012). Alvarez et al. (2009) have also observed intracellular lipofuscin deposits, characteristic of increased oxidative stress, in diabetic brains. Similarly, in another study the contribution of mitochondria to the total ROS production in the brain tissue was evaluated and concluded that mitochondria are the major sources of ROS (Kudin et al. 2008). Furthermore, mitochondrial oxidative damage has been proposed to result in neuronal apoptosis and cognitive dysfunction (Liu et al. 2003).

2.1 HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are a class of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures or other types of stress (Heck et al. 2011, Sharp et al. 2013). This increase in expression is transcriptionally regulated. The dramatic upregulation of HSPs is a key feature of the heat shock response and is induced primarily by heat shock factor (HSF) (Dokladny et al. 2015). HSPs are found in virtually all living organisms, from bacteria to humans, and they are induced in all tissues, including the brain. HSPs are named according to their molecular weight. For instance, HSP60, HSP70 and HSP90 (the most widely-studied HSPs) refer to families of HSPs in the order of 60, 70 and 90 kilo Daltons in size, respectively (Li and Srivastava 2004). Earlier studies indicated that HSPs acts as molecular chaperones, and thus play a critical role in protein folding, intracellular trafficking of proteins, and coping with proteins denatured by heat and other stresses. The roles of HSPs have been expanded to include control of cell signalling (Calderwood et al. 2007, Csermely et al. 2007), modulation of the immune response (Johnson and Fleshner 2006, Chen et al. 2007a) and involvement in some chronic diseases (Kampinga et al. 2007, Schmitt et al. 2007).

The 70-kDa HSP (HSP70) family of proteins includes a constitutive 73-kDa protein (HSC73) and a highly stress-inducible 72-kDa protein (HSP72) (Moseley 1996) also, HSPA2, Grp78, HSP70B and GRP75 (Tavaria et al. 1996, Daugaard et al. 2007), which are located in the cytosol and nucleus. They have also been detected in the lysosomes and endoplasmic reticulum (Daugaard et al. 2007). GRP 75 has also been detected in the mitochondria (Tavaria et al. 1996). Two members of this family, HSP78 (glucose-regulated protein 78 [GRP78]) or immunoglobulin–binding protein and HSP75 (mitochondrial HSP70), perform chaperone functions in the endoplasmic reticulum and mitochondria, respectively (Hood 2001). HSP72 is essential for cellular recovery after stress as well as survival and maintenance of normal cellular function. Furthermore, HSP72 prevents protein aggregation and also refolds damaged proteins. Importantly, expression of high levels of HSPs has been associated with an increased ability of cells to withstand challenges that would otherwise lead to cell injury or death (McKay 1993, Moseley 1997). Moreover, HSP72 is also capable of inhibiting stress-induced apoptosis (Mosser et al. 2000), even after the activation of effector caspases (Jaattela et al. 1998). Although HSP70 is expressed at low levels in normal brain, it is induced in all neuronal cells following ischaemia and serves to refold misfolded or unfolded proteins (Sharp et al. 2013). The constitutive heat shock cognate, HSC70 which is also called HSP73, and its inducible isoform, HSP70 or HSP72 have received more attention.HSP72 is readily inducible with stress, whereas HSC70 is less so (Tanguay et al. 1993). Recent studies have demonstrated that HSC70 is also inducible (Liao and Tang 2014). In addition, HSC70 plays important roles in the aging process and aging-related diseases

(Liao and Tang 2014). It appears that HSC70 mainly acts as a housekeeping protein, whereas HSP72 affects the ability of cells to respond to stress however, both have redundant roles in ensuring cell survival (Havik and Bramham 2007). Studies have also suggested that both HSP70 isoforms, as well as other HSPs, work in concert to protect developing skeletal muscle myotubes (Maglara et al. 2003, Kayani et al. 2008).

HSP60 is a mitochondrial chaperone involved in synthetic processes in the mitochondria (Bukau and Horwich 1998) and is also stress inducible (Yenari et al. 2010). However, 25% to 30% of HSP60 can be found in the cytoplasm, where it is linked to the regulation of signal transduction and especially protects against mitochondrial apoptosis, which seems to be crucial for its cytoprotective function (Gupta and Knowlton 2002, 2005, Arya et al. 2007, Lai et al. 2007). Microglial activation is one of early responses to brain ischaemia and several other stressors in CNS. Microglias continuously monitor and react to changes in brain homeostasis and to specific signalling molecules including HSP60 (Yenari et al. 2010). In has been previously shown that, HSP60 can also move to the cell surface under appropriate conditions to act a ligand to modulate cellular immune response (Soltys and Gupta 2000). Therefore, HSP 60 has a complicated role in innate immune response (Vabulas et al. 2002) and in mitochondrial protein biogenesis (Moseley 2000, Voos and Rottgers 2002). It may inhibit caspase-3 (Gupta and Knowlton 2002) or facilitate the maturation of procaspase-3 to its active form (Xanthoudakis et al. 1999).

The HSP90 family comprises of HSP90α and HSP90β, which are among the most abundant proteins in mammalian cells is found in both the cytoplasm and (Haverinen et al. 2001) and nucleus (Picard 2006), and is a key component in the regulation of steroid-receptor function and the repression of the heat shock response (Zou et al. 1998). HSP90/HSP70-based chaperone mechanism plays a major role in ubiquitination and proteasomal degradation of proteins that have undergone oxidative or other toxic damage, where HSP90 regulates signalling by modulating ligand-binding breaks (Pratt et al. 2014).Therefore, HSP90 is responsible for catalysing the interaction with several substrate proteins and co-chaperones involved in cell regulation and intracellular signalling (Whitesell and Lindquist 2005). Major aberrant proteins that misfold and accumulate in neurodegenerative diseases are target proteins of HSP90 for elimination, including tau (AD), α-synuclein (PD), huntingtin (HD), and the expanded glutamine androgen receptor (polyQ AR) (SBMA) (Prat et al. 2014). In addition, HSP90 is also a potent autoantigen and thought to have a role in various inflammatory diseases, including arteriosclerosis (Rigano et al. 2007). HSP90 also plays an important role in the activation of endothelial nitric oxide synthase (eNOS) resulting in increased synthesis of vasoregulatory NO and concomitant reduction of the eNOS-derived radical, the superoxide anion (Pritchard et al. 2001).

Transcriptional regulation of heat shock transcription factor response

Heat shock transcription factor (HSF) is evolutionarily conserved from yeast to humans, and acts as a major regulator of HSP expression. In mammalian cells, three related HSFs, HSF-1, HSF-2, and HSF-4, are involved in different, but in some cases overlapping, biological functions. HSF-1 is ubiquitously expressed and functions as a key regulator for stress-induced transcription of HSP genes and for acquisition of thermotolerance (McMillan et al. 1998, Pirkkala et al. 2001, Voellmy 2004). Many HSPs function as molecular chaperones that aid the folding of damaged proteins, and increased accumulation of HSPs is essential for survival of cells exposed to protein-damaging stresses, including heat shock. The structure of HSF comprises a conserved DNA-binding domain (DBD), which binds to the 5 bp sequence nGAAn, and two hydrophobic repeat (HR) regions (HR-A and HR-B), which are necessary for homotrimer formation. Trimeric HSF recognizes a heat shock

element (HSE) comprising at least three inverted repeats of the 5 bp unit (Pirkkala et al. 2001, Voellmy 2004). Biochemical and genetic evidence indicates that HSF regulates the expression of genes encoding proteins involved not only in stress resistance but also in cell maintenance and developmental processes (Westerheide and Morimoto 2005, Akerfelt et al. 2007). Under physiological conditions, HSF-1 monomers are colocalized with HSP72 in the nucleus and it is activated by cellular stress (Anckar and Sistonen 2007). The activation process involves trimerization of HSF-1 monomers, translocation of the trimers, hyperphosphorylation and binding to the promoter of heat shock genes (Baler et al. 1993, Sarge et al. 1993, Sarge 1998). The end-products of this process, such as HSP72, exert negative feedback regulation (Abravaya et al. 1992). The posttranscriptional mechanism involves stabilization of HSP72 mRNA (Kaarniranta et al. 1998).

Rapid induction of stress protein expression is accomplished through mechanisms of transcriptional activation and preferential translation. HSFs (HSF-1 through HSF-4) regulate the inducible synthesis of HSPs during development, growth, and adaptation. Whereas essential single-copy genes encode HSF in Saccharomyces cerevisiae and Drosophila, multiple HSFs have been identified in chicks, plants, mice and humans. Two HSFs (HSF-1 and HSF-2, encoding proteins of 75 and 72 kDa, respectively) have been identified in the mouse. Neither HSF-1 nor HSF2 is heat inducible, but HSF-1 is hyperphosphorylated in a ras-dependent manner by members of the MAPK subfamilies (ERK1, JNK/SAPK, and p38 protein kinase) during physiological stress. The acute synthesis of HSPs in skeletal muscle is controlled by the transcription factor heat shock factor (HSF-1), which is expressed constitutively in mature skeletal muscle cells (Broome et al. 2006). In response to stress, the appearance of partially unfolded or oxidized cellular proteins triggers the release of HSP72, HSP90, and other chaperones from HSF-1 due to increased affinity for binding of these HSPs for unfolded proteins compared with that for HSF-1 (Shi et al. 1998, Zou et al. 1998). HSF-1 then trimerizes prior to translocation to the nucleus, where it undergoes further modifications, including phosphorylation to activate transcription of heat shock protein genes (Santoro 2000).

A blunted HSP response and decreased HSP expression in insulin-resistant tissue could be the result of inflammatory inhibition of the primary HSP transcription factor, heat shock factor 1 (HSF-1). HSF-1 has several layers of regulation including negatively regulated feedback control through interaction with HSPs (Morimoto 1998) and phosphorylation by protein kinases. Over activity of stress kinases capable of phosphorylating HSF-1 on serine residues may repress HSF-1 activation in insulin-resistant tissue. Glycogen synthase kinase 3 (GSK-3), extracellular regulated kinase (ERK), and c-Jun NH2-terminal kinase (JNK), protein kinases closely associated with the development of insulin resistance, are known to negatively regulate HSF-1 by phosphorylation on serine residues 303, 307, and 363, respectively (Kline and Morimoto 1997). Constitutive phosphorylation of HSF-1 on serine residues holds HSF-1 in an inactive state under normal physiological growth conditions. The release of HSF-1 serine phosphorylation causes HSF-1 to cotranslocate to the nucleus, trimerize, and bind to heat shock elements (HSE) which is necessary for new HSP synthesis.

Small heat shock proteins molecular mechanisms in the central nervous system

Small HSPSs are presented between 12-40 kDA in mammals; αB-crystalin and HSP25/27 are among the best characterized. However, αB-crystalin has been most closely associated with cataract formation and damage to the eye lens (Sun and MacRae 2005), it can also stabilize muscle structure, as suggested by the onset of a desmin-related myopathy, when it is mutated (Vicart et al. 1998) and the loss of protection against ischaemia-reperfusion injury when it is absent (Morrison et al. 2004). Like αB-crystalin, HSP25/27 is also involved in cell

protection, possibly through both direct and indirect mechanisms (Arrigo 2007). HSP25/27 directly stabilizes microfilaments (Paulsen et al. 2007), acts as a reservoir for non-native proteins, thereby preventing their aggregation until they can recover proper conformation (Ehrnsperger et al. 1997), and like several other HSPs, they might be involved in the prevention of apoptosis (Arya et al. 2007). Interestingly, some HSPs are also expressed in the nervous system (Verschuure et al. 2003, Quraishe et al. 2008). Kirbach et al (2011) demonstrated that seven small HSPs, namely, HSPB1, B2, B3, B5, B6, B8, and B11,are expressed in the brain, however, only three of them were induced by heat shock, namely, HSPB1 (HSP25), HSPB25 and HSPB8 (HSP22) (Kirbach and Golenhofen 2011). It seems more and more mall HSPs seem to play an important function in the brain, most likely protecting it from diverse stress conditions.

Diabetes can promote oxidative stress in the brain and increase the presence of reactive oxygen species (ROS) in both hippocampal and cortical cell cultures, and decrease basal activity of cerebral antioxidant enzymes (Camm et al. 2011). The small HSPs (HSPB) family includes 11 members in the human and mouse genomes and the expression of HSPB1 (HSP27, HSP25), HSPB5, HSPB6 (HSP20) and HSPB8 (HSP22) are confirmed to be expressed in the brain tissue (Quraishe et al. 2008), and HSPB1 and HSPB2 expression has been demonstrated in smooth muscle in vessel walls of the human brain (Wilhelmus et al. 2006). HSPB1 and HSP8 are expressed in motor and sensory neurons in the brainstem, cranial nerve nuclei and cerebellum.

As in the case of other cell types, central nervous system (CNS) cells initiate a stress inducible heat shock response to elevated body temperature, and glial cells show induction of HSP72. It has also been demonstrated that HSPs may be transferred between cell types in the nervous system. Additionally, motor and sensory neurons show a high level for HSP72 induction, which was associated with a failure in the activation HSF-1 (Calderwood et al. 2007). HSPC/HSP 90 is commonly expressed in rat brain and almost all neurons (Izumoto and Herbert 1993, Gass et al. 1994). Additionally HSPC/HSP90 mRNA was found abundant in limbic system-related structures, such as the hippocampus, amygdale and maxillary body, as well as in the Purkinje cell layer of the cerebellum (Izumoto and Herbert 1993). HSP90 has been found in cell bodies, also in dentries and nuclei (Gass et al. 1994) and is also localized to the cell surface in the developing nervous system (Cid et al. 2004, Sidera et al. 2004).

2.2 HEAT SHOCK PROTEINS IN DIABETES

Diabetes is a metabolic disorder that can produce changes in various organs of the body including heart, liver, kidney and the brain. Diabetes affects the CNS and produce disturbances such as neurobehavioral changes, autonomic dysfunction, altered neuroendocrine function and neurotransmitter alterations, thus leading to end organ damage (Nishikawa et al. 2000, Brands et al. 2004).

Oxidative stress has been ascribed a role in the pathogenesis of diabetes and its complications, and stress proteins have been shown to protect organisms *in vitro* and *in vivo* against oxidative stress. Moreover, a number of disease states are associated with oxidative stress. Aging, hyperlipidaemia and type 2 diabetes are associated with a reduced heat stress response. The low HSP levels in diabetes makes tissues vulnerable to stress, and impairs the repair processes, which could contribute to the excessive mortality and organ failure associated with this disease. Furthermore, the essential cellular functions of HSPs such as aiding protein folding, “life guarding” organelles like mitochondria, reducing apoptosis, and diminishing endoplasmic reticulum stress become impaired in type 2 diabetes (Hooper 2007). Also, HSPs play a role as antioxidants and inhibition of apoptosis and inflammation.

Hence, in diabetes, the expression of HSPs can be impaired (Atalay et al. 2004, Atalay et al. 2009). Studies showed that HSP72 gene expression is down regulated in skeletal muscle by insulin resistance in type 2 diabetes (Kurucz et al. 2002, Bruce et al. 2003). Moreover, Chung et al. (2008) reported that HSP72 protein expression is decreased in skeletal muscle from obese insulin-resistant humans (Chung et al. 2008).In animal experimental diabetes models it has been demonstrated that the levels of HSP72 and HSP25 are decreased in SID rats (Atalay et al. 2004). In addition, HSP60 has been shown to be reduced in the heart (Shan et al. 2003, Oksala et al. 2006) and increased in the kidney and liver of diabetic animals (Oksala et al. 2006, Oksala et al. 2007), whereas HSP90 was increased in the heart and decreased in the liver (Atalay et al. 2004). Elevated levels of HSP60, HSP90 and GRP78 have also been reported in the skeletal muscle in diabetic patients (Hojlund et al. 2003). On the contrary, (Yamagishi et al. 2001) found significantly decreased levels of the constitutive HSC70 in the liver. The available studies indicate that diabetes may exert variable and tissue-specific effects on HSP expression.

In diabetes hyperglycaemia can induce oxidative stress in various brain regions(Kaur and Bhardwaj 1998). Using streptozotocin (STZ)-induced hyperglycaemia in a rat model of diabetes, a significant decrease in the activities of mitochondrial electron chain complexes 1-IV in various brain regions has been demonstrated (Kaur and Bhardwaj 1998). The increase in free radical generation along with depletion of antioxidants is the mechanism involved in diabetes-induced oxidative stress (Rochette et al. 2014). Oxidative stress, lipid peroxidation and production of ROS occur at an increased rate in diabetes (Raza et al. 2004). For instance, lipid peroxidation, in brain, liver, and kidney tissue is increased by the induction of both acute (Raza et al. 2004) and chronic (Aragno et al. 1999) hyperglycaemia in rats.

Brain cells synthesize the inducible 70 kDa form of HSP (HSP72) in response to a variety of stressors, including hyperthermia (Walters et al. 1998, Leoni et al. 2000), ischaemia (Simon et al. 1991), hypoxia (Murphy et al. 1999) and energy depletion (Wang et al. 2005). The hippocampus of the brain, a major limbic structure in the brain is very sensitive to stress, is rich in glucocorticoid receptors and is strongly affected by diabetes (Alvarez et al. 2009). In diabetes, Yuan et al. (2006) reported that levels of mitochondrial HSP60 elevated in the hippocampal region in the brain. Moreover, overexpression of HSP72 was shown to protect against both local and global cerebral ischaemia in vivo (Kelly et al. 2002, Tsuchiya et al. 2003). On the other hand, HSC70 and HSP90 expressions remained unchanged (Yamagishi et al. 2001, Hojlund et al. 2003). Experimental evidence suggests a protective effect of HSP72 in peripheral diabetic neuropathy (Biro et al. 1997), although decreased (Atalay et al. 2004, Chen et al. 2005) or unchanged (Yamagishi et al. 2001) levels have also been described in diabetic tissue, including the brain. Also, the mitochondrial specific stress protein cpn60 enhanced diabetic rat brain (Yuan et al. 2006). Interestingly, HSP72 has been suggested to have anti-inflammatory properties, which may partly explain its neuroprotective function in the postischemic brain (Zheng et al. 2008). The expression of HSPs that maintain or enhance expression of HSPs could be a powerful tool in the prevention of insulin resistance and diabetes.

2.3 HEAT SHOCK PROTEINS AND EXERCISE

HSPs are highly conserved proteins that are expressed both constitutively and under various stressful conditions, including during and after exercise (Walsh et al. 2001, Febbraio et al. 2002, Atalay et al. 2004). The exercise-related factors, including heat stress, hypoxia, reduced intracellular pH, reactive oxygen and nitrogen species (ROS and RNS) production, depletion of glucose and glycogen stores, increase in cytosolic calcium levels, myocyte stretching and inflammation induce HSP response. These metabolic factors after both acute and chronic exercise stimulate the synthesis of HSP not only in active skeletal muscle, but

also in cardiomyocytes, liver, peripheral blood leukocytes, in pulmonary macrophages and also in the brain (Mastracola et al. 2012, Powers et al. 2001, Atalay et al. 2004, Lancaster et al. 2004), which lead to an overall increase in the HSP levels in the peripheral circulation.

Physical exercise is known to increase the expression of a wide variety of HSPs in striated muscle (Milne and Noble 2002), and in the heart and to play cardioprotective roles through HSPS10, HSP40, HSP60, and HSP90 (Locke and Noble 1995, Locke et al. 1995, Milne et al. 2006). It appears that exercise causes few changes in the levels of small HSPs, unless the exercise is of the type eccentric exercise that can induce more muscle damage. HSP60 appears to be induced primarily by non-damaging exercise, it probably plays an import role in the mitochondria. HSP72 is strongly induced by non-damaging exercise, including high-intensity potentially damaging exercise and it also plays an important role in protein synthesis and refolding of denatured proteins (Milne and Noble 2002, Morton et al. 2006; Paulsen et al. 2007). Therefore, HSPs expression depends on several factors, such as the type of exercise (Morton et al. 2006, Paulsen et al. 2007), whether it is damaging exercise or not, but more importantly, the intensity and duration of exercise (Milne and Noble 2002, Fehrenbach et al. 2005), gender (Thorp et al. 2007), age (Demirel et al. 2003), and training status (Gjovaag et al. 2006). Data from exercise studies suggest that long-term or intense exercise may induce many heat shock proteins such as inducible HSP70; HSP72 (Walters et al. 1998, Sumitani et al. 2002, Lancaster et al. 2004, Horowitz and Robinson 2007), and HSP60 and HSP8 (HSC71) (Ding et al. 2006). Aerobic training increases HSP72, HSP60 and alpha B-crystallin content in human skeletal muscle (Liu et al. 1999, Liu et al. 2000b, Morton et al. 2008). Furthermore, the blood level of HSP72 was elevated in rowers, soccer players, and endurance runners (Banfi et al. 2006, Fehrenbach et al. 2000, Liu et al. 2000).

Regular endurance training influences HSP expression as demonstrated by Fehrenbach et al. (2005), who compared the expression of a variety of HSP in the cytoplasm and on the surface of leukocytes in trained athletes before and after a half marathon to levels in untrained persons at rest (Fehrenbach et al. 2000). After the race, a significantly greater percentage of leukocytes in the athletes expressed cytoplasmic HSP27, HSP60, and HSP72, whereas heat shock cognate protein 70 (HSC70) whilstHSP90 remained unchanged. Strenuous exercise increased HSP expression in the blood immediately after the run, suggesting a protective role of HSP in leukocytes of athletes in order to maintain function after heavy exercise. An acute exercise-induced increase in HSP72 expression (Magalhaes Fde et al. 2010), on the other hand, Watkins et al,(2007) did not observe any alteration in HSP72 expression in the vastus lateralis, although the subjects exercised for only 30 min daily, which might not have been sufficient to induce adaptation at the cellular level (Watkins et al. 2007). It is possible that body temperature elevation during exercise is important for exercise-induced HSP72 (Ogura et al. 2008). Endurance swimming training increased amount of cardiac HSP60 and HSP72 (Ascensao et al. 2005). McArdle et al. demonstrated that the extensor digitorum longus muscle of adult and aged mice overexpressing HSP72 is significantly protected against damage induced by lengthening contractions (McArdle et al. 2001). Training induces increases in HSP72 levels of the vastus letaralis, however, when the training stimulus is reduced, HSP levels appear to return to baseline values (Morton et al. 2008), Moreover basal HSP72 and HSC70 levels displayed no significant differences in trained and untrained muscle (Morton et al. 2008).

Regular endurance exercise in athletes has been shown to modulate different HSPs following exercise, especially those that are implicated in cyto-protective and anti-oxidant function. Accordingly, Fehrenbach et al (2000) demonstrated that in vitro heat shock of human peripheral blood leukocytes significantly stimulated HSP27 and HSP72 mRNA, with physically active individuals exhibiting the greatest increases (Fehrenbach et al. 2000). Additionally, Ganazalez et al. (2000) demonstrated that HSP72 was expressed at higher

levels in the skeletal muscles of treadmill-trained rats than in sedentary animals after an exhaustive exercise challenge (Gonzalez et al. 2000). Furthermore, physically active rats have both greater and faster HSP72 responses to exhaustive exercise than sedentary rats (Atalay et al. 2004, Campisi and Fleshner 2003, Campisi et al. 2003) in nearly every tissue tested. In contrast, sedentary stressed rats had increased HSP72 only in pituitary, adrenal, liver and spleen, this increase was smaller in the physically active-stressed rats. In healthy human subjects, HSP60 and HSP90 expression levels increased during or following post-exercise period, in contrast, HSP27 in control subjects remained relatively constant before and following exercise (Thambirajah et al. 2008). In addition, basal expression of these HSPs was lower among trained athletes than in untrained control subjects. It is unclear how physical activity modulates HSP72 induction, but trained athletes demonstrate higher leukocyte and skeletal muscle HSP72 mRNA expression at rest than sedentary individuals (Liu et al. 1999, Fehrenbach et al. 2000). Hence, it has been proposed that trained cells provide high HSP72 transcript levels for immediate translation when necessary (Fehrenbach et al. 2000).

However, several studies suggest that elevations in muscle and core temperature may not be the sole factors responsible for exercise-induced HSP expression. For example, HSP72 content was increased in rat soleus and gastrocnemius muscles after treadmill running, independent of an increase in core temperature (Skidmore et al. 1995), and soleus muscle HSP72 production after exercise was enhanced when exercise was performed under elevated ambient temperature. If the HSP response to exercise occurs through a pathway independent of heat stress, then the combination of heat treatment and exercise, or pharmacological induction of HSPs in combination with exercise, may have an additive effect on HSP induction and dramatically improve insulin action in skeletal muscle. One possible mechanism for the exercise mediated HSP response is via inhibition of GSK-3 and subsequent activation of HSF-1. Previous studies suggest that the activation of HSP72 in the heart occurs through phosphatidylinositol 3-kinaseY-mediated activation of Akt and subsequent inhibition of GSK-3 (Shinohara et al. 2006). Given the fact that the variable expression and response of HSPs in insulin-sensitive tissue, distinct regulatory pathways for the HSP response are likely for the tissues where insulin action is crucial for the glucose uptake. On the other hand because in brain glucose uptake occurs also without insulin stimulation, classification of brain as an insulin-sensitive tissue is questionable.

2.4 EFFECTS OF EXERCISE ON HEAT SHOCK PROTEINS IN BRAIN

Regular physical exercise has beneficial effects on the brain, improvement of hippocampal plasticity (O'Callaghan et al. 2007), and cognitive function in humans (Lautenschlager et al. 2008) and in rodents (Vaynman et al. 2004). In contrast, severe long-term exercise may be deleterious to hippocampal neurons (Sumitani et al. 2002). Regular exercise has many beneficial effects on brain integrity and memory (Radak et al. 2001a, Radak et al. 2001b). On the other hand, oxidative stress tends to increase especially during high intensity of exercise (Leeuwenburgh and Heinecke 2001). It has been shown that induction of HSPs by mild stress has a protective effect against higher levels of stress in the brain (Latchman 2004). In rat models of strenuous training and over-reaching, regular exercise had beneficial effects on brain function and lowered accumulation of reactive carbonyl derivatives, a biomarker of oxidative protein damage (Radak et al. 1998). A higher intensity of exercise generated more oxidative stress and induced HSPs, while HSPs have a protective effect against the level of stress in the brain (Latchman 2004). For instance, HSP72 was reported to be increased in the hippocampus after severe exercise in a rodent model (Sumitani et al. 2002). The exercise-induced expression of HSP72 was higher in the brains of trained rats compared with the sedentary rats (Chen et al. 2007b). Moreover, in physically active

animals levels of HSP72 returns to baseline levels more quickly than in sedentary animals, which may suggest a more efficient response of HSP72 (Nickerson et al. 2005). In addition progressive exercise induced HSP72 overexpression in cerebral regions of the brain (Campisi et al. 2003) where the alterations in receptor systems and neurotransmitter regulation of neurons is associated with chronic exercise training (De Souzo et al. 2001, Mueller and Hasser 2006). Overall, the induction of brain HSP72 with physical exercise (Belter et al. 2004) indicates that HSP72 expression is a good marker of metabolic activity changes, especially in the brain regions engaged in cognitive processing (Ambrosini et al. 2005). Intracellular HSP72 increased by chronic adaptations to exercise training and, plays an anti-inflammatory role, while extracellular heat shock protein (eHSP)72 binding to toll-like receptors (TLR-2/4) represents a pro-inflammatory stimulus that may result in a fatigue signal to the CNS during higher load bouts of acute or chronic exercise (Heck et al. 2011). Therefore, exercise-induced HSP72 expression could play a beneficial role in protecting the hippocampus and prefrontal cortex. This effect may improve the cellular stress resistance in brain and contribute to the underlying protective mechanism of swimming. Exercise in humans results in the release of HSP72 from the brain and the hepatosplanchnic region (Febbraio et al. 2002, Lancaster et al. 2004). Although intense and repetitive exercise training can lead to an overtraining syndrome and decreased athletic performance (Smith 2000), HSP72 induction is enhanced in exercise that is more strenuous and of longer duration types (Fehrenbach et al. 2005). Thus, regular exercise, even high-intensity, may improve brain function and could play an important preventive ant therapeutic role in oxidative stress-associated conditions (Mattson and Magnus 2006).

2.5 EFFECTS OF EXERCISE ON HEAT SHOCK PROTEINS IN DIABETES

Regular physical exercise has beneficial effects in the primary and secondary prevention of several chronic diseases, including cardiovascular disease, diabetes, cancer, hypertension and premature death (Warburton et al. 2006). Moderate physical activity is recommended for the prevention of type 2 diabetes and for the management of both type I and type 2 diabetes(ADA 2014). Physical training can attenuate diabetes-induced changes in energy metabolism, increase muscle mass, the rate of protein synthesis and IGF-1. Exercise elicits a number of metabolic adaptations and is a powerful tool in the prevention and treatment of type 2 diabetes (Morrison et al. 2014, Sanz et al. 2010).

Exercise training is also a stimulus for increased HSP expression. Exercise-associated hyperthermia is commonly suggested as the stimulus responsible for inducing an increase in HSP after exercise (Lancaster and Febbraio 2005). Therefore, regular exercise can show a protective effect against oxidative stress and serve as a non-pharmacological therapeutic modality in T2DM (de Lemos et al. 2007). Furthermore, it has been demonstrated that although endurance exercise training up-regulated HSP72 expression in STZ rats, the response was significantly blunted compared with non-diabetic rats (Atalay etal.2004). Moreover, long-term exercise and diet reduced oxidative stress as shown by decreased serum uric acid, protein carbonyls, and cytoprotection was improved in the skeletal muscle tissue as increased mitochondrial HSP60 and GRP75 was observed in impaired glucose tolerance subjects while no response was found in the cytoplasmic chaperones HSP72 and HSP90 (Venojarvi et al. 2008). In addition, 8 weeks of endurance training increased GRP75 expression in red gastrocnemius muscle of SID rats (Atalay et al. 2004). The available information on the effects of exercise on heat shock proteins in diabetes is still limited.

2.6 BIOLOGICAL ANTIOXIDANTS AND THEIR EFFECTS ON BRAIN AND DIABETES

In mammals endogenous antioxidant system includes enzymatic catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxins and non-enzymatic vitamins E and C, glutathione, thioredoxin and uric acid that offer protection to cells and tissues against glucose-induced oxidative injury in diabetes (Bonnefont-Rousselot 2002, Atalay and Laaksonen 2002, Styskal et al 2012). To protect the tissues against increased oxidative stress in diabetes, superoxide dismutase, glutathione peroxidase and catalase, which are central enzymes in the antioxidant defence mechanisms of cells, are induced under hyperglycaemic conditions (Ceriello et al. 1996).

Within the body, tissues with a higher oxygen consumption rate, such as liver, heart, and brain, constitutively express greater antioxidant enzymes than those with lower oxygen consumption (Radak et al. 2013).An increase in oxidative stress in diabetes (Dandona et al. 2002, Hartnett et al. 2000) has been implicated in diabetic vascular complications including vascular damage to the cerebral artery (Auslander et al. 2002) leading to vascular cerebral diseases, e.g., stroke and brain infarction (Saudek et al. 1979). Although the brain consumes 20% of oxygen in the body, it has a low content of antioxidants and high content of unsaturated fatty acids and catecholamines that are easily oxidized (Serafini et al. 1992, Husain et al. 1996), making it more vulnerable to oxidative damage than any other organ in the body (Hong et al. 2000). Lipid peroxides and DNA oxides generated by oxidation (Mecocci et al. 1993) cause cell dysfunction and necrosis, leading to inflammation and functional degeneration of the central nervous system. The central nervous system has a high oxygen requirement and contains unsaturated lipid content: for these reason brain cells are vulnerable to oxidative stress. It has been stated that oxidative stress is elevated in diabetes mellitus (Ates et al. 2006) which is mainly due to the associated hyperglycaemia (Sano et al. 1998), hyperglycaemia induces oxidative stress in various brain regions. Possibly, oxidative stress in diabetes decreases tissue GSH level, impairs antioxidant enzymes activities and generates ROS by glucose auto-oxidation (McLennan et al. 1991).

Diabetes decreases activity of the key antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and reduced glutathione (GSH) levels in rat brain (Ozkaya et al. 2002, Yanardag et al. 2006, da Costa et al. 2013). A decrease in the activity of these enzymes can lead to an excess availability of superoxide anion and hydrogen peroxide in the biological systems, which in turn generate hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation (Costa et al. 2013, Mukherjee et al.1994). In addition, diabetes reduced the levels of GSH, TGSH (Tachi et al. 2001, Baydas et al. 2002, Kamboj et al. 2008b) and decreased SOD activity and catalase in rat hippocampus and cerebral cortex (Kuhad and Chopra 2007, Kamboj et al. 2008b). A concomitant decrease or increase in tissue GST activity and GSH content has been well documented in diabetic rats (Mak et al. 1996, Gupta et al. 1999). Glutathione peroxidase activity increased in cerebral cortex, cerebellum and brain stem of diabetic animals (Ulusu et al. 2003, Kamboj et al. 2008b). Moreover, it has been reported that TRX-1, which is a major intracellular antioxidant, is induced in diabetes (Kakisaka et al. 2002, Miyamoto et al. 2005). TRX is cytokine-like factor with radical-scavenging functions (Ceriello et al. 1996, Lu and Holmgren 2014a), and it has been suggested that the regulation of cellular reduction/oxidation (redox) by TRX plays an important role in signal transduction and cytoprotection against oxidative stress (Ceriello et al. 1996, Nakamura et al. 1997, Hamada and Fukagawa 2007).

2.6.1 Thioredoxin and TXNip system

Thioredoxins (TRX) are low–molecular–weight proteins containing a conserved dithiol motif that supports a range of biological functions (Holmgren 2010). The thioredoxin system consists of TRX, and NADPH dependent thioredoxin reductase (TRxRd) and regulates cellular redox balance through the reversible oxidation of its redox-active cysteine residues (-Cys-Gly-Pro-Cys-) to form a disulphide bond that in turn is reduced by thioredoxin reductase and NADPH (Holmgren 2010, Lee et al, 2013, Lu 2014). Other members of the TRX family are GSH transferases, GSH peroxidases, peroxiredoxins (Prxs), chloride intracellular channels, and the copper-ion binding protein Scol (Pedone et al. 2010).

The TRX system protects the cell against oxidative stress by scavenging ROS through a variety of direct or indirect mechanisms. Also TRX system plays a crucial role in the regulation of the intracellular redox state by reducing numerous protein substrates. In mammalian systems, thioredoxin-1 (TRX1) is found in cytoplasm and nuclei, while TRX2 is found in mitochondria. These proteins were recognized as central regulators of cellular functions in response to redox signals and stress, for instance by the modulation of various signalling pathways, transcription factors and the immune response (Lillig and Holmgren 2007). In addition, TrxR1 can directly reduce a number of substrates, in particular lipid hydroperoxides, hydrogen peroxide (H_2O_2), dehydroascorbate and lipoic acid (Zhong and Holmgren 2000).

TRX interacting protein is a regulator of TRX function that was originally identified as a vitamin D up-regulated protein 1 (VDUP1) in HL-60 cells treated with 1,25-hydroxyvitamin D(3). Txnip binds to reduced TRX, but not to oxidized TRX and is thought to be a negative regulator of TRX (Nishiyama et al. 1999). In addition, TXNip expression was increased in rat hearts in response to acute myocardial infarction (Xiang et al. 2005), hypobaric hypoxia (Karar et al. 2007), and pressure overload (Yoshioka et al. 2007).TXNip is a protein expressed that binds and inhibits TRX and thereby can induce oxidative stress and modulate the cellular redox state (Nishiyama et al. 1999, Junn et al. 2000, Patwari et al. 2006). Therefore, TXNip may represent an important therapeutic target associated with oxidative stress disorders (World et al. 2006).

Increased TXNip expression in diabetes has been described in human pancreatic islets, human aortic smooth muscle cells (Schulze et al. 2002), human mesangial cells (Shah et al. 2013), in kidneys from diabetic mice (Shalev et al. 2002, Kobayashi et al. 2003, Schulze et al. 2004) and in CNS as well. In diabetes, TXNipis a gene that is significantly up regulated in dorsal root ganglia of diabetic rats (Price et al. 2006). Diabetes induced TXNip expression and it is possible that TXNip is activated in response to high glucose in diabetic animals (Minn et al. 2005).

Thioredoxin plays a pivotal role in the antioxidant defence of cells. Increased expression and subsequent binding of TXNip to TRX inhibits the reducing activity of TRX. In brain, the TRX may provide protection against various hypoxic or ischemic events (Stroev et al. 2004, Li et al. 2005), especially different kinds of oxidative stress (Chen et al. 2002, Ueda et al. 2002). In particular, in experiments with transgenic mice it has been shown that overexpression of TRX protects the brain cells against damage during focal ischaemia (Takagi et al. 1999), and that addition of TRX to cultural medium significantly reduces the damaging effects of hypoxia/reoxygenation in cell culture (Isowa et al. 2000). In contrast, inhibition of TRX increases oxidative stress (Yamamoto et al.2003). On the other hand, decreased TRX activity has been reported in diabetes without significant changes in the levels of TRX protein (Schulze et al. 2004). The induction of the expression of TRX1 can rescue diabetic myocardium from diabetes and oxidative stress-related impairment of myocardial angiogenesis by reducing oxidative stress and enhancing the expression of HO-

1 and VEGF (Soman et al. 2011). Additionally, TRX therapy decreased expression of proappoptic proteins (p-JNK and p38MAPkalpha) and increased expression of the TRX-1, HO-1, VEGF and antiapoptotic protein p38MAPK which is down-regulated in diabetes (Soman et al. 2011).

Apoptosis plays an essential role in the development, homeostasis and pathogenesis of multiple diseases, including neurodegenerative disorders (Kroemer et al. 1997). TRXis not only an antioxidant but it can also regulate cell survival by binding and inhibiting the activity of apoptosis signal-regulating kinase 1 (ASK-1) by formatting TRX-ASK-1 "inhibitory complex, while TRX has been identified as a member of MAPKK family (Lu and Holmgren 2012). Thus, under stress condition, TRX can dissociate from ASK-1 and gain a kinase activity to activate JNk and p38 MAPK signalling pathway leading to apoptosis (Nishiyama et al. 1999). In this regard, TRX-1 exerts its potent antiapoptotic effect by direct (TRX-1/ASK1 interaction) and indirect (antioxidant) mechanisms (Lu and Holmgren 2012).

Thioredoxin superfamily proteins contribute to protein homeostasis through protein folding. These proteins, including protein disulphide isomerases and the Dsb protein family, contain TRX folds, and are major players in oxidative protein folding especially in oxidative cellular compartments such as the endoplasmic reticulum (Lu and Holmgren 2014b).

2.6.2 Glutathione system

The glutathione (GSH) system (GSH, glutathione reductase and glutathione peroxidase) is the most abundant intracellular thiol-based antioxidant system. GSH is present in millimolar concentrations in all living aerobic cells. It primarily acts as a sulfhydryl buffer, but it also aids in the detoxification of compounds through conjugation reactions that are catalysed by glutathione S-transferase (van Bladeren 2000, Nordberg and Arner 2001). GSH is known to directly detoxify hydrogen peroxide in the glutathione peroxidase (GPx) - catalysed reaction. In reactions where GSH is involved, it gets oxidized to GSSG. The oxidized glutathione (GSSG) is then reduced by the NADPH-dependent glutathione reductase (GR) (Kanzok et al. 2000). In some instances as in the case of E.coli and Drosophila, GSSG is reduced by TRX (Kanzok et al. 2000). Glutathione peroxidase and reductase are found in the cytoplasm, mitochondria and nucleus. Glutathione peroxidase metabolises hydrogen peroxide and also other peroxides by using reduced glutathione as a hydrogen donor (Radak et al. 2013, Santini et al. 1997). Glutathione disulphide is recycled back to glutathione by glutathione reductase, using the cofactor NADPH generated by glucose 6- phosphate dehydrogenase.

Hyperglycaemia induces oxidative stress in various brain regions (Komboj et al. 2008a). The increase in free radical generation along with depletion of antioxidants contributes to diabetes-induced oxidative stress. Oxidative stress-induced decrease in tissue or systemic GSH and damage to the vascular endothelium of the blood-brain barrier such as in diabetes or stroke has important implications for brain homeostasis (Buşu et al. 2013). A large reduction in GSH content was found in the cerebral cortex(27%), followed by the brain stem (19%) and cerebellum (18%) (Kamboj et al. 2008b). GSH protects tissue from oxidative damage in diabetes, which impairs the antioxidant system. Oxidation of GSH to GSSG results in decreased intracellular GSH levels. Because GSSG is actively transported from the intracellular to extracellular compartment (Sen et al. 1993), in diabetes, glutathione concentration has been decreased in the liver (Thompson and McNeill 1993, Melo et al. 2000, Rauscher et al. 2001, Sanders et al. 2001), kidney (Aragno et al. 1999) or increased in pancreas (Abdel-Wahab and Abd-Allah 2000), plasma (Montilla et al. 1998), red blood cell (Montilla et al. 1998), nerve (Obrosova et al. 1999) of chemically induced diabetic animals.

Most importantly, diabetes leads to decreased levels of GSH in brain tissue (Baydas et al. 2002, Celik and Erdogan 2008, Kamboj et al. 2008a). Furthermore, glutathione peroxidase activity was elevated in liver (Aragno et al. 1999, Rauscher et al. 2000b, 2001), kidney (Aragno et al. 1999, Rauscher et al. 2000a, 2000b, 2001), aorta (Kocak et al. 2000), pancreas (Jang et al. 2000), blood (Kedziora-Kornatowska et al. 1998), and red blood cells (Sailaja Devi et al. 2000), whereas decreased activity was found in the heart (Kaul et al. 1995, Kaul et al. 1996), retina (Obrosova et al. 2000), and brain (Celik and Erdogan 2008) in diabetic animals. Similarly, glutathione reductase and cellular glutathione is reduced in retina (Melo et al. 2000) and plasma (Mohan and Das 1998) but increased in heart (Rauscher et al. 2000b, 2000a, 2001, Sanders et al. 2001) of diabetic animals. In brain tissue, the GPx and GRD enzyme response to diabetes has been reported either unchanged, increased or decreased (Aliciguzel et al. 2003, Ulusu et al. 2003, Kamalakkannan and Prince 2006, Nazaroglu et al. 2009). Variations of GSH and GSH-related enzyme responses to diabetes among different tissue may partly be explained by the diverse levels and activities of these antioxidants in various tissues. It has been demonstrated that GSH level was greater in the liver than the brain and muscle (Saicic et al. 1997).

2.6.3 Other antioxidant enzymes

The other antioxidant systems include the antioxidant scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT). Isoforms of SOD are variously expressed within the cell. Cu,Zn-SOD is found in both the cytoplasm and the nucleus. Mn-SOD is confined to the mitochondria, but can be released into extracellular space, while most of SOD activity in extracellular fluids belongs to a different type of Cu,Zn-SOD called extracellular SOD (Reiter et al. 2000). Catalase, located in peroxisomes, decomposes hydrogen peroxide to water and oxygen (Winterbourn 1993). Catalase is responsible for the catalytic decomposition of H_2O_2 to O_2 and H_2O.

The published data on the effects of experimental diabetes on Cu,Zn-SOD or CAT activities in tissues have been inconsistent (Sechi et al. 1997, Huang et al. 1999, Hunkar et al. 2002, Ozkaya et al. 2002, Aliciguzel et al. 2003) and are likely to be dependent on several factors, including differential baseline expression of these enzymes and techniques used to generate diabetes in animals. The decreased catalase activity in diabetes might reduce the protection against free radicals. It is clear that the simultaneous reduction in the activity of both SOD and catalase makes the brain more vulnerable to hyperglycaemia-induced oxidative stress. Previous studies demonstrate that that catalase activity elevated in heart (Kaul et al. 1995, Kaul et al. 1996, Rauscher et al. 2001) and aorta (Ozansoy et al. 2001) as well as brain (Aragno et al. 1999) of diabetic animals. Another study showed that the SOD activity decreased in the striatum and amygdala, whereas the CAT activity increased in the hippocampus in diabetic animals (Ceretta etal.2012). A decrease in SOD and an increase inCAT activities in the brain of rats submitted to the chronic mild stress procedure has also been demonstrated (Lucca et al. 2009), while Samarghandian et al. (2014) reported lower SOD and CAT activities in hippocampus of diabetic rats. In fact, the activities of antioxidant enzymes depend on the brain portion. The effect of diabetes on the activity of SOD, in liver was depressed by the third or fourth week (Aragno et al. 1999, Maritim et al. 2002) of diabetes, but was either normal (Mekinova et al. 1995) or elevated (Melo et al. 2000) at 8 weeks of STZ induced diabetes. Cardiac SOD activity decreased after 4-8 weeks of diabetes (Kaul et al. 1995, Kaul et al. 1996), on the other hand it is reported to be elevated at 32 weeks (Kocak et al. 2000, Ozansoy et al. 2001). Another study showed that decreased SOD activity in rat hippocampus and cerebral cortex after 10 weeks hyperglycaemia was detected

(Kuhad and Chopra 2007). An imbalance in the SOD/CAT ratio indicates the generation of reactive oxygen species and could closely be associated to the pathophysiology of diabetes.

Exercise training resulted in increased activities of SOD and GPx in the brain such as the stem and corpus striatum (Somani et al. 1995). On the other hand a single bout of exercise, caused oxidative damage to skeletal muscle (Radak et al., 1995), liver and kidney (Radak et.al.1996), but had no damage to the brain (Radak et al 1995). In addition, the activities of antioxidant enzymes Cu,Zn-SOD, Mn SOD, CAT, GPx had no alterations by an exercise session in brain of rats (Radak et al. 1995). However, exercised rats with diabetes have shown decreased Cu,Zn-SOD and GPx activities in brain (Ozkaya et al. 2002).

2.6.4 Alpha-lipoic acid

Alpha-lipoic acid (ALA), also known as thioctic acid, is a naturally occurring compound that is synthesized in small amounts by plants and animals, including humans (Reed 2001).Endogenously synthesized LA is covalently bound to specific proteins, which function as cofactors for several important mitochondrial enzyme complexes. In addition to the physiological functions of protein–bound LA, there is increasing scientific and medical interest in potential therapeutical uses of pharmacological doses of free LA (Smith et al. 2004). LA contains two thiol (sulphur) groups, which may be oxidised or reduced. The reduced form is known as dihydrolipoic acid (DHLA), while the oxidized form is known as LA (Smith et al. 2004). LA also contains an asymmetric carbon, meaning there are two possible optical isomers that are mirror images of each other (R-LA and S-LA). In endogenous biosynthesis, LA is synthesised de novo from an 8-carbon fatty acid (octonoic acid) in mitochondria, where protein-bound LA functions as an enzyme cofactor. Exogenous LA from the diet can be activated with ATP or GTP by lipoate activating enzyme, and transferred to LA-dependent enzymes by lipoyl transferase (Fujiwara et al. 2001). Lipoic acid is found in almost all foods, but significantly more so in kidney, heart, liver, spinach, broccoli and yeast extract. LA has been used as a dietary supplement for humans since 1990s, typically at dose in the range of 60-200 mg/day and this is approved by NOAEL (Cremer et al. 2006).

As for biological activities, R-LA is an essential cofactor for several mitochondrial enzyme complexes that catalyse critical reactions related to energy production and the catabolism of alpha-keto acids and amino acids (Sen and Packer 2000, Packer and Cadenas 2011). ALA and its reduced form dihydrolipoate (DHLA) are powerful antioxidants and have shown the ability to react with reactive oxygen and nitrogenspecies (RONS) such as hydroxyl radical, hypochlorous acid and single oxygen and reduce glutathione disulphide, tocopherol radicals and ascorbate. Moreover, ALA works as a redox regulator of myoglobin, prolactin, TRX, glucose transporter protein (GLUT4) and NF-kB transcription factor. The addition of LA protected cortical neurons against cytotoxicity induced by Abeta peptide and H_2O_2 and has been found to activate PKB/Akt-dependent signalling, resulting in increased survival of neurons (Zhang et al. 2001) and inhibited NF-KB nuclear translocation(Zhang et al. 2001). Moreover, LA has been found to enhance the nuclear translocation of Nrf2 and the transcription of genes containing AREs in vivo, including genes for GCL, the rate-limiting enzyme in glutathione synthesis (Suh et al. 2004).

DHLA is also a potent reducing agent with the capacity to reduce the oxidized forms of several important antioxidants, including vitamin C and glutathione (Jones et al. 2002). DHLA may also reduce the oxidised form of alpha-tocopherol (the alpha-tocopheroxyl radical) directly or indirectly, by reducing the oxidized form of vitamin C (dehydroascorbate), which is able to reduce the alpha-tocopheroxyl radical (May et al. 1998a, May et al. 1998b).

LA is also a water soluble biological antioxidant (Sen and Packer 2000, Packer and Cadenas 2011) that can increase glucose metabolism by enhancing glucose transport and the activity of pyruvate dehydrogenase (Konrad et al. 2001), as well as improve cognitive function and reduce brain oxidative stress (Liu et al. 2002, Farr et al. 2003) and amyloid peptide beta (Aß) induced neurotoxicity in hippocampal neurons (Lovell et al. 2003). Neuroprotective role of LA has also been confirmed in recent studies. Intraperitoneal supplementation of LA at a dose range of 50-150 mg/ kg was as effective as methylprednisolone sodium succinate (30 mg/kg) in neuroprotection after spinal cord injury (Sayin et al. 2013). LA treatment demonstrated beneficial effects, including decreasing capspase activity, lipid and protein oxidation, to protect neurons from central neurotoxicity caused by Ifosfamide (Ozturk et al. 2014). Moreover, LA is a potential antioxidant that acts as a cofactor in the pyruvate dehydrogenase complex and has shown to be beneficial in conditions associated with increased oxidative stress, such as peripheral diabetic neuropathy (Ziegler et al. 1999).

All the above mentioned properties make LA a very promising drug for treatment of neurological diseases whose ethology is related to oxidative stress (Biewenga et al. 1997, Farr et al. 2003). LA can protect cells from the deleterious effects of oxidative stress (Evans et al. 2002)and appears promising in terms of diabetes therapy, as it has been reported to act not only as an antioxidant, but also as a regulator of glucose metabolism (Konrad et al. 2001), including enhanced insulin sensitivity and accelerated glucose utilization by peripheral tissues (Muellenbach et al. 2008, Singh and Jialal 2008) as well as inhibition of hepatic gluconeogenesis (Konrad 2005). LA can directly modulate glucose metabolism in both insulin-sensitive and insulin-resistant muscle tissues. Interestingly, short-term exposure of L6 myocytes to LA causes a transient increase in p38 MAPK phosphorylation (Konrad et al. 2001), a finding we have confirmed in isolated rat skeletal muscle (Saengsirisuwan et al. 2001). Furthermore, it has been demonstrated that chronic treatment with LA elicits improvements in whole-body glucose tolerance and insulin sensitivity, as well as in insulin action on skeletal muscle glucose transports in insulin resistant obese Zucker rats (Saengsirisuwan et al. 2001, Saengsirisuwan et al. 2004). Chronic administration of LA to rats made hypertensive and insulin resistant as a result of high glucose feeding, results in reductions in systolic blood pressure, an increase in whole body insulin sensitivity, and a reduction in markers of oxidative stress (El Midaoui and de Champlain 2002). It has also been demonstrated that chronic administration of LA to diabetes-prone Otsuka Long-Evans Tokushima Fatty (OLETF) rats prevents the age-dependent development of hyperglycaemia, hyperinsulinemia, dyslipidemia, and plasma markers of oxidative stress (Song et al. 2005).

LA is a potent thiol redox modulator that has been used to treat diabetic complications such as neuropathy (Packer and Cadenas 2011, Rochette et al. 2014). It has decreased lipid peroxidation in liver, pancreas (Dincer et al. 2002), kidney (Obrosova et al. 2003), brain (Baydas et al. 2004) and blood vessels (Kocak et al. 2000) of STZ diabetic rats as well as in blood of diabetic patients with neuropathy (Androne et al. 2000). LA appears to prevent diabetes-evoked disturbances in glutathione homeostasis in blood, liver and kidney (Winiarska et al. 2008). Furthermore, LA has been shown to strengthen the GSH defence system by enhancing levels of GSH, GPx, GST and other endogenous antioxidant levels in many tissues(Khanna et al. 1999a, Obrosova et al. 2003, Winiarska et al. 2008), including the brain (Bilska et al. 2007, Derin et al. 2009). Treatment with lipoic acid increases GSH levels in vivo and in vitro (Han et al. 1997). GSH is an important water-soluble endogenous antioxidant and exists in reduced thiol (GSH) and oxidised disulphide forms (GSSG). Studies with human cells have provided insights into the mechanism through which lipoic acid increases GSH levels.

LA disulphide formation may play a key role in triggering the heat-shock response. It is likely that LA achieves this by catalysing the formation of intramolecular disulphides in certain signalling proteins that function as detectors of oxidants or electrophiles (McCarty 2001). In this regard, disulphide bond formation in certain proteins results in the denaturation of these proteins, and this in turn triggers the trimerisation of heat-shock factor-1 and the subsequent transcription of heat-shock genes (McCarty 2001). Indeed, heat shock proteins can suppress activation of the transcription factor, induction of these proteins may have an important function for preservation of macrovascular health in diabetes. Similarly, lipoic acid has been shown to down-regulate expression of inflammatory markers in vascular endothelial cells challenged with TNF (Zhang et al. 2001). As an antioxidant and regulator of carbohydrate metabolism, ALA modulates the activity of the NO/HSP system (HSP72 synthesis), one of the principal body protective system (Strokov et al. 2000). It has been suggested that LA might activate certain, yet unidentified, signalling intermediates by inducing intramolecular disulphide bond formation, a signal for oxidant exposure and function as an HSP inducer (McCarty 2001, 2006).

LA supplementation prevented exercise-induced oxidative stress in rat skeletal muscle (Chae et al. 2008) and exercised horses (Kinnunen et al. 2009a). In addition LA supplementation up-regulated the synthesis of HSP in the skeletal muscle of horses (Kinnunen et al 2009a). Simultaneously, LA supplementation up-regulated HSP60 levels in the myocardial tissue of nondiabetic animals and was capable of compensating the deleterious effects of oxidative stress and upregulating HSP synthesis in experimental diabetes in the liver and heart tissue (Oksala et al. 2006, Oksala et al. 2007). LA increased levels of HSF-1 mRNA and protein and HSP72mRNA, and reversed the induction of HO-1 and TGF-beta protein expression (Oksala et al. 2007). In addition, it has been shown that LA supplementation combined with aerobic exercise inhibited lipid peroxidation in skeletal muscles by increasing vitamin E, SOD and GPx activities in rats. On the other hand, it had no effect on heat shock-induced expression of HSP72 in Raw cells (Demarco et al. 2004). Moreover, LA-treated diabetic rats had lower levels of oxidative stress, as measured by lipid peroxidation and lower glycation levels of serum proteins and haemoglobin, while the RBCs exhibited increased activities of antioxidant enzymes and elevated levels of reduced glutathione. In RBCs, this was accompanied by decreased post-translational glycosylation by O-bound β-N-acetylglucosamine (O-GlcNAc) of the antioxidant enzymes superoxide dismutase and catalase and of heat shock proteins HSP72 and HSP90 (Mirjana et al. 2012).

2.7 Exercise-induced oxidative stress thiol antioxidant protection and redox regulation

Exercise-induced oxidative stress is associated with increased formation of free radicals, mainly due to increased O_2 consumption by active tissues. In biological tissues free radicals are increased after acute exercise, which coincides with the presence of tissue damage (Bloomer and Goldfarb 2004). Most of the O_2 consumed is used in the mitochondria for oxidative phosphorylation, where it is the reduced to water. However, a small amount of the O_2 consumed may leave the electron transport chain to produce ROS (Radak et al. 2013). ROS production due to heavy exercise training has shown to cause tissue damage, increase lipid peroxidation, protein carbonylation, increase serum creatine kinase and alter glutathione redox status. Regular exercise training at a moderate intensity positively alters the oxidative homeostasis of cells and tissues, by decreasing oxidative damage and increasing resistance to oxidative stress (Cooper et al. 2002). Also, regular exercise training causes adaptations in the antioxidant capacity, protecting cells against the harmful effects of oxidative stress, thus preventing cellular damage (Henriksen and Saengsirisuwan 2003)due

to the fact that exercise-induced ROS production is necessary for oxidative stress-related adaptations (Radak et al. 2013). Adaptation to oxidative stress in trained individuals is clearly evidenced by a decrease in DNA damage, by sustained levels of protein oxidation and by an increment of resistance against chronic administration of H_2O_2 (Radak et al. 2000). Therefore, endurance exercise protects various tissues against oxidative stress (Poso et al. 2002, Noble et al. 2008). In this context, response to regular exercise training represents elevated levels of several cytoprotective proteins, such as heat shock proteins and antioxidant enzymes (Smuder et al. 2011).

Endogenous thiol antioxidants thioredoxin and glutathione systems control the cellular redox state (Ji 2008, Radak et al. 2013). Among the antioxidant systems, GSH and its redox enzymes play important protective role in the cellular protection and were even proposed to be determinant of oxidative stress. Oxidative stress has been quantified in humans as the redox ratio of plasma GSH/GSSG (Jones 2006). Plasma GSH redox state in humans becomes oxidised with age, in response to oxidative stress under the chemotherapy, smoking, diabetes and cardiovascular disease. However, the redox ratio of plasma GSH/GSSG is not equilibrated with the larger plasma cysteine/cystine (Cys/CySS) pool, indicating that the balance of pro-oxidants and antioxidants cannot be defined by a single entity. The cellular thiol/disulphide systems, including GSH/GSSG, thioredoxin-1(-SH2/-SS-), and Cys/CySS, are not in redox equilibrium and respond differently to chemical toxicants and physiologic stimuli. Individual signalling and control of events occur through discrete redox pathways rather than through mechanisms that are directly responsive to a global thiol/disulphide balance such as that conceptualized in the common definition of oxidative stress. Therefore glutathione and thiol redox status can be a modulator of redox regulation not only in the basal state, but also physiologically increased oxidative stress condition during exercise-induced oxidative stress and its adaptations (training response).

During strenuous physical exercise, GSH is oxidised when oxygen consumption is increased many folds, and as a consequence, GSSG accumulates. The balance GSSG/GSH acts as a redox control to regulate sulphur switches in response to acute exercise-induced oxidative stress and also develop adaptation mechanisms to regular physical training (Sen 2000). GSSG levels and GSSG/GSH ratios are sensitive markers of oxidative stress, but the levels depend on the exercise type, intensity and training status of the organism. For instance, in the regularly trained horse GSSG and GSH-redox ratio in muscle did not change in response to exercise (Kinnunen et al. 2009ab). However, data on the role of endogenous GSH on endurance exercise performance are limited. Probably, GSH not only plays an important role in exercise-induced oxidative stress, but also affects exercise performance (Sen et al. 1994).

Among antioxidant enzymes in skeletal muscle, SOD, MnSOD and GPx activities increase with exercise training in an intensity-dependent manner (Higuchi et al. 1985, Powers et al. 1994a, Powers et al. 1994b, Ji 2008). GPx activity has also been shown to increase after endurance training (Lawler et al. 1993, Leeuwenburgh and Heinecke 2001). The training effect on CAT activity has been inconsistent and controversial (Meydani et al. 1993). Exercise training adaptation of antioxidant activity is due to altered gene expression, and both mRNA and enzyme protein levels are upregulated. The elevated antioxidant enzyme activities in response to training has demonstrated clear benefits in preventing oxidative stress in a variety of experimental models and pathogenic conditions, including SID in rats (Coskun et al. 2004).

The TRX (thioredoxin) system is a key player in redox homoeostasis in mammals, along with the GSH system. TRX system and glutathione are the main antioxidant system that reduce thiol (-SH) groups. An important effect of oxidative stress and inflammation is the upregulation of protective antioxidant genes (Radak et al. 2013). Similarly, in response to

oxidative stress, while TRX translocates from the cytosol into the nucleus to regulate the expression of various genes, the transcription factors require TRX reduction for DNA binding (Lillig and Holmgren 2007). Nevertheless, loss of cellular TRX-1 is known to result in elevated GSH levels (Carmel -Harel et al. 2000).

Moreover, after acute exercise, during recovery, TRX-1 activity correlated negatively with the GSSG /TGSH (ratio of oxidised GSH to total GSH) in skeletal muscle of horses (Kinnunen et al, 2009b).TRX is cytokine-like factor with radical-scavenging functions, and the regulation of cellular reduction/oxidation (redox) by TRX plays an important role in signal transduction and cytoprotection against oxidative stress and apoptosis (Lu and Holmgren 2012). Limited number of studies has shown that TRX contributes to antioxidant defence against oxidative stress induced by acute exercise of variable intensity and duration. In mouse peripheral blood mononuclear cells, 30 min of swimming exercise induced TRX protein expression 12 and 24 h after exercise (Sumida et al. 2004). Similarly, plasma TRX concentrations increased continuously during an ultra-marathon race (Marumoto et al. 2010).

There are conflicting results on the influence of exercise training on TRX levels. Short-term daily exercise for 3 weeks prior to transient brain ischaemia in rats decreased the severity of paralysis and impairment in forelimb motor coordination. Furthermore, exercise induced superoxide dismutase activity and reduced the infarct volume and the immunepositive brain areas for 4-hydroxy-2-nonenal-modified proteins and 8-hydroxy-2'-deoxyguanosine, with no influence on tissue TRX levels (Hamakawa et al. 2013). On the other hand, peripheral levels of TRX seem to respond more sensitively to exercise training: low-volume exercise training for 12 weeks attenuated oxidative stress and increased circulating TRX concentrations, and glutathione peroxidase activities in older adults (Takahashi et al. 2013).

TXNip inhibits the antioxidant function of thioredoxin, leading to inhibition of thioredoxin function. The interaction results in a shift of the cellular redox balance that promotes increased intracellular oxidative stress. Increased oxidative stress is associated with TXNip induction in STZ-induced diabetic nephropathy (Hamada and Fukagawa 2007). Therefore, TXNip may be a therapeutic target in diabetic nephropathy. Nevertheless, although, TXNip is implicated as a negative regulator of glucose uptake in skeletal muscle in vivo (Muoio 2007), there is a lack of information in the literature on the TXNip responses to acute exercise and physical training.

3 Aims and Objectives

The general aim of this thesis was to clarify the effects of exercise training and alpha-lipoic acid on brain endogenous thiol antioxidant homeostasis and HSP response in acute and chronic models of metabolic and oxidative stress induced by acute exhaustive exercise and experimental diabetes in rats.

The more specific objectives of these series of studies were:

1. To clarify whether experimental diabetes impairs the TRX and GSH systems in the brain and to assess if exercise training improves cellular redox status and antioxidant protection in brain.
2. To clarify if exercise training up-regulates the HSP response in brain tissue, and whether training can offset the adverse effects of experimental diabetes on the HSP response.
3. To test the antioxidant effects of the thiol LA on brain antioxidant protection, including the redox-sensitive thiol–based GSH and TRX systems during exercise–induced acute and diabetes-induced chronic metabolic and oxidative stress.
4. To test the potency of LA in enhancing the brain HSP response in diabetes and to gain insight on the mechanisms of diabetes-induced disruptions in brain protein synthesis.

4 Materials and Methods

The study design is presented in Fig 1 and Fig 2.Animal care and experimental procedure were in accordance with the Guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH publication N. 85-23, Revised 1985). The experimental protocol was approved by the Ethics Committee for Laboratory Animal Research of University of Kuopio, Finland.

4.1 ANIMALS

Original papers I and II. Twelve- week-old outbred male Wistar rats were used in both studies. The animals (n=24) were maintained at 22°C ± 2 °C with 12:12-h light-dark cycles and had free access to standard rat chow and water.

Original papers III and IV. Twelve weeks old male Wistar rats (n=48) were maintained at22°C ± 2 °C with 12:12-h light-dark cycles and access to standard chow and water ad libitum.

4.2 PREPARATION OF DIABETIC RATS

The animals were first randomly assigned to a non-diabetic control and a diabetic group. Diabetes was then induced by a single intraperitoneal injection of streptozotozin (STZ) (60 mg/kg, prepared in 0.1 mol/L citrate buffer, pH 4.5) as previously described (Atalay et al., 2004), which destroys pancreatic B cells, and is used in experimental models of type 1 diabetes (Wang et al., 1994). Diabetes of the STZ-injected animals was confirmed by glucosuria using glucose test strips (BM-Test-5L, Boehringer Mannheim, Mannheim, Germany) 1 week after the injection, and routinely repeated once a week throughout the study. In addition, blood glucose levels were measured at the end of the study in truncal blood collected immediately after decapitation using a commercial kit (Glucoquant Glucose/HK, Boehringer Mannheim) using the hexokinase reaction. Animals with sustained diabetes (glucosuria of at least 20 mmol/L 2 weeks after the STZ injection) and the non-diabetic control animals were further divided into respective training and non-training groups.

4.3 EXERCISE AND TRAINING PROTOCOL

Acute exercise to exhaustion was performed on a treadmill as described previously (Khanna 1999). A mild electrical shock was used intermittently to coerce the rats run. Exhaustion was identified as the loss of righting reflex of the animals when they were laid on their backs.

Exercise training protocol: The rats were trained on a treadmill for 8 weeks, 5 days a week as described previously (Gul et al., 2002). Briefly, after 1 week of familiarizing to the treadmill, training (5 days a week with 1.5 h/day) continued for a total of 8 weeks. All animals tolerated the training well and were able to increase the running distance and intensity according to the training protocol throughout the study. Response to exercise training was confirmed by increased citrate synthase activity in skeletal muscle as reported previously (Atalay et al., 2004).

4.4 TISSUE HARVESTING

The animals were killed by decapitation at rest or ~72 h after the last training session. Following decapitation, whole brains (cerebrum) of the animals were quickly removed, rinsed in ice-cold saline, blotted, placed in liquid nitrogen, and stored at -70°C until use.

4.5 LA SUPPLEMENTATION

LA was administered orally (150 mg · kg–1 · day–1) for 8 weeks, while the control animals received a matched volume of saline. Half the LA-supplemented and non-supplemented rats were sacrificed at rest by decapitation and the other half immediately after acute exhaustive exercise.

4.6 BIOCHEMICAL METHODS

Analysis of antioxidant enzyme proteins by Western blot. The expression of antioxidant enzymes at protein level was analysed by a Western Blot technique. First, the frozen whole brains were pulverized and homogenized under liquid nitrogen with a mortar and sonicated in a buffer containing 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM HEPES, 5 μM DTT, and 5 μM PMSF at 4°C. Protein levels of the brain extracts were quantified by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Equal amounts of total protein (30 μg/lane) were electrophoresed together with molecular weight markers on a SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Whatman, Kent, UK). Next, after blocking with 5% fat-free milk solution at 37°C for 60 min, the membranes were treated with the following antibodies. A rabbit polyclonal antibody to mouse thioredoxin-1 (TRX1), which recognizes both rat and mouse cytosolic TRX1 in Western blot application (IMCO, Stockholm, Sweden), was used. For the detection of glutathione peroxidase-1 (GPx1), an isoform-specific rabbit polyclonal antibody was used and tested not to recognize the GPx isoforms -2, -3, or -4, and for CAT, a rabbit polyclonal antibody was used (both polyclonal antibodies were purchased from Abcam, Cambridge, UK). For glutaredoxin (GRX), affinity-purified goat polyclonal antibody against human GRX-1 was used that also recognizes rat GRX-1 in Western assay (IMCO). Rabbit polyclonal Cu,Zn-SOD and Mn-SOD antibodies were purchased from StressGen (Victoria, CA). A polyclonal rabbit antibody against TXNip and the Cy5-conjugated secondary antibodies were from Zymed Laboratories (San Francisco, CA). For normalization of the data, a mouse monoclonal antibody to β-actin (Sigma, St. Louis, MO) was used as an endogenous control. The membranes were developed using an infrared imaging system (Odyssey, LI-COR Bioscience, Lincoln, NE). For clarity, the data are normalized to β-actin and expressed relative to values from the untrained nondiabetic group.

Analysis of gene expression. To analyse mRNA expression of TRX-1, TXNip, GRX-1, Cu,Zn-SOD, CAT, and cyclophilin B (CypB), a quantitative real-time RT-PCR was applied. Briefly, 100 mg of whole brain was first homogenized with Ultra-Turrax (Janke and Kunkel), and total cellular RNA was isolated using the Eurozol reagent (Euroclone, West York, UK) according to manufacturer's instructions. Nucleic acid concentrations were determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), and their integrity was checked with gel electrophoresis. One microgram of 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 set not to amplify genomic DNA. The primers were synthesized by Oligomer Oy (Helsinki, Finland) as follows (with GenBank accession number): TRX-1 (NM_053800) forward primer (-F) 5′-TTCCTTGAAGTAGACGTGGATGAC-3′ and reverse primer (-R) 5′-AGAGAACTCCCCAACCTTTTGAC-3′; TXNip (NM′001008767)-F 5′-CCTAGAAGACCAGCCTACAGGTGA-3′ and TXNIP-R 5′-CACAGCCATATTTCCCTTTGAAG-3′; GRX-1 (NM′022278)-F 5′-CGTGGTCTCCTGGAATTTGTG-3′ and GRX-1-R 5′-AAGACCCGAGGAACTGTTCTTG-

3′; Cu,Zn-SOD (NM′012880)-F 5′- AACGTTCTTGGGAGAGCTTGTC-3′ and Cu,Zn-SOD-R 5′-GGTCAAGCCGGTCTGCTAAG-3′; CAT (NM′012520)-F 5′-TTCAGAGGAAAGCGGTCAAG- 3′ and CAT-R 5′-CATTCTTAGGCTTCTGGGAGTTG-3′;CypB (NM′022536)-F 5′-GCCTTAGCTACAGGAGAGAAAGGA-3′and CypB-R 5′-TCCACCCTGGATCATGAAGTC-3′.For PCR analysis, the samples were amplified in duplicate using Brilliant SYBR Green Master Mix (Stratagene, La Jolla, CA, ,USA) with 200 nM of gene-specific primers and run on Mx3000P System (Stratagene) with the following program: a 10-min preincubation at 95°C, followed by 40 cycles of 15 s at 95°C, 20 s at 60°C, and 25 s at 72°C. The data were normalized relative to expression of CypB by the previously introduced algorithm (Pfaffl 2001). Unique amplification products and absence of primer-dimers were evaluated by melt curve analysis. To analyse mRNA expression of HSP60, HSC70, HSP72, HSP90, GRP75 and cyclophilin B (CypB) in brain tissue, a quantitative real-time RT-PCR was applied. Briefly, 100mg of brain tissue was first homogenized with Ultra-Turrax and total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, Maryland, USA). Nucleic acid concentrations were determined by a NanoDrop spectrophotometer (Nano- Drop Technologies, Wilmington, Delaware, USA) and their integrity was checked with gel electrophoresis. One microgram of RNA from each sample was then converted to cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, California, USA) and oligo(dT) primers (Promega, Madison, Wisconsin, USA). For PCR primer design, the annotated nucleotide sequences were retrieved from the GenBank database (National Center for Biotechnology Information, Bethesda, Maryland, USA), and BLAST searches were performed to identify unique stretches of nucleotide sequence, and not to amplify genomic DNA. The primers were synthesized by Oligomer Oy (Helsinki, Finland) as follows (shown in 50-30 orientation): HSP60 forward primer (-F) AAAGCTG AACGAGCGACTTG and reverse primer (-R) ATCACTT GTCCCTCCAACCTTC; HSC70-F AGCACCCAGGCCAG TATTG and HSC70-R CAGCATTCAACTCCTCAAATCG; HSP72-F CAACTGGCTTGACCGAAACC and HSP72-RAGCGCAAGCCTAGTCCACTTC; HSP90-F GTACGAAA CAGCACTCCTGTCTTC and HSP90-R ATCCTCATCAATACCTAGACCAAGC; GRP75-F ACGAGGATGCCCAAGGTTC and GRP75-R TGAATGGCAGCTCCAATGG;CypB-FGCCTTAGCTACAGGAGAGAAAGGA and CypBRTCCACCCTGGATCATGAAGTC.The samples were amplified in duplicate using Brilliant SYBR Green Master Mix (Stratagene) with 200nM of gene-specific primers, and run on an Mx3000P System (Stratagene) with the following program: a 10-min pre-incubation at 95 ^{0}C, followed by 40 cycles of 15 s at 95^{0}C, 20 s at 59 ^{0}C, and 25 s at 72^{0}C. The data were normalized relative to expression of CypB by the previously introduced algorithm (Pfaffl, 2001). Unique amplification products and absence of primer-dimers were evaluated by melt-curve analysis.

Assays for glutathione levels and antioxidant enzyme activity. For the determination of total (TGSH) and oxidized glutathione (GSSG), the whole brains were first homogenized on ice in brief burst by Ultra-Turrax in a 1:6 (wt/vol) dilution with cold 5% meta-phosphoric acid, centrifuged at 10,000 *g* for 15 min at 4°C, and the supernatants were collected and stored at -80°C. On the day of measurement, the supernatants were diluted in water and measured spectrophotometrically for TGSH and GSSG. Total GPx activity was determined with cumune hydroperoxide as substrate (Tappel 1978). Glutathione reductase (GRD) activity was determined as described by Carlberg and Mannervick (1985) in the presence of 50 mM Tris·HCl buffer with 1 mM EDTA, 2 mM NADPH, and 20 mM GSSG by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH per minute. TRX1 and TRX reductase-1 (TrxR1) activities were assayed using a commercially available kit

according to manufacturer's instructions (IMCO). Total SOD activity was determined according to Beuchamp and Frodovich (1971) and CAT activity essentially as described by Aebi (1984). Results are expressed as units (U) per milligram of protein, as nanomoles per minute per milligram of protein, or as micromoles per gram wet weight, where appropriate.

Analysis of stress proteins and eukaryotic elongation factors by Western blot. To analyse protein expression in brain, standard Western Blot techniques were used as previously described (Atalay et al., 2004; Oksala et al., 2006). First, the frozen whole brains were pulverized under liquid nitrogen with a mortar and sonicated in a buffer containing 25% glycerol, 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 20 mmol/L HEPES, 5 mmol/L DTT and 5 mmol/L PMSF at 4^0C. Protein extracts (30 mg of protein per lane) were electrophoresed together with molecular weight markers on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Millipore, Bedford, Massachusetts, USA). Next, after blocking with 5% (w/v) fat free milk solution at 37^0C for 60 min, the membranes were treated with monoclonal antibodies (Ab) against HSPs (all from StressGen, British Columbia, Victoria, Canada) recognizing the 60 kDa HSP (HSP60), HSP72 and the constitutive cognate form of the 70 kDa HSP (HSC70), the 90 kDa HSP (HSP90) and glucose-regulated protein 75 (GRP75). The polyclonal Ab for eukaryotic elongation factor (eEF)-1a and eEF-2 and EF-2 kinase were purchased from Santa-Cruz Biotechnology (Santa Cruz, California, USA). As secondary Ab, horseradish peroxidase-conjugated anti-mouse (Santa- Cruz Biotechnology) and anti-rat immunoglobulins (Zymed Laboratories, San Francisco, California, USA) were used, respectively. The membranes were developed with the enhanced chemiluminescence method (NEN Life Sciences, Boston, Massachusetts, USA) and quantified using image-analysis software (ScionCorp, Frederick, Maryland, USA). For clarity, all results are expressed relative to values obtained from the respective untrained non-diabetic control group. Analysis of protein carbonyls as indices of oxidative injury to evaluate the potential effect of diabetes or exercise training on markers of oxidative stress, protein carbonyls were measured using an ELISA method, as described previously (Oksala et al., 2007b), in brain tissue homogenates. The carbonyl contents are expressed as nmol of protein carbonyl in mg of total protein (nmol/mg protein).

Fig.1 Study plan for paper I and II

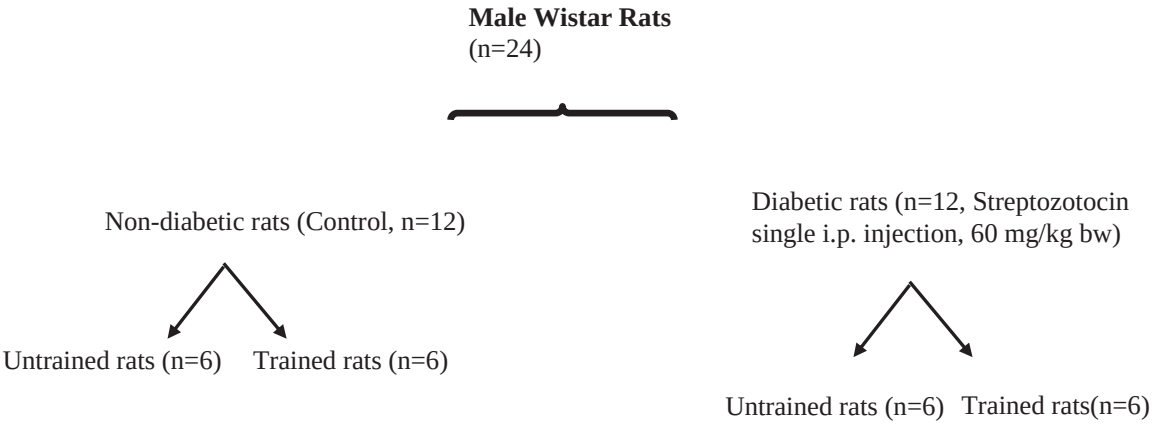
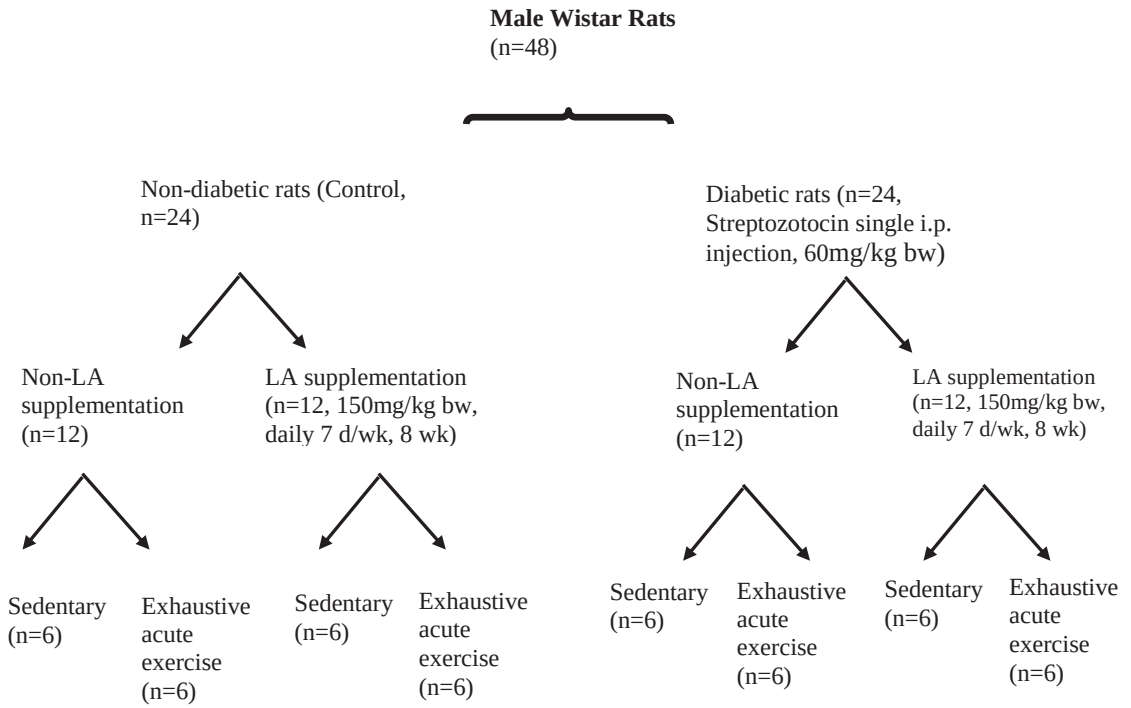


Fig 2. Study plan for paper III and IV



4.7 STATISTICAL METHODS

The data were analysed by SPSS software (SPSS, Chicago, IL). Means and standard errors of means (SEM) were calculated in all studies and multiple comparisons were performed. In original papers I and II.; Differences in continuous variables between the groups were assessed using Student's *t*-test. The effect of diabetes and endurance training was tested with two-way ANOVA with Bonferroni's correction was used. Correlation analyses were performed using the Pearson product-moment correlation coefficient. Statistical significance was considered at $P< 0.05$. In original papers III and IV; Differences between the groups were assessed using Student's *t* test, and effect of diabetes, exercise, and LA, using two-way ANOVA. Statistical significance was set at $p <0.05$.

5 Results

5.1 EFFECTS OF EXERCISE TRAINING ON THIOREDOXIN RESPONSE AND GLUTATHIONE STATUS AND ANTIOXIDANT PROTECTION IN EXPERIMENTAL DIABETES BRAIN (ORIGINAL PAPER I)

Blood glucose level. In animals with diabetes, blood glucose levels at rest were higher than in the non-diabetic rats (19.2 ± 3.8 vs. 8.8 ± 0.7 mmol/l, respectively) (P <0.001) and those described in rats without diabetes (7.0 ± 1.0 mmol/1).

Effects of diabetes and training on TRX System. TRX1 and TXNip levels did not differ between non-diabetic and diabetic animals, but exercise training increased TRX1 protein (P = 0.024) without affecting TXNip levels. On the other hand, diabetes inhibited the effect of training on TRX1 protein and also increased TXNip mRNA (P = 0.027), although training or diabetes had no effect on TRX1 mRNA, TXNip protein, or TRX1 and TrxR1 activities.

Effect of diabetes and training on glutathione and glutathione- related enzymes. Diabetes had no effect on TGSH levels but increased GSSG and the GSSG/TGSH ratio (P = 0.004 and P < 0.0001, respectively), whereas exercise training had no effect on these values. GPx activity was significantly increased by diabetes (P = 0.003), but GPx1 protein levels were not affected by diabetes or exercise training. On the other hand, both diabetes and exercise training significantly decreased GRD activity (P < 0.0001 and P < 0.0001, respectively), without affecting GRX1 protein and mRNA levels.

Effect of diabetes and training on other antioxidant enzymes. Exercise training on Cu,Zn-SOD, Mn-SOD, and CAT protein, and total SOD activity. The levels of Cu,Zn-SOD protein were increased in diabetic animals (P = 0.048), and exercise training increased this protein in both diabetic and non-diabetic animals (P = 0.005). However, neither diabetes nor exercise training had any effect on Cu,Zn-SOD mRNA, Mn-SOD protein, total SOD activity, or CAT.

Correlations between TRX protein and antioxidant enzymes. In nondiabetic untrained animals, TRX1 protein showed a strong positive correlation with Mn-SOD (r = 0.90) and GPx1 protein (r = 0.92), whereas in exercise-trained animals, TRX1 correlated strongly with Cu,Zn-SOD (r = 0.93) and Mn-SOD (r = 0.86), TXNip (r = 0.83), GRX1 (r = 0.82), and CAT (r = 0.96), but not with GPx1 (r = 0.148). Interestingly, GRD activity showed a strong negative correlation with TRX1 protein in exercise-trained animals (r = - 0.90). In untrained animals with diabetes, a significant correlation was only observed between TRX1 protein and total SOD activity (r = 0.88), and Cu,Zn-SOD protein (r = 0.96). In exercise-trained animals with diabetes, TRX1 showed a significant positive correlation only with CAT protein (r = 0.97) and a negative correlation with CAT activity (r = -0.98). No significant correlations with TRX1 and GSH or GSSG/GSH ratio were found in either diabetic or non-diabetic animals.

5.2 EFFECTS OF EXERCISE TRAINING ON OXIDATIVE STRESS PROTEIN RESPONSE IN EXPERIMENTAL DIABETES BRAIN (ORIGINAL PAPER II)

Effect of diabetes and training on protein carbonyls. The protein carbonyl contents were similar in diabetic and non-diabetic animals, and exercise training had no effect on these values.

Effect of diabetes and training on HSP mRNA and protein in the brain. To determine whether the expression of stress proteins is altered in diabetic brain, we first analysed the tissue levels of HSPs in non-diabetic and diabetic rats. At rest, the levels of all HSPs investigated did not differ between the groups, except GRP75 mRNA, which was slightly depressed (P = 0.03) in diabetic animals.

We also observed that in response to 8-week endurance training, all HSP mRNAs, excluding HSC70, were significantly up-regulated in diabetic animals, whereas HSP60, HSP90 and GRP75 mRNAs were increased in non-diabetic animals. Interestingly, diabetes significantly inhibited the effect of training on HSP72 and HSP90 proteins, whereas the levels of constitutive HSC70 protein were slightly, but non-significantly (P = 0.08) lower in diabetic animals. Training and diabetes showed an interaction only for HSP72 and HSP90 proteins, whereas at the mRNA level, this interaction was observed on HSP72, HSP90 and GRP75.

Effect of diabetes and training on key elongation factors. The levels of elongation factor eEF-1 and eEF-2 were similar in diabetic and control animals (P = 0.39). Exercise training increased eEF-1 and eEF-2 kinase levels slightly, but non-significantly, in diabetic animals only (P = 0.07 and 0.097, respectively).

5.3 EFFECTS OF ALPHA LIPOIC ACID SUPPLEMENTATION ON BRAIN REDOX STATUS AND THIOL ANTIOXIDANT PROTECTION RESPONSE AFTER STRENUOUS ACUTE EXERCISE IN DIABETES (OROGINAL PAPER III)

The TRX system. Exhaustive exercise increased TRX-1 mRNA levels in both diabetic and non-diabetic animals ($p < 0.001$), whereas TXNip was not significantly altered. LA supplementation increased TXNip mRNA only in diabetic animals ($p = 0.013$). Diabetes increased brain TRX-1 and also TXNip mRNA ($p = 0.025$ and $p = 0.003$, respectively), but had no effect on the protein levels (Original paper I). Moreover, TRX-1 and TrxR1 activities were not altered by exercise, LA, or diabetes.

GSH and GSH-related enzymes. Total GSH levels were not affected by acute exhaustive exercise, diabetes, or LA. Similarly, neither exercise nor LA had any effect on brain GSSG or the GSSG:TGSH ratio. Increased levels of GSSG and the GSSG:TGSH ratio in diabetes (Original paper I) were not altered after treatment with LA. The protein levels of GSHPX were not significantly affected by acute exercise, LA, or diabetes. In contrast, diabetes significantly increased GSHPX enzyme activity ($p = 0.006$) and decreased GRD activity ($p = 0.001$), as previously reported (Original paper I). On the other hand, LA decreased GRD activity in both diabetic and non-diabetic animals ($p = 0.0048$). Acute exercise decreased the activity of GRD in non-diabetic animals ($p = 0.031$) but showed additive effects with LA by increasing GRD activity in animals with diabetes ($p = 0.001$). GRX-1 mRNA levels were increased by diabetes ($p = 0.029$) and by LA in diabetic animals ($p = 0.031$), but LA, acute exercise, and diabetes had no effect on the protein level.

Other antioxidant enzymes. Diabetes increased Cu,Zn-SOD mRNA expression ($p = 0.039$) but had no effect at the protein level. On the other hand, acute exercise increased Cu,Zn-SOD protein in non-diabetic animals ($p < 0.001$) without significant effect at the mRNA level. Although LA increased Cu,Zn- SOD mRNA in diabetic animals ($p = 0.039$), an increasing effect of LA and acute exercise on Cu,Zn-SOD protein was only observed in non-diabetic animals ($p = 0.014$). Nevertheless, Mn-SOD protein, total SOD activity, and CAT mRNA, protein, and enzyme activity were not affected by exercise, LA, or diabetes.

5.4 EFFECTS OF ALPHA LIPOIC ACID SUPPLEMENTATION ON STRESS PROTEIN RESPONSE TO ACUTE EXERCISE IN DIABETIC BRAIN (ORIGINAL PAPER IV)

Diabetes increased mRNA levels of the constitutively expressed HSC70 (p = 0.042) and decreased HSP90 (p = 0.046) and GRP75 (p = 0.039) mRNAs, but had no effect on the

respective protein levels. In addition, diabetes decreased eEF-2 protein levels ($p = 0.001$), whereas those of eEF-1 were not affected by any of the experimental conditions.

Stress protein response. Acute exercise was not found to affect HSP72 and HSP60 mRNA expression or protein levels, but to increase HSP90 protein levels ($p = 0.012$) and HSP90 mRNA ($p = 0.044$), and also GRP75 mRNA ($p = 0.042$) in non-diabetic animals. In addition, HO-1mRNA expression was significantly increased by exercise in non-diabetic animals ($p = 0.005$), but not in diabetic animals. LA supplementation increased HSC70 mRNA expression in diabetic animals ($p = 0.015$; interaction without exercise $p = 0.032$, and with exercise $p = 0.021$), but decreased in non-diabetic controls $p = 0.012$). On the other hand, LA supplementation had no effect on the levels of all analysed proteins as they remained unchanged. Similarly, HSP90 and GRP75 mRNA and protein levels, and HO-1 mRNA expression were not affected by LA supplementation, except for the slightly increased HO-1 mRNA expression in diabetic animals ($p = 0.045$), suggesting a possible interaction of LA with diabetes.

6 Discussion

6.1 EFFECTS OF EXPERIMENTAL DIABETES AND EXERCISE ON THE THIOREDOXIN SYSTEM

The findings of the present thesis (original paper I) showed that the levels of TRX1 and TXNip protein in brain were not affected by experimental diabetes. Importantly, exercise training increased TRX1 protein levels in nondiabetic brain, without any effect on TXNip levels. Moreover, acute exhaustive exercise induced TRX-1 mRNA expression in both diabetic and nondiabetic brain, but had no effect on TRX-1 protein levels (original paper III). Therefore, regular physical exercise may provide a safe and physiological manner to improve brain antioxidant status and redox regulation and possibly benefit brain health.

To our knowledge, our study is the first to measure the level of TRX in brain tissue in response to regular exercise training, and recent studies confirm our results. Consistent with our results, Hamakawa et al. (2013) showed that cerebral levels of TRX did not change with three weeks of treadmill exercise prior to stroke induced by transient middle cerebral artery occlusion. The same study also revealed a reduction in the infarct size, lipid and protein oxidation in response to endurance training (Hamakawa et al. (2013). On the other hand, peripheral levels of TRX seem to respond more sensitively to exercise training: low-volume exercise training for 12 weeks decreased systemic oxidative stress and increased circulating TRX levels in older adults (Takahashi et al. 2013). Nevertheless, TRX plays an essential role in cell function and protection by limiting oxidative stress directly via its antioxidant effects, and also indirectly by protein-protein interactions with key signalling molecules. It is also pivotal for growth promotion, neuroprotection, inflammatory modulation, antiapoptosis, and immune function (Mahmood et al. 2013). This aspect may be crucial for the maintenance of redox control and to trigger physiological adaptations during strenuous physical exercise when peroxide production is increased and redox control circuits are prone to be disrupted (Jones, 2006). TRX expression is affected by stress and protects against oxidative stress-induced apoptosis (Nakamura et al. 1997). On the other hand, unaltered TRX activity has been reported in brain in rats in response to ischaemia, although TRX protein levels were increased and sustained during ischaemia (Siu et al. 2004, Ma et al. 2012). We also found that TRX1 or TrxR1 enzyme activities were not affected by exercise training (original paper I). Thus elevations of TRX1 protein in response to exercise training may serve a normal physiological function in brain tissue, without concomitant increase inTRX1 or TrxR1 activity. On the other hand, it has been suggested that TrxR may be transiently or permanently inactivated by oxidants such as hydrogen peroxide (Arner and Holmgren 2000).

Diabetes inhibited the effect of training on TRX1 protein and also increased TXNip mRNA levels (original paper I). Experimental diabetes also seemed to be associated with an incomplete response to exercise training of TRX and TRX1 mRNA translation into protein , because the beneficial effects of exercise training on the TRX system were absent in diabetes. TXNip mRNA and protein levels have previously been reported to increase in peripheral neuronal cells of diabetic animals without an effect on TRX levels (Price et al. 2006). Increased TXNip levels has been found in response to high glucose concentrations in vivo in streptozotocin-induced diabetic mice kidney (Kobayashi et al. 2003) and in the skeletal muscle of prediabetic and diabetic humans (Parikh et al. 2007). In diabetes the overproduction TXNip impairs endothelial function, including the abnormal vascular blood flow resistance (Wongeakin et al. 2014). Elevated TXNip levels induce β-cell apoptosis,

whereas TXNip deficiency protects against diabetes by promoting β-cell survival (Shalev 2014).The redox-active site of TRX interacts with TXNip (Yamanaka et al. 2000) and TRX-TXNip, a redox-sensitive signalling complex, is a regulator of cellular redox status and has emerged as a key component in the link between redox regulation and the pathogenesis of diabetes (Yoshihara et al. 2014). Therefore, the increased binding of TXNip to TRX may account for the functional inhibition of TRX activity and contribute to oxidative stress in diabetes (Schulze et al. 2004, Yoshihara et al. 2014).This is of importance as TRX has also been shown to play a key role in regulating redox activation of some proteins, including those involved in DNA binding (Sumida et al. 2004).

6.2 EFFECTS OF EXPERIMENTAL DIABETES AND EXERCISE ON GLUTATHIONE REDOX-STATUS AND RELATED OTHER ANTIOXIDANT ENZYMES

In this thesis, increased levels of GSSG and the GSSG/GSH ratio in animals with diabetes were found, indicating altered redox status and increased oxidative stress (original paper I), but diabetes and exercise training did not have a significant effect on total GSH levels. Due to the fact that GSH and TRX systems would appear to compete with each other for intracellular reducing equivalents, there may be an underlying specificity and organization of the GSH and TRX –dependent redox-signalling events (Jones, 2008). Furthermore, GRXs catalyse introduction and removal of GSH, and an isotype of TrxR have been shown to display activity towards both TRX and GSH (Su et al. 2005). It is not well known, however, to what extent cross-talk with GSH might affect the ability and affinity of TRX to bind and interact with its protein targets, or its subcellular localization. Nevertheless, perturbations in thiol redox status and changes in antioxidant pools have been observed in clinical and in experimental diabetes, and diabetes induces alterations to GSH metabolism by decreasing total GSH (Jain and McVie 1994, Aouacheri et al. 2014) or increasing GSSG (Murakami et al. 1989, Winiarska et al. 2014) and the GSSG/ GSH ratio (Grunewald et al. 1993) in brain tissue (Mastrocola et al. 2005, Ashafag et al. 2014, Winiarska et al. 2014).

With respect to exercise training, increased (Somani and Husain 1996, Devi and Kiran 2004, Acikgoz et al. 2006, Zhang et al. 2012) or unchanged (Hara et al. 1997, Liu et al. 2000a, Coskun et al. 2005) GSH and GSSG levels have been reported in brain tissue. Furthermore, induction of oxidative stress by physical exercise may regulate the signalling pathways through TRX system (Hirota et al. 1997, Yodoi et al. 2002, Radak et al. 2013), possibly with involvement of the GSH system. The metabolism of GSH and TRX may also follow a tissue-specific pattern. Indeed, the GSH response in brain to exercise-induced oxidative stress was quite different from other tissues, with no significant response during exercise training (Liu et al. 2000a). However, it has been previously pointed out that the activity of antioxidant enzymes and their response to exercise training may differ according to the brain region (Somani and Husain 1996).

Experimental diabetes increased total GPx activity without affecting GPx1 protein levels or GRX mRNA and protein, whereas GRD activity decreased (Original paper I). GRD regenerates GSH from GSSG, and GPx works in concert with GSH in the decomposition of hydrogen peroxide or other organic hydroperoxides, resulting in the oxidation of GSH to GSSG. Hence, an increased GPx activity coupled with decreased GRD activity may explain the observed increased GSSG levels and GSSG/TGSH ratio in diabetic animals. Therefore, induction of GPx activity may be a response to increased peroxidative stress in diabetes despite the depressed GRD activity. Previously, variable antioxidant enzyme responses to diabetes have been noted in brain tissue, with findings of either unchanged, increased, or decreased GPx and GRD (Aliciguzel et al. 2003, Ulusu et al. 2003, Kamalakkannan and Prince 2006, Nazaroglu et al. 2009).

We also found that experimental diabetes increased Cu,Zn-SOD protein in brain, without any effect on total SOD activity or CAT (original paper I). The published data on the effects of experimental diabetes on Cu,Zn-SOD or CAT activities in tissue have been very inconsistent (Sechi et al. 1997, Huang et al. 1999, Hunkar et al. 2002, Aliciguzel et al. 2003, Vieira et al. 2014) and are likely to be dependent on several factors, including differential baseline expression of these enzymes and techniques used to induce experimental diabetes in animals. Furthermore, according to our results TRX1 correlated with other antioxidant enzymes, especially with SOD isoforms and CAT, which represent much of the overall antioxidant enzyme capacity. Also, correlation values appeared to be different between exercise-trained and untrained groups, but we cannot conclude that exercise training specifically targets CAT or SOD in addition to TRX1 (original paper I). Mn-SOD can be induced by TRX, with increasing the removal of highly reactive superoxide anions (Das et al. 1997). Thus, exercise training has the potential of increasing TRX1 levels in brain, which in turn may induce Mn-SOD. It is therefore possible that the cytoprotective effects of TRX1 could also be mediated in part through Mn-SOD.

Exercise training increased GPx activity in nondiabetic animals, whereas decreased GRD activity in both exercise trained groups (original paper I). GRD may therefore be more susceptible to inhibition due to the increased pro-oxidant tone during exercise. Nevertheless, the levels of Cu,Zn-SOD protein were slightly increased in all exercise-trained animals, but CAT mRNA, protein levels, and activity did not change. Consistent with exercise training effects, acute exhaustive exercise decreased GRD activity in the brain of nondiabetic animals, but not in diabetic animals (original paper III). This result again suggests a possibly higher aerobic performance and more exhaustive exercise in non-diabetic animals, which may result in a higher pro-oxidant tone and inhibition of GRD activity.

Similar to endurance training response acute exhaustive exercise increased Cu,Zn-SOD protein levels in nondiabetic animals but not in diabetic animals (original paper III). A few studies have indicated that acute exercise does not significantly alter antioxidant enzyme status in brain (Radak et al. 2001b), whereas others have found that exercise training differently altered CAT, GPx, GRD, and SOD activity in brain depending on the region investigated (Somani and Husain 1996, Somani and Rybak 1996). On the other hand, acute exhaustive exercise had no effect on SOD and GPx activities in the hippocampus, prefrontal cortex and striatum (Acikgoz et al. 2006). However, exercise training increased the SOD and GSH-Px activities during hypoxia (Li et al. 2013). Moreover, exercise training with caloric restriction elevated the GSH level but did not alter SOD activity in rat hippocampus (Santin et al, 2011). These results taken together indicate that antioxidant enzyme activity seems to vary from tissue to tissue, and the presence of diabetes may also affect enzyme activity.

6.3 HEAT SHOCK RESPONSE TO EXPERIMENTAL DIABETES AND EXERCISE

We found the levels of HSPs (original paper II) did not differ in non-diabetic and diabetic brain, except GRP75 mRNA which was decreased in diabetes. In study IV, we found that diabetes increased only HSC70 mRNA levels and decreased HSP90 and GRP75 mRNAs expression, but had no effect on the respective protein levels. In diabetes, Yuan et al. (2006) reported that levels of mitochondrial HSP60 levels were elevated in the hippocampal and hypothalamus region (Kleinridders et al. 2013) of the brain. Moreover, overexpression of HSP72 was shown to protect against both local and global cerebral iscahemia in vivo (Kelly et al. 2002, Tsuchiya et al. 2003). HSP72 has also been shown to protect against severe degenerative diseases of the nervous system such as Alzheimer's, Parkinson's, and polyglutamine diseases (Ingemann and Kirkegaard 2014). Kim et al. (2013) showed that the

HSP72 overexpression improved neurological function and decreased brain haemorrhage in experimental traumatic brain injury. On the other hand, HSC70 and HSP90 expressions remained unchanged (Yamagishi et al. 2001, Hojlund et al. 2003). Recent studies showed that hippocampal HSP 70 and HSP 90 expression were similar in diabetic and nondiabetic mice (Mastrrocola et al. 2012). On the contrary, HSP 25 protein expression was greater in nondiabetic mice brain (Mastrocola et al. 2012). However, experimental evidence suggests a protective effect of HSP72 in peripheral diabetic neuropathy (Biro et al. 1997), although decreased (Atalay et al. 2004, Chen et al. 2005) or unchanged (Yamagishi et al. 2001) levels have also been reported in diabetic tissue, including the brain. Therefore, studies indicated that diabetes may exert variable and tissue-specific effects on HSP expression.

Results of study II showed unaltered HO-1 mRNA expression in the diabetic brain. In contrast, it has been reported previously that HO-1 mRNA expression levels decreased in the skeletal muscle of type 2 diabetic subjects whilst levels increased in the peripheral blood lymphocytes (Bruce et al. 2003, Calabrese et al. 2007) and in the kidneys of diabetic rats (Oksala et al. 2006). Also, the chronic induction of HO-1 reduced hyperglycemia, improved glucose metabolism and, at least in part, protected the renal tissue from hyperglycaemic injury, possibly through the antioxidant activity of HO-1 (Ptilovanciv et al. 2013). Despite its crucial role as a mediator of antioxidant and tissue-protecting actions, the functional significance of HO-1 in diabetes remains vague.

In study paper II and IV, exercise and exercise training induced brain HSP72, HSP60, HSP90 and HSC70 synthesis in non-diabetic rats, but not in diabetic rats, and this effect was blunted in diabetes. In contrast to our results, in an earlier studyHSP72 expressions in the heart and nucleus tractus solitarii of the brain were significantly increased in diabetic rats with exercise training (Hung et al. 2008). Furthermore, after administration of lipopolysaccharide, the survival time was significantly longer in diabetic rats with exercise training (Hung et al. 2008). These discrepancies between our results could be attributed to several factors, including severity and duration of diabetes, training intensity, time of sampling and assay procedures. In the study of Hung et al. (2008) the total duration of diabetes was 4 weeks, while in our study it was a minimum of 9 weeks. In contrast to our sampling time of 72 hours after the last training bout, in the study of Hung et al. (2008) sample collection was performed only 24 hours after the last training session, which may cause a mixed effect of both acute and chronic exercise on HSP levels.

In study IV findings showed that acute exhaustive exercise induced brain HSP90 protein and mRNA synthesis and also HO-1 and GRP75 mRNA expression in non-diabetic animals, but had no effect on diabetic animals. On the other hand, our study IV showed that the levels of HSC70, HSP72 and HSP60 protein or mRNA did not change in response to acute exhaustive exercise. Consistent with our result, other studies have also shown increased HSC70 levels in brain after intensive exercise (Sumitani et al. 2002) and also increased HSP72 levels after prolonged exercise (Lancaster et al. 2004). Therefore, acute exercise (Walters et al. 1998, Leoni et al. 2000, Febbraio et al. 2002, Lancaster et al. 2004), and habitual exercise induces HSP72 expression in specific brain areas (Campisi et al. 2003), suggesting a potentiating effect of physical activity on HSP72 expression. Our findings support a role for exercise-induced HSP72 response in brain tissue.

Mechanisms underlying the increased HSP response may include elevated body temperature, muscle damage and membrane stability changes that occur with exercise. During exercise, the temperature increase, oxidative stress and inflammatory response after endurance exercise stimulates the synthesis of HSP in peripheral blood leukocytes as well as in pulmonary macrophages (Fehrenbach et al. 2000). Thus, endurance training may act as a potential tool for enhancing chaperone-mediated cellular regulation in the brain.

Interestingly, we found that diabetes inhibited the effect of exercise training on HSP72 induction at the protein level, whereas the mRNA level was up-regulated. Although no comparable study result on training is available in brain tissue, other studies have shown an accumulation of myocardial HSP72 following heat stress in both diabetic and non-diabetic animals (Joyeux et al. 1999, Swiecki et al. 2003). This discrepancy may be explained by tissue-specific differences, the duration or severity of diabetes, type of stressor, or other still unknown factors. Moreover, because endurance training up-regulated HSP72 and HSP90 protein levels in non-diabetic animals, it is likely that these proteins play an important role in protecting brain tissue during stress as suggested earlier (Stahnke et al. 2007), but in diabetes, this response was inhibited. We also found significantly increased HSP60 and GRP75 mRNA expression in response to training in both diabetic and nondiabetic animals, although the protein levels remained unchanged, providing additional evidence that not all mRNAs are translated into protein. Therefore, experimental diabetes impairs the HSP response at the protein level.

The results of study II presented in this thesis show that protein carbonyl content in the brain was not affected by training or diabetes. This suggests that oxidative stress may not explain the differential effect of exercise training on HSP expression in the brain of diabetic and non-diabetic animals, although exercise training induced protein carbonyl content in the heart, liver and skeletal muscle in SID rats in a previous study by our group (Atalay et al. 2004). However, our findings are consistent with a previous study (Radak et al. 1995) suggesting that brain tissue may be less susceptible to exercise-induced to oxidative damage.

The elongation step of protein synthesis may also be affected, especially with oxidant compounds (Parrado et al. 2003). Many pathologic states, including diabetes, have been associated with changes in the elongation factors (Kimball and Jefferson 1994). The initial level of mRNA translation into protein has been acknowledged for its crucial role in controlling net protein synthesis (Proud et al. 1982). In fact, experimental diabetes was shown to decrease the rate of peptide chain elongation, which was further associated with reduced levels of the elongation factor eEF-2 (Bergstedt et al. 1993). Nevertheless, in original paper II, the decrease (on average 7.5%) in total eEF-2 protein in diabetic animals was not significant, and exercise training had no significant effect on this protein. This result was also supported by original paper IV, in which acute exhaustive exercise had no effect on eEF levels. The eEF-2 kinase is highly specific for phosphorylation of eEF-2 and also inactivates eEF-2, and thus can modulate the rate of polypeptide chain elongation during translation. As such, diabetes may compromise stress protein response through impaired levels of eEF-2. Our findings also support this hypothesis as a defective mRNA translation in diabetes may contribute to the impaired HSP synthesis. Unfortunately, we could not analyse any functional modifications of this protein, such as phosphorylation.

6.4 EFFECTS OF LA SUPPLEMENTATION ON REDOX SYSTEM

In this thesis, study III showed that LA increased mRNA levels of TXNip and GRX-1 in diabetic animals, but had no effect in nondiabetic animals. LA supplementation increased the activities of thioredoxin reductase and glutathione reductase and total glutathione levels in horse muscle during the recovery from an acute bout of exercise (Kinnunen 2009b). The TRX and GRX antioxidant systems are in the first line of defence for maintaining cellular redox homeostasis (Holmgren et al. 2005). Moreover, a functional link between the two has been described in yeast, whereby a lack of TRX decreased GSH levels, which was compensated for by increased GRX expression (Garrido and Grant 2002). Nevertheless, our findings indicate that the potential effects of LA are unlikely to be mediated through the TRX system. Furthermore, in diabetic animals LA induced transcription of TXNip, an endogenous inhibitor of TRX-1, which is considered to have prodiabetogenic effects. In agreement with our results, the increase of TXNip gene expression in peripheral neuronal cells of diabetic animals is reported to be sustained after administration of LA (Price et al. 2006).

6.4.1 GSH and GSH-related enzymes

In study III LA did not improve GSH and GSSG levels or the GSSG:TGSH ratio under any of the experimental conditions, which is in agreement with previous reports in brain (Bilska et al. 2007, Derin et al. 2009), but in contrast to the effects in other tissues (Khanna et al. 1999a, Obrosova et al. 2003, Suh et al. 2004, Winiarska et al. 2008, Dinic et al. 2013) and in the brain of nondiabetic animals (Suh et al. 2004). On the contrary, LA has previously been reported to increase brain GSH levels in aged rats (Suh et al. 2004), raising the possibility that this discrepancy may be explained by age. However, we found that both LA and exhaustive exercise in fact decreased GRD activity in nondiabetic animals, but not in diabetic animals. LA has previously been shown to have beneficial effects on lipid peroxidation and to increase other GSH-related antioxidants, including GRD and GSHPX, as well as CAT and SOD in the skeletal muscle (Shin et al. 2008) and brain (Arivazhagan et al. 2001, Arivazhagan et al. 2002). Some of the differences in the effect of LA are likely to be tissue specific, although this does not explain the discrepancy between the current results and those reported by Arivazhagan et al. (2002). On the other hand, it has been proposed that orally administered LA may not readily cross the blood–brain barrier, thereby accounting for the lack of GSH response to LA in the current study (Chng et al. 2009). Nevertheless, it is very likely that systemic antioxidant and metabolic effects of LA indirectly influence brain antioxidant protection. Alternatively, LA may be subjected to direct metabolic modification in tissues, but another possibility for the variable effect on the GSH system may be related to dosage and administration route of LA.

In this thesis, LA and diabetes had an additive effect on Cu,Zn-SOD mRNA levels in the brain. On the other hand, contrasting results have been published on the effects of diabetes and LA on the enzymatic activity of GSHPX, GRD, SOD, and CAT in the kidney and liver (Obrosova et al. 2003, Winiarska et al. 2008, Dinic et al. 2013). It is thus likely that LA has a limited effect on these antioxidant enzymes in the brain, possibly because of poor blood–brain permeability for LA, and that acute exhaustive exercise produces a greater response in nondiabetic animals.

6.5 EFFECTS OF LA SUPPLEMENTATION ON HEAT SHOCK RESPONSE

Findings in study IV suggest that, except for increased HSC70 mRNA expression, oral LA treatment has no significant effect on brain HSP synthesis. Similarly, LA had no effect on the levels of HSP60, HSP25, or GRP75 in skeletal muscle in horse (Kinnunen et al. 2009b). On the other hand, LA does not seem to have negative effects via the blunted HSP response, as has been reported with some antioxidant supplementations (Khassaf et al. 2003). Interestingly, in original paper IV we found that LA supplementation increased HO-1 mRNA expression in diabetic animals. LA has previously been shown to increase HO-1 levels in vitro (Fujita et al. 2008), but reports in diabetic tissue are limited. LA also decreased HSC70 mRNA in non-diabetic rats, but had no effect on protein levels. Earlier, it has been postulated that high LA doses may enhance heat shock response via increased disulphide formation in certain target proteins at least in tissues other than brain (McCarty 2001). However, our findings suggest that LA supplementation has only limited effects on brain HSPs in non-diabetic and diabetic animals. It is, therefore, likely that the effects of LA are tissue and dose specific. On the other hand, we found that LA did not compromise the HSP induction, which is in contrast to reports using other antioxidants (Atalay et al. 2006). For example, vitamin C and E have previously been shown to attenuate or completely inhibit the exercise-induced increase of HSPs in circulation and in the skeletal muscle (Khassaf et al. 2003; Fischer et al. 2006).

LA had no effect on the levels of elongation factors, which is in line with the present data on HSP induction. Similarly, we observed that exercise had no effect on eEF levels, which is dependent on intensity and muscle fiber type during endurance exercise (Rose et al. 2009). Depressed levels of the key elongation factor in diabetes are suggestive of impaired HSP induction through decreased mRNA translation into the respective protein. Furthermore, antioxidant supplements may, in fact, prevent the induction of molecular regulators of insulin sensitivity and endogenous antioxidant defences during physical exercise in humans.

7 Future Aspects

The results reported in this thesis provide further insight for investigations on the molecular mechanisms of TRX-TXNIP-mediated redox signalling and tissue defence disturbances in diabetic brain. Additionally, because TXNip may promote apoptosis and LA has been previously shown to reduce apoptosis in some tissues, future studies could gain further insight into whether LA can protect against TXNip-induced apoptosis in diabetic brain. Moreover, further studies are needed not only to define the potential benefits of LA on brain cytoprotection, but also to determine the dose and timing for its maximum effectiveness induced by diabetes and exercise of the metabolic stress.

8 Conclusions

The present thesis has reported new information on the response of redox regulation systems and antioxidant and HSP defences during acute exhaustive exercise and exercise training in experimental diabetic and non-diabetic brain.

1. Exercise training up-regulated the HSP response in non-diabetic brain, but the HSP response was inhibited by diabetes in the brain.
2. Regular exercise training improved brain antioxidant status and redox regulation, but the beneficial effects of regular exercise on redox regulation were decreased by diabetes in the brain.
3. Endurance training may act as a potential tool for enhancing chaperone-mediated cellular protection and may provide means for improving brain health.
4. LA supplementation appeared to neither exert any benefits against the impaired antioxidant defence in diabetic brain nor to have any impact on the response of brain antioxidant proteins induced by a bout of exhaustive exercise.
5. LA induced transcription of TXNip, which may be considered an unfavourable effect.
6. LA did not seem to have any negative effects on the HSP response in the diabetic and non-diabetic brain.

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Zekine Pündük

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Protection against oxidative stress, a disruption of redox control of signalling and cellular events, depends on an orchestrated synergism between exogenous micronutrients and endogenous antioxidant defences. This study aimed to clarify the effects of exercise training and thiol supplementation on exercise-induced oxidative stress and protection, including endogenous antioxidant homeostasis and heat shock proteins in diabetic and non-diabetic rat brain.

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Publications of the University of Eastern Finland
Dissertations in Health Sciences

ISBN 978-952-61-1831-4