Genetic and Nutritional Preconditioning against Ischemia/Reperfusion Injury

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Genetic and Nutritional Preconditioning against Ischemia/Reperfusion Injury

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Voor Ronald en mijn ouders

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General introduction

Organ transplantation and ischemia/reperfusion injury

Organ transplantation is considered to be one of the greatest achievements in modern medicine during the last decades. Improvements in tissue typing for the human leukocyte antigens (HLA) class II antigens, organ preservation, surgery, and the use of immunosuppressive drugs made organ transplantation the treatment of choice for patients with end-stage organ failure.[1]

The success of organ transplantation has led to a marked increase in the number of transplantations. However, with only a limited number of transplantable organs available from living donors,[2] the demand for donor organs has far exceeded the supply. To expand the organ donor pool, the transplant community searched for alternatives e.g. through the use of donors after cardiac death.[3] Organs donated after cardiac death are considered less than optimal. The extended time from the termination of the circulation, to the actual moment of death, to organ recovery, and, finally, to the initiation of perfusion with cold preservation solutions[3, 4] reduces the quality of the graft by the initiation of irreversible ischemia/reperfusion (I/R) injury.

I/R injury is a complex phenomenon which is initiated by the insufficient supply of oxygenated blood to the organs (ischemia), followed by the re-initiation of oxygenated blood flow (reperfusion). The reduction in the energy source adenosine triphosphate (ATP) is thought to be one of the earliest consequences of ischemia.[5] ATP depletion compromises ATP-dependent processes including the activity of ATP-driven membrane pumps that normally maintain ion homeostasis by the generation of electrochemical gradients.[6] In turn, uncontrolled calcium fluxes enter the cell upon reperfusion,[7] and this ultimately results in cellular death.[8] Furthermore, the hydrolysis of ATP by ATP synthase (complex V of the mitochondrial oxidative phosphorylation [OXPHOS] system)[9] during ischemia may lead to the generation of reactive oxygen species (ROS) by the enzyme xanthine oxidase at the early reperfusion phase.[10] Alterations in the enzymatic activity of complexes I and III of the mitochondrial OXPHOS system by ischemia can result also in enhanced ROS formation upon reperfusion.[11-13] Excessive generation of ROS to levels above the normal scavenging capacity of the antioxidant defense systems[14, 15] induces oxidative damage to local macromolecules such as lipids, proteins[16] and DNA.[17] Accordingly, these oxidative modifications result in cellular pathology and eventually cell death.

During the late reperfusion phase, the production of pro-inflammatory cytokines, such as interleukin-6 and the expression of adhesion molecules such as P-selectin, facilitate the infiltration of neutrophils.[18] Neutrophils are another potential source for ROS generation. The influx of these cells during the late reperfusion phase exacerbates the tissue injury after the ischemic insult.[19]

Taken together, the damaging mechanisms of I/R result in profound tissue injury of the graft after transplantation, which can severely impair organ function. I/R injury during organ transplantation is considered to be a risk factor for the development of primary, or delayed non-function of the graft.[4, 20-22] In addition, graft dysfunction following organ transplantation

is associated with decreased graft and patient survival, and acute transplant rejection.[20, 23, 24]

Many studies have searched for means to reduce I/R injury, and identified a number of preventative mechanisms. For example, brief non-lethal periods of I/R, known as ischemic preconditioning (IPC), has been shown to protect organs from subsequent prolonged periods of I/R in several animal species,[25-27] as well as in humans.[28, 29] Conversely, other studies demonstrated that IPC fails to induce protection against I/R injury.[30, 31] Pharmacological preconditioning is another preventative mechanism that can induce protection against I/R injury in several animal models.[32-34] Results obtained from clinical trials, however, often fail to show a significant beneficial effect by pharmacological compounds that induce protection against I/R injury in laboratory animal models.[35] Since the treatment of I/R injury is still unsatisfactory, new therapeutic interventions to reduce or prevent I/R injury remain warranted.

Genetic preconditioning

Oxidative damage to DNA is an unavoidable consequence of ROS generated by normal cellular metabolism.[36] Such damage is thought to underlie normal aging.[37, 38] The relevance of oxidative modifications is illustrated by the conservation of elaborate intracellular defense mechanisms against ROS, including antioxidant enzymes.[14, 15] When DNA damage does occur, a number of mechanisms are available for repair.

Nucleotide excision repair (NER) is one of the most versatile repair mechanisms due to its ability to eliminate a broad spectrum of structurally unrelated lesions, including oxidative damage, by removing damaged nucleotides, and filling the gap with newly synthesized nucleotides.[39] The NER mechanism can be subdivided in 2 different sub-pathways: global-genome nucleotide excision repair (GG-NER; removes lesions from the entire genome), and transcription-coupled nucleotide excision repair (TC-NER; repairs lesions from the transcribed DNA strand).

A deficiency in one of the 2 sub-pathways of NER can lead to a variety of diseases of which some are characterized by symptoms of premature aging. For example, a mutation in the *CSB* gene results in a defective TC-NER mechanism. Humans carrying this mutated *CSB* gene develop Cockayne syndrome (CS); a photosensitive autosomal recessive disorder with characteristics of premature aging such as neurodegeneration, hearing loss, thin hear, and thin skin.[40, 41] In support of oxidative stress hypersensitivity in the etiology of CS, an increase in photoreceptor cell loss following whole-body ionizing radiation was observed in both $CSA^{-/-}$ and $CSB^{-/-}$ mice.[42] Thus, at least particular CS cell types appear hypersensitive to the effects of oxidative DNA damage.

Evidence of a beneficial adaptive response predicted to be protective against oxidative stress has been reported in short-lived NER-deficient mice. In addition to cerebellar ataxia and reduced lifespan, phenotypes of these mice include reduced body weight, body temperature, blood glucose and serum insulin-like growth factor-1 (IGF-1).[43-45] These latter physiological attributes are usually associated with extended longevity and enhanced resistance to stress, as in endocrine-deficient dwarf mice.[46] For example, hypopituitary Ames and Snell dwarf mice

have a lifespan significantly longer than their control littermates and are more resistant to acute oxidative stress, such as that induced by paraquat injection.[46]

Although the underlying mechanisms of protection from acute stress and extended longevity are unknown, they correlate with alterations in glucose metabolism including improved insulin sensitivity, and up-regulation of antioxidant enzyme defense mechanisms. This can be interpreted as an adaptive response to unrepaired endogenous DNA damage engaged to protect from further oxidative stress injury. One important prediction of this interpretation is that a TC-NER deficiency in a mouse model of CS genetically preconditions these mice, thereby increasing their resistance to acute oxidative stress, such as the stress caused by I/R. When a defective TC-NER mechanism indeed is associated with acute oxidative stress resistance, further elucidation of the underlying mechanisms may allow translation of these benefits to the clinic.

Nutritional preconditioning

Dietary restriction (DR) is the reduction of food intake while maintaining adequate nutrition. Life-long DR is considered to be the only non-invasive intervention that prolongs lifespan in a variety of organisms such as yeasts, worms, fruit flies, mice, and rats.[47] It is likely that these observations bear significance to humans since human volunteers on a low-calorie diet show alterations in their physiological and biochemical parameters that resemble those of food restricted laboratory animals.[48, 49] The fact that life-long DR increases the lifespan of a given species indicates that it slows down the aging process.

In 1956, Harman proposed a theory of aging, based on the recognition that metabolic use of oxygen is a major source of ROS.[36] Oxidative damage to cellular macromolecules including DNA, proteins, and lipids, is now accepted as a primary basis for aging.[37, 38] The attenuation of such oxidative damage by DR may be due either to a decreased rate of mitochondrial ROS production, an increased protection against oxidative stress, or an improved repair of oxidative damage. Many studies have shown that DR increases the activity of ROS scavenging enzymes such as catalase and superoxide dismutase.[50, 51] Moreover, it has been shown that long-term DR induces the ability of mitochondria to consume less oxygen and produce less ROS, while at the same time maintain their ATP production.[52] This indicates that DR improves the bioenergetic efficiency of mitochondria and the ability to repair oxidatively damaged DNA,[53] two factors which may be critical in lifespan extension.

A pathway critically involved in lifespan extension of all animal species that have been studied is the insulin/IGF-1 pathway. DR decreases both glucose and insulin and IGF-1 plasma levels throughout life.[54, 55] By maintaining low plasma insulin levels, DR effectively reduces insulin/IGF-1 signaling. The importance of insulin/IGF-1 signaling is shown by the fact that loss-of-function mutations of the insulin/IGF-1 system results in lifespan extension in worms,[56] and fruit flies.[57] Mice with a selective disruption of individual components of the growth hormone/IGF-1 axis, including growth hormone-deficient mice[58] and IGF-1 receptor-deficient mice,[59] also display an increase in lifespan.

It has been shown that embryonic fibroblasts isolated from IGF-1 receptor deficient heterozygous female mice are more resistant to H_2O_2 and paraquat toxicity than fibroblasts isolated from wild-type mice.[60] Livers from growth hormone-deficient mice contain elevated antioxidant enzyme levels, which are suppressed by exposure to growth hormone or IGF-1.[58] These findings, among others that have been documented, provide evidence that a reduction in insulin/IGF-1 signaling induces a beneficial adaptive response to oxidative stress. This adaptive stress response may not be limited to lifespan extension, but may occur also in response to the acute oxidative stress after I/R. In fact, preoperative pharmacological inhibition of rat sarcoma (ras), a protein functioning downstream of the insulin and IGF-1 receptors, with chaetomellic acid A, reduces an ischemic insult after renal I/R in uninephrectomised rats.[61]

Taken together, these findings suggest a common mechanistic basis between lifespan extension by DR and I/R injury, since both show enhanced resistance to oxidative stress mediated by downregulation of the insulin/IGF-1 signaling pathway. In addition, long-term DR has been shown to reduce focal ischemic brain damage[62] and myocardial oxidative stress[63] in rats. Whether DR is also able to reduce an ischemic insult to the kidney or liver is not known. Moreover, the length of time required to achieve such beneficial effects, as well as its application to short-term, clinically relevant endpoints remain largely unexplored.

Aims and outline of the thesis

The studies reported in this thesis describe two novel treatments to ameliorate I/R injury: genetic and nutritional preconditioning.

In **chapter 2**, we tested the hypothesis that a TC-NER deficiency in mouse models of Cockayne syndrome results in increased resistance to the acute oxidative stress following renal I/R injury. In addition, attempts were made to characterize the mechanism of protection by genetic preconditioning against renal I/R injury. In **chapter 3**, the effect and the kinetics of nutritional preconditioning on acute oxidative stress resistance were investigated in male C57BL/6 mice which either were restricted in their daily food intake (30% dietary restriction) for 2 or 4 weeks or fasted for 1, 2 or 3 days before their kidneys were subjected to renal I/R injury. In addition, by fasting mice for 1 to 3 days before induction of hepatic I/R injury, we studied the effects of nutritional preconditioning on the liver. To unravel the protective mechanism of nutritional preconditioning, we compared the transcriptional profiles of kidneys from 4-week 30% dietary restricted mice and 3-day fasted mice with those of *ad libitum* fed control mice at baseline (= before induction of renal I/R injury). In **chapter 4**, we investigated whether the protection against renal I/R injury by three days of fasting was mediated by preoperative changes in mitochondrial function. We studied the number of mitochondria, mitochondrial OXPHOS enzyme activity, mitochondrial respiration, mitochondrial permeability transition pore opening and voltage-dependent anion channel protein expression. In **chapter 5**, we investigated whether preoperative ingestion of a glucose solution during a 3-day fasting regimen interfered with fasting-induced protection against renal I/R injury, in order to allow a possible translation of the least intrusive form of food restriction to the clinic. In **chapter 6**, we aimed to study the effect of individual dietary nutrients in protection against renal I/R injury in mice by feeding these animals diets deficient in a specific dietary macro or micro-nutrient. A review of the literature on nutritional interventions in acute stress resistance of the liver in the clinical and animal experimental settings is described in **chapter 7**. In **chapter 8**, we investigated the mechanism of protection induced by three days of preoperative fasting on I/R injury of the liver, and determined its effect on liver regeneration after one-third or two-thirds partial hepatectomy. In **chapter 9**, a summary and discussion of the results of this thesis is presented and suggestions are made for future research.

References

1. Linden PK. History of solid organ transplantation and organ donation. Crit Care Clin 2009; 25: 165-184, ix
2. Klein AS, Messersmith EE, Ratner LE, et al. Organ donation and utilization in the United States, 1999-2008. Am J Transplant 2010; 10: 973-986
3. Steinbrook R. Organ donation after cardiac death. N Engl J Med 2007; 357: 209-213
4. Nicholson ML, Metcalfe MS, White SA, et al. A comparison of the results of renal transplantation from non-heart-beating, conventional cadaveric, and living donors. Kidney Int 2000; 58: 2585-2591
5. Jennings RB, Hawkins HK, Lowe JE, et al. Relation between high energy phosphate and lethal injury in myocardial ischemia in the dog. Am J Pathol 1978; 92: 187-214
6. Fekete A, Vannay A, Ver A, et al. Sex differences in the alterations of Na(+), K(+)-ATPase following ischaemia-reperfusion injury in the rat kidney. J Physiol 2004; 555: 471-480
7. McCoy CE, Selvaggio AM, Alexander EA, et al. Adenosine triphosphate depletion induces a rise in cytosolic free calcium in canine renal epithelial cells. J Clin Invest 1988; 82: 1326-1332
8. Halestrap AP. What is the mitochondrial permeability transition pore? J Mol Cell Cardiol 2009; 46: 821-831
9. Jennings RB, Reimer KA, Steenbergen C. Effect of inhibition of the mitochondrial ATPase on net myocardial ATP in total ischemia. J Mol Cell Cardiol 1991; 23: 1383-1395
10. Xia Y, Zweier JL. Substrate control of free radical generation from xanthine oxidase in the postischemic heart. J Biol Chem 1995; 270: 18797-18803
11. Rouslin W. Mitochondrial complexes I, II, III, IV, and V in myocardial ischemia and autolysis. Am J Physiol 1983; 244: H743-748
12. Paradies G, Petrosillo G, Pistolese M, et al. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. Circ Res 2004; 94: 53-59
13. Petrosillo G, Ruggiero FM, Di Venosa N, et al. Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin. Faseb J 2003; 17: 714-716
14. Plotnikov EY, Vysokikh MY, Tsvirkun DV, et al. Mitochondrial regulation of production of reactive oxygen species and nitrogen in rat cells of kidney during ischemia/reperfusion. Dokl Biochem Biophys 2005; 400: 80-83
15. Paller MS, Hoidal JR, Ferris TF. Oxygen free radicals in ischemic acute renal failure in the rat. J Clin Invest 1984; 74: 1156-1164
16. Erdogan H, Fadillioglu E, Yagmurca M, et al. Protein oxidation and lipid peroxidation after renal ischemia-reperfusion injury: protective effects of erdosteine and N-acetylcysteine. Urol Res 2006; 34: 41-46
17. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 2003; 25: 207-218
18. Monson KM, Dowlatshahi S, Crockett ET. CXC-chemokine regulation and neutrophil trafficking in hepatic ischemia-reperfusion injury in P-selectin/ICAM-1 deficient mice. J Inflamm (Lond) 2007; 4: 11
19. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. Faseb J 1990; 4: 3355-3359
20. Ojo AO, Wolfe RA, Held PJ, et al. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation 1997; 63: 968-974
21. Briceno J, Marchal T, Padillo J, et al. Influence of marginal donors on liver preservation injury. Transplantation 2002; 74: 522-526
22. Uemura T, Randall HB, Sanchez EQ, et al. Liver retransplantation for primary nonfunction: analysis of a 20-year single-center experience. Liver Transpl 2007; 13: p. 227-233
23. Terasaki PI, Cecka JM, Gjertson DW, et al. High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med 1995; 333: 333-336

24. McLaren AJ, Jassem W, Gray DW, et al. Delayed graft function: risk factors and the relative effects of early function and acute rejection on long-term survival in cadaveric renal transplantation. Clin Transplant 1999; 13: 266-272
25. Joo JD, Kim M, D'Agati VD, et al. Ischemic preconditioning provides both acute and delayed protection against renal ischemia and reperfusion injury in mice. J Am Soc Nephrol 2006; 17: 3115-3123
26. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136
27. Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. Circulation 1997; 96: 1641-1646
28. Teoh LK, Grant R, Hulf JA, et al. The effect of preconditioning (ischemic and pharmacological) on myocardial necrosis following coronary artery bypass graft surgery. Cardiovasc Res 2002; 53: 175-180
29. Jassem W, Fuggle S, Thompson R, et al. Effect of ischemic preconditioning on the genomic response to reperfusion injury in deceased donor liver transplantation. Liver Transpl 2009; 15: 1750-1765
30. Rahman IA, Mascaro JG, Steeds RP, et al. Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? Circulation 2010; 122: S53-59
31. Iliodromitis EK, Kremastinos DT, Katritsis DG, et al. Multiple cycles of preconditioning cause loss of protection in open-chest rabbits. J Mol Cell Cardiol 1997; 29: 915-920
32. Sayan H, Ozacmak VH, Sen F, et al. Pharmacological preconditioning with erythropoietin reduces ischemia-reperfusion injury in the small intestine of rats. Life Sci 2009; 84: 364-371
33. Yang CW, Ahn HJ, Han HJ, et al. Pharmacological preconditioning with low-dose cyclosporine or FK506 reduces subsequent ischemia/reperfusion injury in rat kidney. Transplantation 2001; 72: 1753-1759
34. Amersi F, Buelow R, Kato H, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 1999; 104: 1631-1639
35. Dirksen MT, Laarman GJ, Simoons ML, et al. Reperfusion injury in humans: a review of clinical trials on reperfusion injury inhibitory strategies. Cardiovasc Res 2007; 74: 343-355
36. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956; 11: 298-300
37. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science 1996; 273: 59-63
38. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature 2000; 408: 239-247
39. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature 2001; 411: 366-374
40. van der Horst GT, Meira L, Gorgels TG, et al. UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. DNA Repair (Amst) 2002; 1: 143-157
41. van der Horst GT, van Steeg H, Berg RJ, et al. Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell 1997; 89: 425-435
42. Gorgels TG, van der Pluijm I, Brandt RM, et al. Retinal degeneration and ionizing radiation hypersensitivity in a mouse model for Cockayne syndrome. Mol Cell Biol 2007; 27: 1433-1441
43. van de Ven M, Andressoo JO, Holcomb VB, et al. Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. PLoS Genet 2006; 2: e192
44. van der Pluijm I, Garinis GA, Brandt RM, et al. Impaired genome maintenance suppresses the growth hormone-insulin-like growth factor 1 axis in mice with Cockayne syndrome. PLoS Biol 2007; 5: e2
45. Niedernhofer LJ, Garinis GA, Raams A, et al. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 2006; 444: 1038-1043
46. Bartke A, Brown-Borg H. Life extension in the dwarf mouse. Curr Top Dev Biol 2004; 63: 189-225
47. Weindruch R, Walford RL: The Retardation of Aging and Disease by Dietary Restriction, Charles C Thomas Pub Ltd 1988

48. Walford RL, Mock D, Verdery R, et al. Calorie restriction in biosphere 2: alterations in physiologic, hematologic, hormonal, and biochemical parameters in humans restricted for a 2-year period. J Gerontol A Biol Sci Med Sci 2002; 57: B211-224
49. Heilbronn LK, de Jonge L, Frisard MI, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. Jama 2006; 295: 1539-1548
50. Longo VD, Finch CE. Evolutionary medicine: from dwarf model systems to healthy centenarians? Science 2003; 299: 1342-1346
51. Sun J, Folk D, Bradley TJ, et al. Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics 2002; 161: 661-672
52. Lopez-Lluch G, Hunt N, Jones B, et al. Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. Proc Natl Acad Sci U S A 2006; 103: 1768-1773
53. Guo Z, Heydari A, Richardson A. Nucleotide excision repair of actively transcribed versus nontranscribed DNA in rat hepatocytes: effect of age and dietary restriction. Exp Cell Res 1998; 245: 228-238
54. Masoro EJ, McCarter RJ, Katz MS, et al. Dietary restriction alters characteristics of glucose fuel use. J Gerontol 1992; 47: B202-208
55. Breese CR, Ingram RL, Sonntag WE. Influence of age and long-term dietary restriction on plasma insulin-like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. J Gerontol 1991; 46: B180-187
56. Wolkow CA, Kimura KD, Lee MS, et al. Regulation of C. elegans life-span by insulinlike signaling in the nervous system. Science 2000; 290: 147-150
57. Clancy DJ, Gems D, Harshman LG, et al. Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science 2001; 292: 104-106
58. Brown-Borg HM, Rakoczy SG, Romanick MA, et al. Effects of growth hormone and insulin-like growth factor-1 on hepatocyte antioxidative enzymes. Exp Biol Med (Maywood) 2002; 227: 94-104
59. Brown-Borg HM. Longevity in mice: is stress resistance a common factor? Age (Dordr) 2006; 28: 145-162
60. Holzenberger M, Dupont J, Ducos B, et al. IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature 2003; 421: 182-187
61. Sabbatini M, Santillo M, Pisani A, et al. Inhibition of Ras/ERK1/2 signaling protects against postischemic renal injury. Am J Physiol Renal Physiol 2006; 290: F1408-1415
62. Yu ZF, Mattson MP. Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. J Neurosci Res 1999; 57: 830-839
63. Chandrasekar B, Nelson JF, Colston JT, et al. Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2001; 280: H2094-2102

Chapter 2

Congenital DNA repair deficiency results in protection against renal ischemia/reperfusion injury in mice

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Abstract

Cockayne syndrome and other segmental progerias with inborn defect in DNA repair mechanisms are thought to be due in part to hypersensitivity to endogenous oxidative damage. The accelerated aging-like symptoms of this disorder include dysmyelination within the central nervous system, progressive sensineuronal hearing loss and retinal degeneration. We tested the effects of congenital nucleotide excision DNA repair deficiency on acute oxidative stress sensitivity *in vivo*. Surprisingly, we found mouse models of Cockayne syndrome less susceptible than wild-type animals to surgically induced renal ischemia/reperfusion injury, a multifactorial injury mediated in part by oxidative damage. Renal failure-related mortality was significantly reduced in $CSB^{-/-}$ mice, kidney function was improved and proliferation was significantly higher in the regenerative phase following ischemic injury. Protection from ischemic damage correlated with improved baseline glucose tolerance and insulin sensitivity and a reduced inflammatory response following injury. Protection was further associated with genetic ablation of a different Cockayne syndrome-associated gene, *CSA*. Our data provide the first functional *in vivo* evidence that congenital DNA repair deficiency can induce protection from acute stress in at least one organ. This suggests that while specific types of unrepaired endogenous DNA damage may lead to detrimental effects in certain tissues, they may at the same time elicit beneficial adaptive changes in others and thus contribute to the tissue specificity of disease symptoms.

Introduction

The case for the involvement of oxidative damage to macromolecules in the etiology of aging, aging-related disorders and so-called 'premature aging' disorders (here referred to as segmental progerias) is compelling. The free radical theory as originally proposed by Harman[1] and extended in recent years,[2] posits that reactive oxygen species (ROS), by-products of endogenous cellular metabolism, inflict damage on lipids, protein and DNA. Despite defense systems to prevent or repair such damage, oxidized macromolecules accumulate over time *in vivo* and are thought to underlie both normal and pathological aging. When combined with congenital defects in certain of these defense systems, for example, genome stability mechanisms, endogenous oxidative damage may also contribute to the symptoms of segmental progerias, which display some but not all of the characteristics of normal aging in accelerated or exacerbated forms.[3]

Nucleotide excision DNA repair (NER) is one such defense system that removes a range of damages, including helix distorting lesions induced either by exogenous ultraviolet radiation (e.g. pyrimidine dimers) or endogenous oxidative radicals (e.g. cyclopurines).[4] Two basic modes of lesion recognition have been defined. In the first, lesions occurring anywhere in the genome are recognized by damage-binding proteins such as the XPC-HR23A/B-CEN1 complex. This triggers the assembly of the multiprotein NER machinery, which functions via a cut and patch mechanism to remove the damage and fill the remaining single-strand gap.[5] In the second mode of recognition, transcription-coupled (TC)-NER, lesions that block an elongating RNA polymerase trigger assembly of the NER machinery in a process that depends on the chromatin remodelling protein CSB,[6] and a ubiquitin ligase complex containing the TC-NER specific protein CSA.[7, 8]

Defects in the *CSA* or *CSB* gene can give rise to Cockayne syndrome, a severe neuro-developmental disease marked by photosensitivity (but curiously without skin cancer predisposition), dysmyelination within the central nervous system, progressive sensineuronal hearing loss, retinal degeneration and cachectic dwarfism resulting in an aged appearance.[9] Mouse models lacking *CSA* or *CSB* function recapitulate some progeroid characteristics of the human disease, including cachexia and progressive loss of photoreceptor cells, but with a normal lifespan and an overall milder phenotype than in humans.[10-12] In support of the role of oxidative stress hypersensitivity in Cockayne syndrome etiology, an increase in photoreceptor cell loss following whole-body ionizing radiation was observed in both $CSA^{-/-}$ and $CSB^{-/-}$ mice.[13] Thus, at least particular CS cell types appear hypersensitive to the effects of oxidative DNA damage, a property that may underlie tissue-specific disease phenotypes.

Contrary to the notion of hypersensitivity to oxidative stress in Cockayne syndrome is the observation that mouse models of Cockayne syndrome and related NER-deficient segmental progerias (NER progerias) display characteristics of hypopituitary dwarf mutants or dietary restricted wild-type mice.[14-18] In dwarf and dietary restricted mice, these phenotypes, including reduced body weight and temperature, hypoglycemia, hypoinsulinemia and reduced serum

IGF-1, are correlated not only with extended longevity but also with resistance to acute oxidative stress.[19] In progeroid NER mice, these phenotypes have been interpreted as an adaptive response to unrepaired endogenous DNA damage engaged to protect from further oxidative stress injury.[20] One important prediction of this interpretation is that these mice should be resistant rather than hypersensitive to acute oxidative stress.

Ischemia/reperfusion (I/R) injury is a complex insult initiated by loss of blood to an organ. During the ischemic period, the lack of molecular oxygen as an electron acceptor in oxidative phosphorylation prevents ATP generation and compromises processes with high energy demand, such as maintenance of ion gradients across intracellular membranes. Reinitiation of oxygenated blood flow, or reperfusion, results in inappropriate activation of cellular oxidases and ROS generation, which can affect not only the reperfused organ but distant sites in the body as well.[21] Following reperfusion, a maladaptive inflammatory response mediated in part by the release of ROS from neutrophils infiltrating the tissue causes further injury.[22] In the kidney, I/R injury is associated with cell death primarily in the stripe between the cortex and medulla consisting mainly of tubular epithelial cells via necrosis or apoptosis, depending on the severity of the insult.[23] Recovery and a return to proper kidney function depends on regeneration of the tubular epithelial cells and remodeling of the renal tubules, a process which takes place in the days and weeks following the injury.

We used surgically induced renal I/R injury to test whether mouse models of Cockayne syndrome are susceptible to acute oxidative stress, as predicted by photoreceptor sensitivity to ionizing radiation, or protected from it, as predicted by physiological similarities between other short-lived progeroid NER mice and long-lived, stress resistant mice. Here, we report resistance to renal I/R injury in mouse models of Cockayne syndrome.

Materials and Methods

Animals

Mice were allowed free access to food and water throughout the experiments. All experiments were performed with the approval of the appropriate ethical board. Male $CSB^{-/-}$[10] and $CSA^{-/-}$[11] mice in a C57BL/6 background were bred at the animal facility of the Erasmus Medical Center; C57BL/6J mice were purchased from Harlan, Horst, the Netherlands.

Ischemia model

Mice between 12 and 16 weeks of age were anesthetized by isoflurane inhalation. Following a midline abdominal incision, a non-traumatic microvascular clamp was used to occlude the left kidney for 25 or 37 minutes. After the release of the clamp, a contralateral nephrectomy was performed.

Kidney functional measurements

Blood samples were collected by retro-orbital puncture. Serum urea and creatinine levels were measured using QuantiChrom assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICT-500, Gentaur, Brussels, Belgium) according to manufacturer's instructions, or were determined using an ELAN analyzer (Eppendorf Merck, Hamburg, Germany) with Ecoline S+ reagents (Diagnostic Systems GmbH, Holzheim, Germany) according to manufacturer's instructions.

Histology

Kidneys were harvested, bisected longitudinally, fixed for 24 hours in formalin and embedded in paraffin. Three-micrometer sections were stained with Hematoxylin and Eosin (H&E), modified Jones staining or periodic acid Schiff (PAS). Immunohistochemistry was performed on deparaffinized sections following antigen retrieval in boiling 10 mM sodium citrate.

mRNA expression analysis

Total RNA was extracted from frozen kidney using TRIzol reagent (Invitrogen, Breda, the Netherlands) and oligodT or hexamer-primed cDNA synthesized using SuperScript II (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. Quantitative real-time PCR was performed using an Opticon2 DNA Engine (MJ Research, Waltham, MA, USA) or a MyIQ (Bio-Rad B.V., Veenendaal, the Netherlands) with SYBR Green incorporation. Each sample was tested in duplo at least two times.

Glucose tolerance and insulin sensitivity tests

Mice were fasted overnight prior to testing. Following baseline blood glucose determination from tail blood of conscious, restrained mice, animals were injected with a bolus of glucose (1.5 mg glucose/gram body weight) or insulin (0.75 U kg^{-1} body weight) into the intraperitoneal cavity. Blood glucose determinations were performed at the indicated times following injection using a HemoCue glucose 201 RT blood glucose analyzer (HemoCue, Ängelholm, Sweden).

Statistics

The data are expressed as means ± SEM. Statistical analyses of data on urea, creatinine, histomorphology, immunohistochemistry and immunoblot were preformed using Student's t-test. Survival was analysed by Log-rank test (SPSSv11).

Results

Warm ischemia was induced in the left kidney of male wild-type and $CSB^{-/-}$ (subsequently reffered to as WT and CSB, respectively) mice for 37 minutes by clamping the renal artery and

vein with a non-traumatic surgical clamp. The 37-minute time point was chosen based on the results of preliminary experiments in which ischemia times were titrated between 30 and 45 minutes. Following clamp release, the undamaged right kidney was removed so that animal survival depended on the function of the damaged kidney; mice entirely lacking kidney function build up toxic compounds in their blood and die within 2-4 days. Survival of CSB mice was significantly higher than that of WT mice (Figure 1A). On postoperative day (POD) 7, survival was 56% in the CSB group but only 30% in the WT group ($P < 0.0033$).

Kidney function was determined by measuring the concentrations of urea and creatinine in the serum. High levels of these waste products indicate an inability of the kidneys to remove them from the blood and thus correlate inversely with kidney function. Interestingly, preoperative serum urea and creatinine values were slightly, but significantly lower in CSB mice (urea 8.5 ± 1.3 vs. 9.7 ± 1.2 mmol/L in WT mice, $P = 0.0007$; creatinine 95 ± 17 vs. 122 ± 34 µmol/L in WT mice, $P = 0.002$; Figures 1B-C). Following I/R injury, these markers of kidney injury rose with similar kinetics, suggesting maximal dysfunction on POD 2 and a return to function beginning on POD 3. However, average values were significantly lower in CSB animals on POD 1 for urea and on PODs 1 and 2 for creatinine (Figures 1B-C), consistent with better survival in the CSB group.

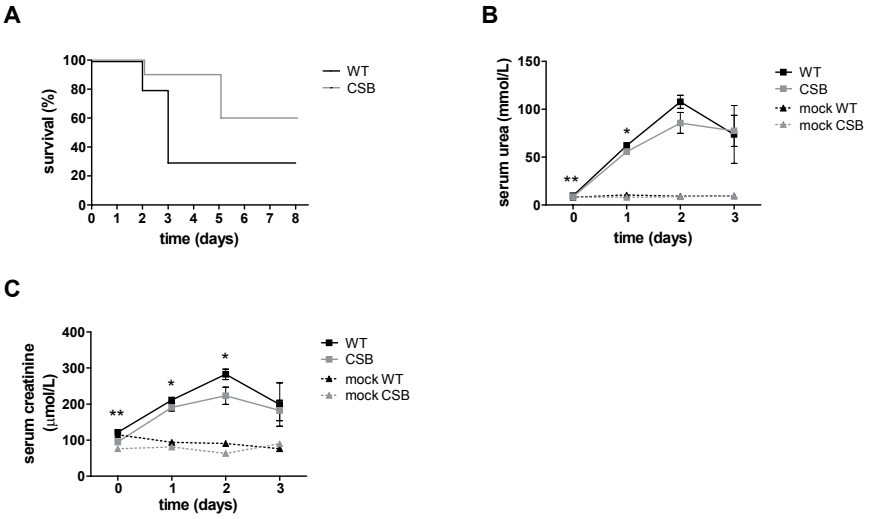

Figure 1. CSB mice are better protected against warm renal I/R injury than wild-type (WT) mice. (A) Kaplan Meier survival curves of CSB (n = 24) and WT (n = 40) animals; a log-rank score of $P < 0.0033$ indicates a highly significant difference between the genotypes. (B-C) Kidney function following I/R injury as determined by serum levels of (B) urea and (C) creatinine. The data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. WT mice at the indicated time point. Groups receiving a mock treatment that did not involve ischemia are indicated.

We next analyzed cell death at various time points after the injury to look for differences that

could explain the observed survival and functional benefits in CSB animals vs. WT controls. We used histology to score for acute tubular necrosis, the major form of cell death due to this type of injury (Figure 2A). In both groups, acute tubular necrosis peaked on POD 2; in the CSB group, average total cell death was lower at all time points, reaching statistical significance on PODs 1 and 2. The high mortality due to kidney dysfunction in the WT group prevented meaningful comparisons beyond POD 3.

Following cell death and clearance of cellular debris by shedding into the lumen of the tubules, kidneys damaged by I/R injury may undergo a regenerative phase in which cellular proliferation can be observed. Since the survival benefit of the CSB mice was likely only partially explained by the lower cell death on PODs 1 and 2, we next asked whether regeneration of the kidney was enhanced. We used three different assays to gauge this parameter: histology, or the presence of mitotic figures in H&E stained sections; immunohistochemistry against the proliferating cell nuclear antigen (PCNA); and PCNA immunoblotting of total kidney homogenates to assess total levels of this protein. In both groups, mitotic figures were first seen on POD 3, with significantly higher scores in CSB mice ($P = 0.025$; Figure 2B). We next looked at PCNA on the single-cell level by immunohistochemistry. The number of PCNA positive cells was significantly increased in the CSB group on POD 1 ($P = 0.035$; Figure 2C), possibly indicative of its additional role in DNA repair. We further analyzed PCNA levels in total

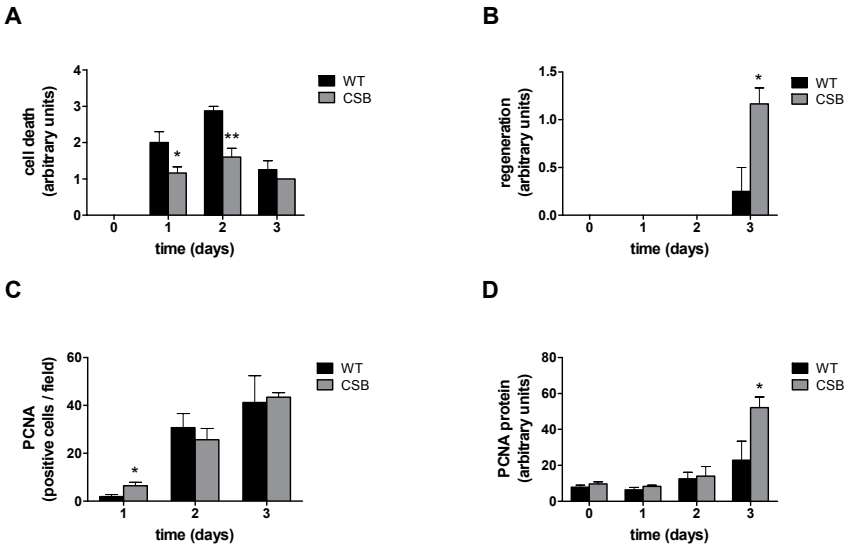

Figure 2. Cell loss and regeneration following 37 minutes of warm renal I/R injury. (A) Cell death as determined by H&E-stained paraffin sections on the indicated day following I/R. Day 0 kidneys were contralateral kidneys harvested immediately after clamp release. (B) Regeneration as determined by mitotic index in H&E stained sections. (C) Relative number of cells expressing PCNA as determined by IHC. (D) Total PCNA protein level as determined by immunoblotting on the indicated days following I/R injury. The data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. WT at the indicated time point.

kidney homogenates by immunoblot. Although preoperative PCNA levels were similar between groups, significantly higher levels of PCNA were observed on POD 3 in the CSB group ($P = 0.042$; Figure 2D). Taken together, these results are consistent with enhanced proliferation leading to better regeneration and survival in CSB vs. WT animals.

Previously, it was reported that wild-type C57BL/6 mice subjected to 30-50 minutes of warm ischemia to one kidney followed by contralateral nephrectomy had a 1-week survival of approximately 80%, with no statistically significant differences between ischemia times.[24] We consistently found mortality of approximately 70% due to kidney failure following 30-37 minutes of warm ischemia (Figure 1A and data not shown), making it difficult to study the course of organ recovery beyond POD3. To circumvent this problem, we repeated our experiments in WT and CSB mice using 25 minutes of warm renal ischemia. With this amount of ischemic damage, survival was 100% in both WT and CSB groups (data not shown). However, 25 minutes of ischemia resulted in significantly less kidney dysfunction in mice lacking the CSB protein relative to WT controls at all time points following ischemic damage as measured by serum urea (Figure 3A, left). A similar trend was seen for serum creatinine (Figure 3A, right).

Analysis of total cell death by histology revealed a significant elevation above preoperative values in CSB and WT (although less than after 37 minutes of warm ischemia) on all PODs examined, but no significant differences between genotypes (data not shown). Despite similar low amounts of cell death between groups, we observed large differences in proliferation following 25 minutes of warm renal ischemia. As with 37 minutes of ischemia, proliferation was elevated in CSB vs. WT mice. Regeneration scored by mitotic figures in histological sections was first seen in wild-type mice on POD 3, while regeneration was already significantly elevated relative to baseline in CSB mice on PODs 2 and 3 ($P = 0.035$ and $P = 0.021$, respectively; Figure 3B). The number of PCNA positive cells on PODs 1 and 2 was also significantly increased in the CSB group ($P = 0.005$ and $P = 0.002$, respectively; Figure 3C). Because PCNA is involved in DNA repair as well as proliferation, we stained sections for an additional proliferative marker, Ki-67, which is expressed during all active phases of the cell cycle (G1, S, G2 and mitosis) but is absent from resting cells (G0).[25] Ki-67 positive cells were also increased in CSB mice, with a maximum on POD 2 ($P = 0.016$; Figure 3D).

The cyclin-dependent kinase inhibitor p21, which binds stoichiometrically to PCNA and inhibits its action in DNA replication, is a key regulator of proliferation following renal ischemic injury. Mice lacking p21 display hyperproliferation and greatly reduced survival.[24] We tested p21 protein levels by immunohistochemistry (Figure 3E). Consistent with increased proliferation in CSB relative to wild-type on PODs 1 and 2, the number of p21 positive cells was significantly reduced in CSB mice (POD 2, $P = 0.016$). Interestingly, p21 staining was equal in wild-type and CSB on POD3, coincident with a decline in the first wave of proliferation following injury.

We next turned to potential mechanisms of protection against renal I/R injury specific to CSB animals. Addition of antioxidants is a proven way to ameliorate the effects of this injury.[26.] Furthermore, antioxidant capacity is increased in multiple organs in both genetic dwarfism

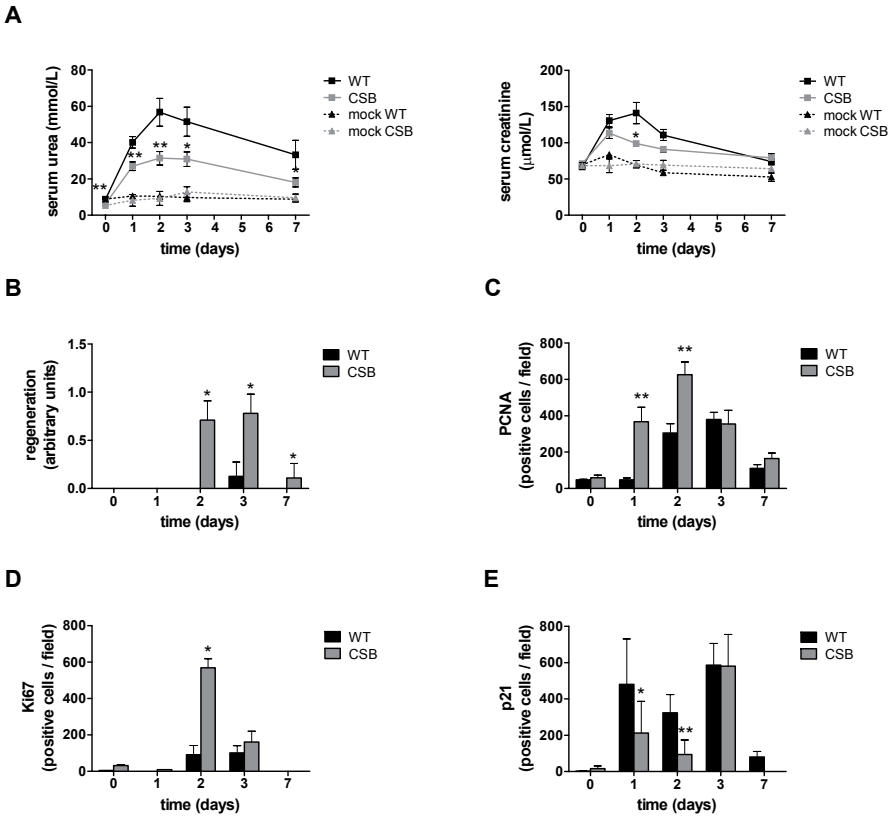

Figure 3. Kidney function, cell death and regeneration following 25 minutes of warm renal I/R injury in CSB and WT animals. (A) Kidney function of the indicated genotypes following I/R injury or mock treatment as determined by concentration of serum urea (left) or creatinine (right). The data are expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 vs. WT mice on the indicated day. (B-E) Regeneration as indicated by (B) mitotic figures in H&E stained sections and (C) immunohistochemistry against PCNA, (D) Ki67, and (E) p21. The data are expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 vs. WT mice at the indicated time point.

and dietary restriction, a property thought to contribute to stress resistance and increased longevity in these mice.[19] We thus looked for evidence of increased antioxidant capacity on the level of gene expression. We used quantitative real time-PCR (qRT-PCR) over a time course before and after renal I/R injury to analyze antioxidant defense capabilities as represented by the steady state levels of candidate mRNAs including superoxide dismutase 1, glutathione reductase and heme oxygenase-1 (Figure 4A and data not shown). At baseline, steady state amounts of these mRNAs were not significantly different between WT and CSB groups. Similarly, over a 24-hour period following 25 or 37 minutes of warm renal ischemia, some of these genes (e.g. heme oxygenase-1) were significantly up-regulated above baseline but none were consistently, significantly differentially regulated between CSB and WT groups (Figure 4A

and data not shown).

Another proven way to reduce the effects of I/R injury is to prevent the inflammatory response that follows tissue injury, for example, by neutralization of cellular adhesion molecules that serve to recruit neutrophils to the site of tissue injury by genetic or antibody-based methods.[27] We examined the expression of P-selectin and ICAM-1; endothelial adhesion molecules that are up-regulated upon tissue injury and whose expression correlates negatively with survival and functional outcomes. We observed a significant increase in both markers following renal I/R injury above baseline levels; this increase, however, was less in the CSB animals (Figure 4A). An area under the curve analysis revealed significant differences in

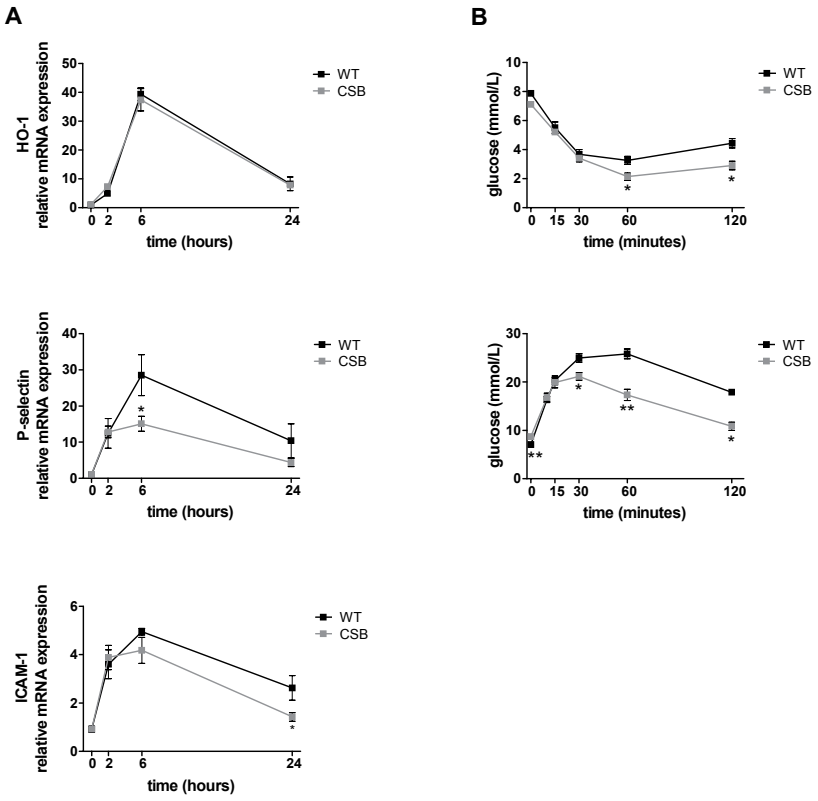

Figure 4. Mechanisms of reduced susceptibility to renal I/R injury in CSB vs. WT mice. (A) Time course of heme oxygenase-1 (HO-1; top), P-selectin (center) and ICAM-1 (bottom) mRNA expression as indicated in the kidney following 37 minutes of renal I/R injury using qRT-PCR. Expression levels at all time points were set relative to the WT group at t = 0 hours. The data are expressed as means ± SEM. **P* < 0.05 vs. WT mice at the indicated time point. (B) Improved insulin sensitivity and glucose clearance in CSB vs. WT mice as indicated. Whole blood glucose levels at the indicated time points following intraperitoneal injection of insulin (n = 11 WT, 7 CSB; top) or glucose (n = 9 WT, 5 CSB; bottom) into overnight fasted animals. The data are expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 vs. WT mice at the indicated time point.

expression of both P-selectin (P = 0.02) and ICAM-1 (P = 0.05) following renal I/R injury, consistent with less damage subsequent to the reperfusion and a better outcome.

Finally, enhanced sensitivity to the effects of insulin on glucose metabolism is a property shared by dwarf and dietary restricted mice that had been proposed to underlie longevity benefits observed in these models.[19] We chose to analyze glucose metabolism by performing both glucose tolerance and insulin sensitivity tests in overnight fasted CSB and WT mice. We found a significant increase in the ability of CSB animals to clear glucose from the circulation in response to a bolus injection of insulin or glucose, respectively (Figure 4B). It should be noted that despite the slight but significant elevation of fasting glucose levels observed at baseline in CSB animals subject to the glucose tolerance test, no significant difference was observed in the insulin tolerance test between CSB and WT (P = 0.34) nor in the combined data set (P = 0.41). This lack of a significant difference is consistent with previous reports of fed glucose levels of single mutant CS and XPCS mice at postnatal day 15 prior to weaning, while in more severe XPA-deficient double mutant animals hypoglycemia was observed.[16, 17]

Cockayne syndrome can also be caused by defects in the *CSA* gene. In mice, $CSA^{-/-}$ (subsequently referred to as CSA) mice are nearly indistinguishable from CSB mice.[11] We tested the resistance of CSA mice to renal I/R injury and found evidence of protection against both 25 and 37 minutes warm ischemia in CSA mice vs. WT controls at the level of kidney function (Figure 5A).

No sex bias has been previously reported for Cockayne syndrome[9] or for mouse models of this disease. To see whether the protective effects we observed are specific to males, we tested CSB females for resistance to 37 minutes of warm renal I/R injury. Female mice are in general less susceptible to this injury than males,[28] and as expected we observed less evidence of kidney dysfunction-related mortality following 37 minutes warm ischemia in females than in males. However, CSB females displayed evidence of increased resistance to injury, including significantly lower urea and creatinine in the serum 24 hours after reperfusion (Figure 5B).

Discussion

Although the genetic lesions causative of Cockayne syndrome are known, the molecular defect(s) leading to the observed symptoms remains unclear. The prominent inability to repair UV-induced DNA lesions in Cockayne cells from both man and mouse models has led to the hypothesis that hypersensitivity to DNA damage, presumably oxidative in nature, is primarily responsible for disease symptoms. Consistent with this, CSB mice are hypersensitive to the effects of whole-body ionizing irradiation, a potent oxidative stress,[29] and both CSA and CSB mice demonstrate an increase in photoreceptor cell loss following ionizing radiation.[13] However, primary CSB cells[16] as well as immortalized CSA cells[30] are not hypersensitive to oxidative stress.

A number of recent reports have described overlap between short-lived segmental

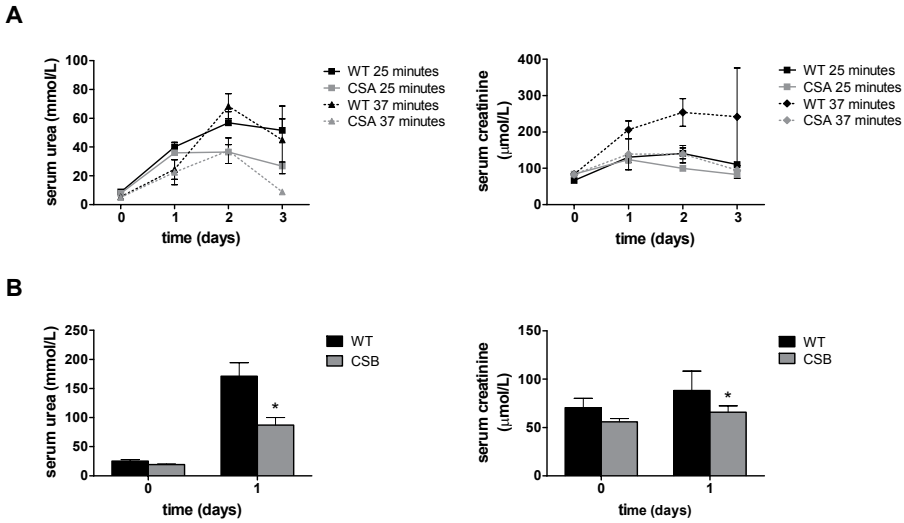

Figure 5. Protection from renal I/R injury correlates with genetic defects in TC-NER and extends to CSB females. (A) Functional response of TC-NER deficient CSA vs. WT animals to 25 and 37 minutes of warm I/R injury according to serum urea (left) and creatinine (right) values after I/R injury. The data are expressed as means ± SEM. **P* < 0.05, ***P* < 0.01 vs. WT mice at the indicated time point. (B) Functional response of WT vs. CSB females to 37 minutes of warm renal I/R injury as measured by serum urea (left) and creatinine (right) values prior to and 24 hours after injury. The data are expressed as means ± SEM. **P* < 0.05 vs. WT mice at the indicated time point.

progeroid NER models and long-lived hypopituitary dwarfs or dietary restricted wild-type mice on the level of physiology and gene expression.[14-17, 20] These phenotypes have been interpreted as an adaptive response to genotoxic stress overlapping with the starvation response. A previously untested prediction of this interpretation is increased resistance rather than hypersensitivity to acute oxidative stress in these models.

Consistent with this prediction, we showed here that mouse models of Cockayne syndrome deficient in either *CSA* or *CSB* are less susceptible to renal I/R injury than wild-type mice. Susceptibility was measured in terms of animal survival, kidney function and histological evidence of damage and organ regeneration. In all cases, CSB mice did significantly better than WT mice. On the molecular level, we observed less up-regulation of the cell-cycle inhibitor p21 after injury in CSB mice, consistent with less damage and/or better proliferative potential. Up-regulation of the inflammatory markers ICAM-1 and P-selectin were also significantly reduced following injury in CSB mice, consistent with either reduced damage and/or a dampened ability to mount an inflammatory response. Finally, improved glucose tolerance and insulin sensitivity at baseline were observed in overnight fasted CSB animals relative to WT controls. Taken together with previous studies demonstrating hypersensitivity to ionizing radiation in certain tissues, these data suggest that hypersensitivity and resistance to oxidative stress can coexist in the same animal, depending on the tissue or cell type involved.

Although the mechanism of protection against acute stress in the long-lived dwarf or dietary restricted models is not known, a number of plausible candidate mechanisms have been identified. These include increased antioxidant capacity, reduced inflammation and improved glucose homeostasis due to heightened insulin sensitivity.[19] The induction of genes including heme oxygenase-1 upon ischemic injury demonstrated that the capacity of CSB animals to mount a transcriptional response is normal, despite a defect in the repair of RNA polymerase-stalling bulky DNA lesions. However, we did not observe any differences in antioxidant capacity on the level of gene expression between WT and CSB mice. We did find a difference in the up-regulation of two markers of inflammation, P-selectin and ICAM-1. Reduced inflammation is associated with improved outcome following renal ischemia,[22] suggesting that these differences may in part underlie the stress resistance observed in the CSB model.

Glucose tolerance and insulin sensitivity tests were both consistent with improved response of CSB animals to the effects of insulin. Improved insulin sensitivity is associated with extended longevity in various models, including genetic dwarfism and dietary restriction, and is usually associated with reduced IGF-1 and improved glucose metabolism. How improved insulin sensitivity might underlie protection from acute stress observed here, or extended longevity observed in other models, remains unknown. As insulin and insulin-like growth factor signaling are pro-survival, and growth factor delivery can improve outcome after ischemic injury to other organs, including the brain,[31] we speculate that increased sensitivity to these growth factors may result in both better cell survival as well as improved proliferation following I/R injury.

Which defective biochemical activity (or activities) of CSB is causative of disease phenotypes, possibly including protection from renal ischemic insult? We speculate that a defect in TC-NER is the likely culprit based on the following genetic argument. CSB and other proteins implicated in Cockayne syndrome are multifunctional and have roles in various cellular processes, defects in any of which could in principle give rise to disease symptoms. Nonetheless the symptoms of Cockayne syndrome are remarkably similar regardless of the underlying genetic lesion, particularly in mice including CSA, CSB, XPCS or TTD models in an XPA deficient background, as well as in XPG, XPF and ERCC1 single mutant animals.[20] We speculate that this common phenotype likely stems from a defect in a shared process in which each of these proteins participates directly. To date, the only common pathway in which each of these multifunctional proteins has a direct role is TC-NER. It should be noted, however, that the presumed unrepaired lesion or repair intermediate remains to be identified.

We propose a model in which a congenital DNA repair deficiency results in activation of systemic protective mechanisms, including a reduced inflammatory response and improved insulin sensitivity. These mechanisms would then lend resistance to certain forms of acute stress in some tissues (renal I/R injury to the kidney, as shown here) but not others (ionizing radiation in the photoreceptor layer of the eye).[13] We can envision at least two distinct mechanisms by which this organ/tissue specificity could occur. Based on the neuronal phenotypes in Cockayne patients and mouse models, TC-NER deficiency could in principle result in neuronal deficiencies

causing a state of real or perceived dietary restriction, for example, through defective function of neurons involved in nutrient sensing, or neuronal control of the gut leading to malabsorbtion. Extended periods of dietary restriction in wild-type mice have been shown to protect against ischemic damage to organs, including the heart[32] and the brain.[33] Alternately, unrepaired TC-NER substrates in the genome may elicit an adaptive stress response similar to dietary restriction but in the absence of reduced food intake. Consistent with this latter interpretation is the adaptive response involving transient alterations in glucose homeostasis and serum IGF-1 levels observed in a related TC-NER deficient mouse model during the potentially stressful period of postnatal development.[16]

Hormesis is a common biological phenomenon in which exposure to a low intensity stressor induces a general adaptive response that has net beneficial effects on the cellular and/or organismal level, including protection against subsequent, higher-dose exposures as well as to different types of stress.[34, 35] Dietary restriction has been proposed to act as a mild stressor that extends longevity through hormetic mechanisms.[36, 37] Interestingly, ischemic preconditioning, a procedure used to protect against ischemic insult that entails brief period(s) of ischemia prior to a longer ischemia time, is also thought to function via hormesis.[38] Our data suggest that specific types of unrepaired endogenous DNA lesions may also be hormetic in nature. The interplay between tissue-specific sensitivities to endogenous DNA damage and the resulting adaptive responses may underlie the complex phenotypes observed in segmental disorders such as Cockayne syndrome. Having identified such unexpected benefits associated with DNA repair deficiency, further elucidation of underlying mechanisms may allow exploitation of the benefits without suffering the severe complications associated with congenital DNA repair insufficiencies.

Acknowledgements

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References

1. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956; 11: 298-300
2. Beckman KB, Ames BN. The free radical theory of aging matures. Physiol Rev 1998; 78: 547-581
3. Martin GM, Oshima J. Lessons from human progeroid syndromes. Nature 2000; 408: 263-266
4. Brooks PJ, Wise DS, Berry DA, et al. The oxidative DNA lesion 8,5′-(S)-cyclo-2′-deoxyadenosine is repaired by the nucleotide excision repair pathway and blocks gene expression in mammalian cells. J Biol Chem 2000; 275: 22355-22362
5. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. Nature 2001; 411: 366-374
6. Citterio E, Van Den Boom V, Schnitzler G, et al. ATP-dependent chromatin remodeling by the Cockayne syndrome B DNA repair-transcription-coupling factor. Mol Cell Biol 2000; 20: 7643-7653
7. Groisman R, Polanowska J, Kuraoka I, et al. The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. Cell 2003; 113: 357-367
8. Fousteri M, Vermeulen W, van Zeeland AA, et al. Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. Mol Cell 2006; 23: 471-482
9. Nance MA, Berry SA. Cockayne syndrome: review of 140 cases. Am J Med Genet 1992; 42: 68-84
10. van der Horst GT, van Steeg H, Berg RJ, et al. Defective transcription-coupled repair in Cockayne syndrome B mice is associated with skin cancer predisposition. Cell 1997; 89: 425-435
11. van der Horst GT, Meira L, Gorgels TG, et al. UVB radiation-induced cancer predisposition in Cockayne syndrome group A (Csa) mutant mice. DNA Repair (Amst) 2002; 1: 143-157
12. Dolle ME, Busuttil RA, Garcia AM, et al. Increased genomic instability is not a prerequisite for shortened lifespan in DNA repair deficient mice. Mutat Res 2006; 596: 22-35
13. Gorgels TG, van der Pluijm I, Brandt RM, et al. Retinal degeneration and ionizing radiation hypersensitivity in a mouse model for Cockayne syndrome. Mol Cell Biol 2007; 27: 1433-1441
14. Wijnhoven SW, Beems RB, Roodbergen M, et al. Accelerated aging pathology in ad libitum fed Xpd(TTD) mice is accompanied by features suggestive of caloric restriction. DNA Repair (Amst) 2005; 4: 1314-1324
15. Niedernhofer LJ, Garinis GA, Raams A, et al. A new progeroid syndrome reveals that genotoxic stress suppresses the somatotroph axis. Nature 2006; 444: 1038-1043
16. van de Ven M, Andressoo JO, Holcomb VB, et al. Adaptive stress response in segmental progeria resembles long-lived dwarfism and calorie restriction in mice. PLoS Genet 2006; 2: e192
17. van der Pluijm I, Garinis GA, Brandt RM, et al. Impaired genome maintenance suppresses the growth hormone--insulin-like growth factor 1 axis in mice with Cockayne syndrome. PLoS Biol 2007; 5: e2
18. Schumacher B, van der Pluijm I, Moorhouse MJ, et al. Delayed and accelerated aging share common longevity assurance mechanisms. PLoS Genet 2008; 4: e1000161
19. Bartke A, Brown-Borg H. Life extension in the dwarf mouse. Curr Top Dev Biol 2004; 63: 189-225
20. van de Ven M, Andressoo JO, Holcomb VB, et al. Extended longevity mechanisms in short-lived progeroid mice: identification of a preservative stress response associated with successful aging. Mech Ageing Dev 2007; 128: 58-63
21. Kelly KJ. Distant effects of experimental renal ischemia/reperfusion injury. J Am Soc Nephrol 2003; 14: 1549-1558
22. Friedewald JJ, Rabb H. Inflammatory cells in ischemic acute renal failure. Kidney Int 2004; 66: 486-491
23. Padanilam BJ. Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. Am J Physiol Renal Physiol 2003; 284: F608-627
24. Megyesi J, Andrade L, Vieira JM, Jr., et al. Coordination of the cell cycle is an important determinant of the syndrome of acute renal failure. Am J Physiol Renal Physiol 2002; 283: F810-816

25. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000; 182: 311-322
26. Devarajan P. Cellular and molecular derangements in acute tubular necrosis. Curr Opin Pediatr 2005; 17: 193-199
27. Takada M, Nadeau KC, Shaw GD, et al. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. J Clin Invest 1997; 99: 2682-2690
28. Kher A, Meldrum KK, Wang M, et al. Cellular and molecular mechanisms of sex differences in renal ischemia-reperfusion injury. Cardiovasc Res 2005; 67: 594-603
29. de Waard H, de Wit J, Gorgels TG, et al. Cell type-specific hypersensitivity to oxidative damage in CSB and XPA mice. DNA Repair (Amst) 2003; 2: 13-25
30. de Waard H, de Wit J, Andressoo JO, et al. Different effects of CSA and CSB deficiency on sensitivity to oxidative DNA damage. Mol Cell Biol 2004; 24: 7941-7948
31. Tang BL. SIRT1, neuronal cell survival and the insulin/IGF-1 aging paradox. Neurobiol Aging 2006; 27: 501-505
32. Chandrasekar B, Nelson JF, Colston JT, et al. Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2001; 280: H2094-2102
33. Yu ZF, Mattson MP. Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. J Neurosci Res 1999; 57: 830-839
34. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136
35. Mattson MP. Hormesis defined. Ageing Res Rev 2008; 7: 1-7
36. Turturro A, Hass BS, Hart RW. Does caloric restriction induce hormesis? Hum Exp Toxicol 2000; 19: 320-329
37. Sinclair DA. Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev 2005; 126: 987-1002
38. Arumugam TV, Gleichmann M, Tang SC, et al. Hormesis/preconditioning mechanisms, the nervous system and aging. Ageing Res Rev 2006; 5: 165-178

Chapter 3

Short-term dietary restriction and fasting precondition against ischemia/reperfusion injury in mice

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Abstract

Dietary restriction (DR) extends lifespan and increases resistance to multiple forms of stress, including ischemia/reperfusion injury to the brain and heart in rodents. While maximal effects on lifespan require long-term restriction, the kinetics of onset of benefits against acute stress is not known. Here, we show that 2–4 weeks of 30% DR improved survival and kidney function following renal ischemia/reperfusion injury in mice. Brief periods of water-only fasting were similarly effective at protecting against ischemic damage. Significant protection occurred within 1 day, persisted for several days beyond the fasting period and extended to another organ, the liver. Protection by both short-term DR and fasting correlated with improved insulin sensitivity, increased expression of markers of antioxidant defense and reduced expression of markers of inflammation and insulin/insulin-like growth factor-1 signaling. Unbiased transcriptional profiling of kidneys from mice subject to short-term DR or fasting revealed a significant enrichment of signature genes of long-term DR. These data demonstrate that brief periods of reduced food intake, including short-term daily restriction and fasting, can increase resistance to ischemia/reperfusion injury in rodents and suggest a rapid onset of benefits of DR in mammals.

Introduction

Dietary restriction (DR) encompasses a variety of interventions resulting in reduced nutrient and energy intake without malnutrition. Dietary restriction is best known for its ability to extend lifespan in a wide variety of organisms.[1-3] Longevity effects were first reported in rodents in 1935[4] and extended in subsequent decades to fish,[5] worms,[6] flies,[7, 8] yeast,[9, 10] and nonhuman primates.[11] Effects on human longevity are not known, but prospective studies show a favorable impact on markers of aging and predictors of long-term health, including improved cardiovascular fitness, body-mass index and insulin sensitivity.[12-14]

The kinetics of onset and loss of longevity benefits of DR are best understood in the fruit fly *Drosophila melanogaster*. In young adult flies, maximal effects of DR on longevity, measured as a function of daily mortality rate, are achieved within 1-3 days of switching from a normal to a restricted diet and vice versa.[15] The use of daily mortality rate as an endpoint in young adult rodents would require large numbers of animals because of low daily mortality rates and is thus considered impractical.

In addition to extended lifespan, another common property of organisms on DR is increased resistance to multiple forms of acute stress.[16, 17] In rodents, this includes resistance to paraquat toxicity and ischemia/reperfusion (I/R) injury. Paraquat is a free radical generator that primarily targets the lungs, and mice subject to ~5 or 8 months of 40% DR have a significant survival advantage over *ad libitum* fed animals.[18, 19] I/R injury is initiated by a lack of blood flow (ischemia) resulting in a state of tissue oxygen and nutrient deprivation characterized chiefly by ATP depletion, loss of ion gradients across membranes and buildup of toxic byproducts. Restoration of blood flow (reperfusion) causes further damage at first by inappropriate activation of cellular oxidases and subsequently by inflammatory mediators in response to tissue damage.[20] Rodents on a restricted diet for 3 months to 1 year have reduced damage upon ischemic injury to the heart and brain in models of heart attack and stroke, respectively.[21-23]

The length of time on a restricted diet required for the onset of increased stress resistance is not well characterized in any organism. Here, we examined the kinetics of onset and loss of protection against renal and hepatic I/R injury in mice using brief periods of food restriction, including 2-4 weeks of 30% reduced daily food availability (defined here as short-term DR) or 1-3 days of 100% restriction (water-only fasting).

Materials and Methods

Animals

Male C57BL/6 mice in the age/weight range of 10-14 weeks/22-25 grams were purchased from Harlan, Horst, the Netherlands. Animals were kept under standard laboratory conditions (temperature 20-24°C, relative humidity 50-60%, 12 hour light/12 hour dark) with 3-4 animals

per cage and allowed free access to water and food (Special Diet Services, Witham, UK) except where noted. All experiments were performed with the approval of the appropriate local ethical board.

Dietary regimens

The amount of food eaten *ad libitum* was approximately 3.5 gram per day as determined by weighing the remaining food on a daily basis for 1 week. Dietary restriction was applied for 2-4 weeks by feeding mice 70% of this amount on a daily basis. Fasting was applied by transferring mice to a fresh cage without food for 1-3 days. Despite significant weight loss as a result of short-term DR and fasting, no morbidity or mortality was observed as a function of the diets alone.

Renal ischemia model

Mice were anaesthetized by isoflurane inhalation (5% isoflurane initially and then 2-2.5% with oxygen for maintenance). Body temperature was maintained by placing the animals on heating pads until recovery from anesthesia. Following a midline abdominal incision, the left renal pedicle was localized and the renal artery and vein were dissected. A non-traumatic microvascular clamp was used to occlude the left kidney for 37 minutes. For bilateral occlusion, the procedure was repeated immediately on the right kidney. After inspection for signs of ischemia (purple color), the wound was covered with a phosphate-buffered saline (PBS) soaked cotton and the animal was placed under an aluminum foil blanket to maintain body temperature. After release of the clamp, restoration of blood-flow was inspected by return of the kidney to normal color. In the case of unilateral occlusion, a contralateral nephrectomy was performed immediately following clamp release. The abdominal wound was closed in two layers using 5/0 sutures. Animals were given 0.5 mL PBS subcutaneously for maintenance of fluid balance and kept warm under a heat lamp. All animals were observed to have regained consciousness before moving their cages from the operating room to the stable.

Kidney function analysis

Unlabeled dimercaptosuccinicacid (DMSA) kits were purchased from GE Healthcare (Roosendaal, the Netherlands) and radiolabeled with technetium-99m (^{99m}Tc) according to manufacturer's instructions. Mice were injected in a lateral tail vein with 30 MBq ^{99m}Tc-DMSA. Four hours post-injection, the mice were killed, the kidney was removed and the absorbed radioactivity was measured in a gamma counter (Perkin Elmer, Groningen, the Netherlands) and expressed as percentage of injected dose per kidney (%ID/kidney).

Liver ischemia model

Liver I/R injury was performed by visualizing the liver hilus and clamping the portal vein, hepatic artery and bile duct to the left and median hepatic lobes with a non-traumatic clamp for 75 minutes. In this model, 70% of the liver tissue becomes ischemic and blood outflow from

the small intestine is preserved through the right anterior and caudate liver lobes. Mortality associated with this amount of ischemic damage to the liver was not observed. Liver samples were fixed in formalin for 24 hours prior to embedding in paraffin. Four-micrometer sections were cut and stained with Hematoxylin and Eosin. Liver necrosis was scored blindly on a scale from 0 to 4, with 4 representing 100% of the area covered by hemorrhagic necrosis.

Serum measurements

Blood samples were collected by retro-orbital puncture. Serum urea and creatinine levels were measured using QuantiChrom assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICt-500; Gentaur, Brussels, Belgium). Serum alanine aminotransferase (ALAT) levels were determined using an ELAN analyzer (Eppendorf Merck, Hamburg, Germany) with Ecoline S+ reagents (Diagnostic Systems GmbH, Holzheim, Germany) according to manufacturer's instructions. Serum lactate dehydrogenase (LDH) levels were determined using Ecoline S+ reagents according to manufacturer's instructions in a 96-well format on a Varioskan microplate reader (Thermo Scientific B.V., Breda, the Netherlands).

Histology

Organs were harvested, fixed for 24 hours in formalin and embedded in paraffin. Three-micrometer sections were stained with Hematoxylin and Eosin. Tubular injury was assessed in a blind fashion on a five point scale as described previously.[24]

Insulin sensitivity tests

Following baseline blood glucose determination from tail blood of conscious, restrained mice, animals were injected with a bolus of insulin (Novorapid; 0.75 U / kg body weight) into the peritoneal cavity. Blood glucose determinations were performed at the indicated time points after injection using a HemoCue glucose 201 RT blood glucose analyzer (HemoCue, Ängelholm, Sweden) according to manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from frozen kidney tissue using Ambion mirVana miRNA Isolation Kit and oligodT or hexamer-primed cDNA synthesized using SuperScript II (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. Quantitative real-time PCR was performed using a MyIQ (BioRad B.V., Veenendaal, the Netherlands) with SYBR Green incorporation. Relative expression was calculated using the equation $1.8^{-(\Delta Ct\ sample - \Delta Ct\ control)}$.[25] Each sample was tested in duplo at least two times.

Microarrays

Purified total RNA extracted from frozen kidneys (three per group) was used as a template to generate biotin-labeled cRNA and hybridized to 430 2.0 GeneChips according to manu-

facturer's protocol (Affymetrix UK Ltd., High Wycombe, Buckinghamshire, UK). Raw data were Robust Multichip Average normalized[26] and assessed for differential gene expression using Limma.[27] Annotated pathway over-representation was performed using GAzer.[28] Fisher's exact test was performed using the web application available at http://www.langsrud.com/fisher.htm. Spearman's rho and Pearson's correlation coefficients were calculated using SPSSv11. Affymetrix 430 2.0 probe sets representative of the 28 common dietary restricted genes[29] were obtained from Ensembl (http://www.ensembl.org/), based on the NCBI m37 mouse assembly; except for MGI:1924575 which was obtained from the Mouse Genome Informatics data base (http://www.informatics.jax.org/) given the absence of annotations in Ensembl for this transcript at the time of submission.

Statistics

The data are expressed as means ± SEM. Statistical analyses of data on urea, creatinine, histomorphology, immunohistochemistry and immunoblot was preformed using a Student's t-test unless otherwise indicated. Survival was analyzed by Log-rank test (SPSSv11). The area under the curve was calculated using GraphPad Prism 4.0, and the significance was calculated using a Student's t-test.

Results

Bilateral renal ischemia was induced by clamping both renal pedicles for 37 minutes, followed by clamp removal to reinitiate blood flow. Under these conditions, 60% of the control mice fed *ad libitum* prior to surgery died or were killed as a result of morbidity (including excessive weight loss, drop in body temperature, ruffled fur, decreased activity and hunched body posture), and the associated buildup of toxic waste products (urea and creatinine) in the blood indicative of irreversible kidney failure by the fourth day following surgery (Figures 1A-D). In contrast, mice restricted daily to 70% of their *ad libitum* food intake (30% DR) for 4 weeks prior to challenge with renal I/R injury were protected from mortality, weight loss and kidney dysfunction (Figures 1A-D).

To investigate shorter periods of more severe restriction, we tested the effects of water-only fasting. As with 4 weeks of 30% DR, 3 days of fasting resulted in 100% survival, postoperative weight gain and reduced kidney dysfunction (Figures 1A-D). Protection afforded by both DR and fasting was further confirmed on the level of organ damage by longitudinal assesment of LDH in the serum (Figure 1E). Upon cellular damage or death, cytoplasmic LDH is released into the blood and can thus serve as a marker of acute tissue injury. Although LDH levels were significantly elevated in all groups 2 and 6 hours after reperfusion, they returned to preoperative levels 24 hours after reperfusion only in the DR and fasted groups. LDH levels were also significantly lower in the DR group at the 2-hour time point. Kidney damage was further assessed on the histological level by scoring acute tubular necrosis on a 5-point scale[24]

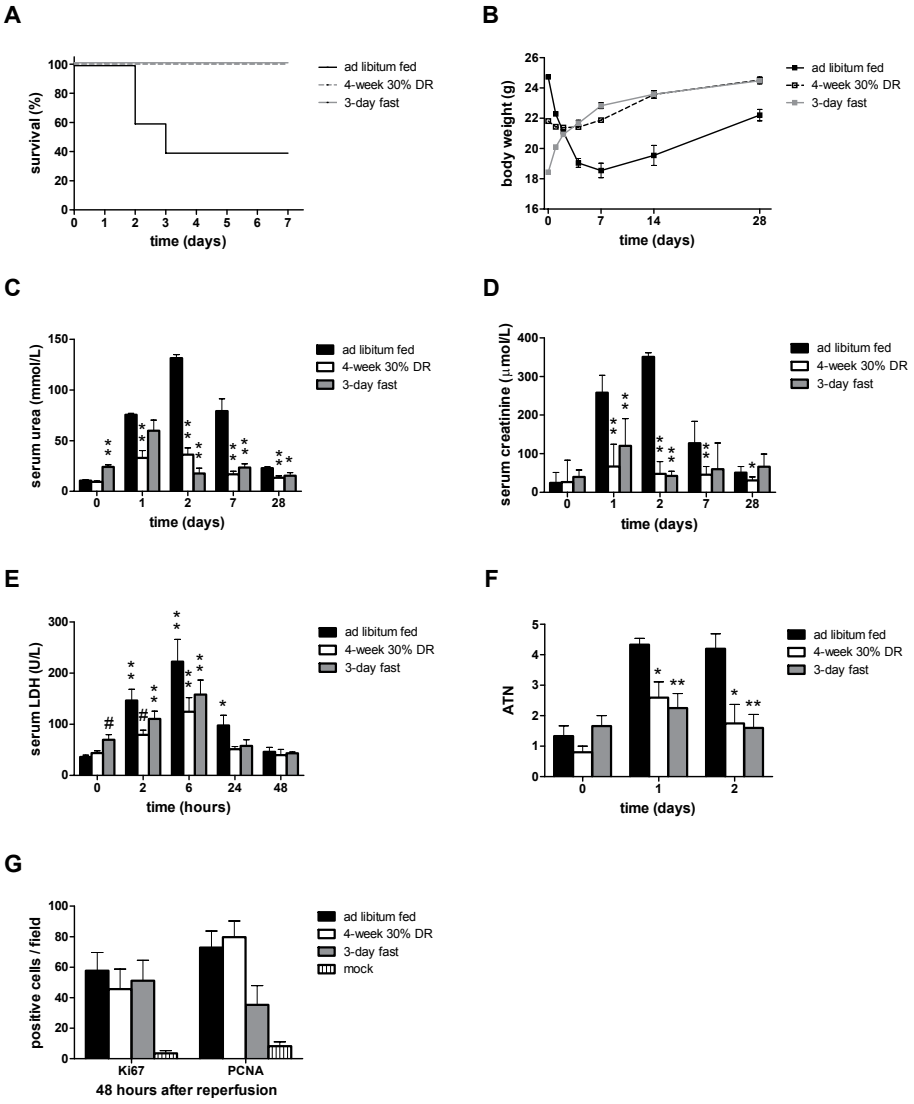


Figure 1. DR and fasting protect against the damaging effects of renal I/R injury. (A) Survival of *ad libitum* fed mice, 30% DR mice, and 3-day fasted mice after induction of 37 minutes of bilateral renal I/R injury (n = 10 per group). Both dietary treatments led to a significant survival advantage ($P < 0.01$). (B) Body weight of mice over a 28-day time course following renal I/R injury. (C-D) Kidney function as measured by (C) serum urea and (D) creatinine concentrations before and after surgery. *$P < 0.05$, **$P < 0.01$ vs. the *ad libitum* fed group at the same time point. (E) Serum LDH of the indicated groups over a time course following reperfusion. *$P < 0.05$, **$P < 0.01$ relative to t = 0 hours of the same treatment; $^{\#}P < 0.05$ relative to the *ad libitum* fed group at the indicated time point. (F) Quantification of acute tubular necrosis on a 5-point scale before and after renal I/R injury. *$P < 0.05$, **$P < 0.01$ relative to the *ad libitum* fed group at the indicated time point. (G) Percentage of cells expressing Ki67 or PCNA proliferative markers in a microscopic field of the indicated group on 2 day following I/R injury. All groups were significantly elevated vs. the mock control. The data are expressed as means ± SEM.

before and after injury (Figure 1F). Both DR and fasted groups had significantly less acute tubular necrosis than the *ad libitum* fed group on days 1 and 2 after injury, consistent with a better outcome. Following ischemic injury, damaged tubules have a limited capacity to regenerate. We measured cellular proliferation on the histological level using the proliferative markers PCNA and Ki67 (Figure 1G) on the second day following reperfusion coincident with the onset of organ regeneration. Both markers were elevated in animals subjected to renal I/R injury relative to mock treated animals. There were no significant effects of dietary pretreatment on absolute proliferation levels. Taken together, these data are consistent with better outcome in the DR and fasted groups.

We next tested the kinetics of onset and loss of benefits of DR and fasting using a unilateral occlusion model in which the left kidney was occluded for 37 minutes followed by removal of the right (undamaged) kidney. This model represents a more severe stress than the bilateral model with the same ischemic time, because recovery of renal function and animal survival depend on regeneration of a single damaged kidney. Under these conditions, 90% of *ad libitum* fed mice died or were killed as a result of morbidity indicative of irreversible kidney failure by the fourthday following I/R injury. Mice on 30% DR for 4 weeks or fasted for 3 days had significantly elevated survival rates (both 100%; Figure 2A) and improved renal function (Figure 2B) as in the bilateral model. Two weeks of 30% DR was similarly effective as 4 weeks with respect to survival and kidney function (Figures 2A-B), indicating a rapid onset of benefits of DR. Similarly, 2 or even 1 day of water-only fasting prior to ischemia significantly elevated survival rates (100% and 90%, respectively; Figure 2A). Interestingly, despite maximal effects on survival within 2 days, protection against kidney dysfunction increased in a dose-dependent manner for up to 3 days, reaching protection levels similar to 2 or 4 weeks of 30% DR (Figure 2B).

Assessment of kidney function by serum-based measures, including creatinine and urea, can in some cases be affected by confounding factors such as differences in muscle mass or diet. We thus sought to confirm the rapid onset of protection afforded by fasting with a more direct measure of kidney function. The uptake of technetium-99m-radiolabeled dimercaptosuccinicacid (^{99m}Tc-DMSA) by the kidneys is directly related to kidney tubular reabsorption. Twenty-four hours after I/R injury significantly less ^{99m}Tc-DMSA accumulated in the *ad libitum* group relative to the mock-treated animals, indicative of kidney dysfunction. In mice fasted for 1 to 2 days prior to I/R injury, this reduction was significantly ameliorated (Figure 2C).

To determine the kinetics of loss of the protective effect of fasting from renal I/R injury, we allowed animals *ad libitum* access to food for variable times following a 3-day fast and tested their resistance to ischemic injury. Significant benefits on animal survival remained for at least 2 days after refeeding but were lost after 4 days (Figure 2D). Despite these lingering survival benefits, protection from kidney dysfunction was lost by as little as 2 hours of refeeding prior to surgery (Figure 2E).

In contrast to *ad libitum* fed mice, food-restricted mice started eating shortly after awaking

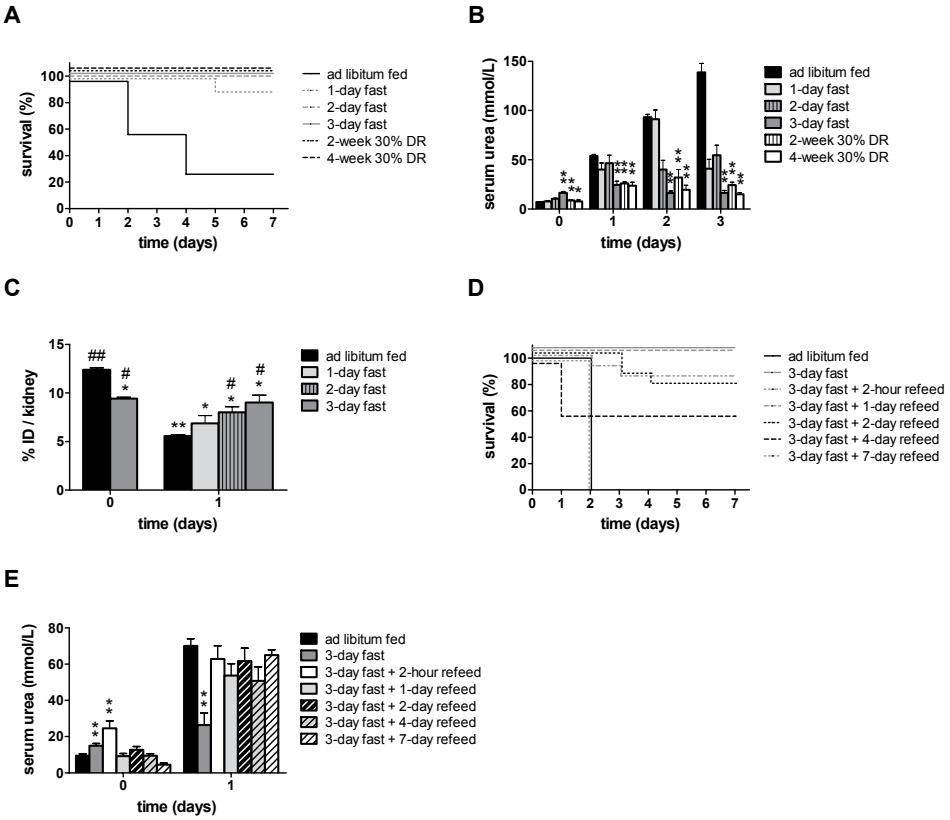


Figure 2. Rapid onset and loss of protective effects of short-term dietary restriction and fasting. (A-C) Rapid onset: (A) Kidney function as measured by serum urea following 37 minutes of unilateral renal I/R injury in the indicated groups (n = 4-10 per group per time point). $^{**}P < 0.01$ vs. the *ad libitum* fed group at the same time point. (B) Survival curves of mice fed *ad libitum*, 30% restricted for 2-4 weeks or fasted for 1 to 3 days prior to induction of 37 minutes of unilateral renal I/R injury with a contralateral nephrectomy (n = 10-18 per group). (C) Mice fasted for 0 to 3 days (4-10 animals per group) were analyzed for kidney function either in the absence of renal ischemia (day 0) or 1 day following 37 minutes of renal ischemia by measuring radioactivity in the kidney with a gamma-counter 4 hours after injection of ^{99m}Tc-DMSA, expressed as a percentage of the injected dose per one kidney (% ID/kidney). Note the reduced percentage of ^{99m}Tc-DMSA in the kidneys of the *ad libitum* fed group 24 hours after renal I/R injury (12.4% to 5.6%), indicative of renal dysfunction. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the *ad libitum* fed group at day 0; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the *ad libitum* fed group at day 1 post-reperfusion. (D-E) Rapid loss: (D) Survival curves of the indicated groups. Survival of animals refed for 2 hours, 1- and 2 days was significantly different than that of *ad libitum* fed animals ($P < 0.002$); survival of animals refed for 4 and 7 days was not significantly different than that of *ad libitum* fed animals. (E) Kidney function as measured by serum urea prior to and 1 day following I/R injury. Data from three independent experiments with 4-12 animals per group are averaged. In the day 0 group, asterisks indicate significant differences vs. the *ad libitum* fed control group; in the day 1 group, asterisks indicate significant difference between 3 days of fasting without refeeding and *ad libitum* fed animals, as well as each of the refed groups ($^{**}P < 0.01$). There were no significant differences between the *ad libitum* fed group and any of the refed groups on day 1 following renal I/R injury. The data are expressed as means ± SEM.

from surgery. As a result, they gained weight rapidly following surgery, whereas the *ad libitum* fed mice did not eat in the first days following surgery and lost weight (Figure 1B). We thus asked if the benefits of fasting were caused by diet-induced changes present at the time of renal I/R injury (preconditioning) or by differences in eating behavior after the injury leading to weight gain in the protected groups. To do this, we prevented access to food for 1 day following reperfusion in the *ad libitum* fed animals, as well as those fasted for 2 days prior to bilateral renal I/R injury. Although both groups continued to lose weight the first day after surgery (Figure 3A), animals fasted for 2 days prior to I/R injury had a significant survival advantage (Figure 3B) and a significantly better renal function on the second day following the injury (Figure 3C). We cannot rule out the possibility that differential feeding after this initial 24 hours postoperative period, or other physiological postoperative differences between fasted and fed mice, contribute to the observed protection in the fasted group. However, our data are consistent with a substantial impact of the diet prior to the onset of injury. We thus conclude that the impact of the diet on the ability to withstand renal I/R injury is largely caused by a preconditioning effect. The best known preconditioning technique is ischemic preconditioning, in which brief periods of ischemia are protective against longer, subsequent bouts of ischemia.[30] Hereafter, we refer to short-term nutritional interventions prior to ischemia as dietary preconditioning.

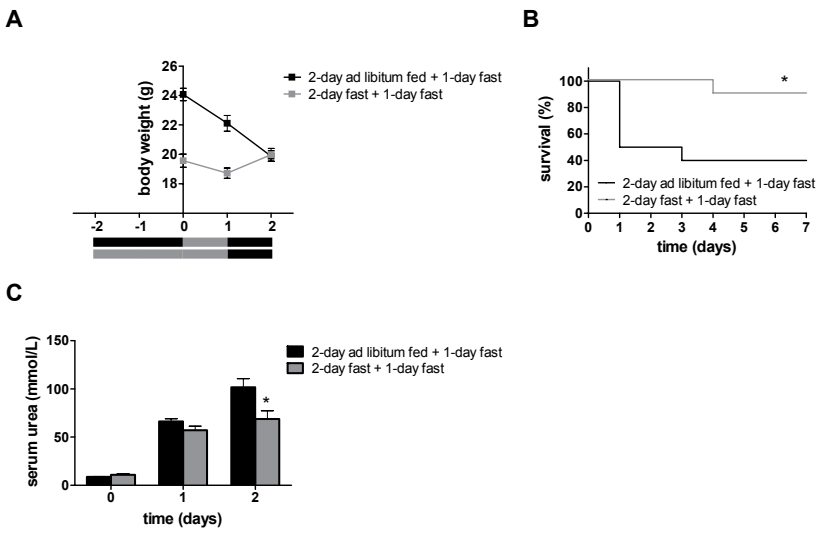


Figure 3. Protection is a preconditioning effect. (A) Black bars indicate periods of free access to chow; gray bars indicate periods without access to chow. Body weights of the two groups at the time of surgery (day 0) are indicated. The data are expressed as means ± SEM. (B) Survival following renal I/R injury. Fasted animals retained their survival advantage despite lack of refeeding for 1 day following renal I/R injury. *$P < 0.05$ vs. 2-day *ad libitum* fed + 1-day fasted mice. (C) Kidney function as measured by serum urea levels before and after I/R injury. *$P < 0.05$ vs. 2-day *ad libitum* fed + 1-day fasted mice on the second day following surgery. The data are expressed as means ± SEM.

To find out if the protection afforded by brief fasting against I/R injury was specific to the kidney or more broadly applicable, we used a model of liver I/R injury. Seventy percent hepatic ischemia was induced by clamping the portal vein, hepatic artery and bile duct to the left and median hepatic lobes for 75 minutes. As no mortality is associated with this model, we monitored ischemic liver damage by measuring the release of the liver-specific enzyme ALAT from dead or damaged cells into the blood for up to 24 hours (Figure 4A). Six hours after reperfusion, serum ALAT levels were elevated above baseline in all groups. However, they were significantly lower in the 2-and 3-day fasted groups than in the *ad libitum* fed group. Twenty-four hours after reperfusion, serum ALAT levels were significantly lower in each of the fasted groups. Histological sections prepared 24 hours after reperfusion showed less hemorrhagic necrosis in the 3-day fasted group than in the *ad libitum* fed group (Figure 4B). Thus, as in the renal ischemia model, 1-3 days of water-only fasting significantly protected against ischemic damage to the liver.

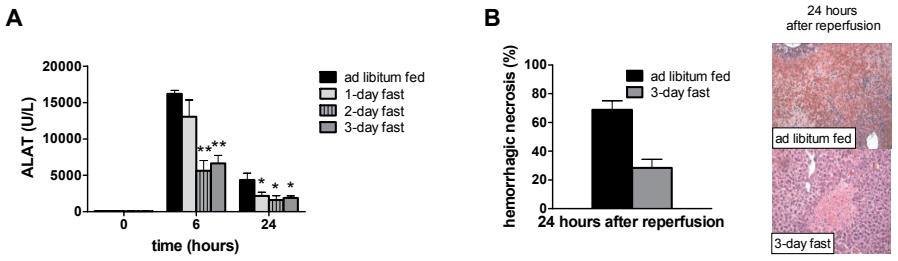


Figure 4. Dietary preconditioning in the liver. (A) Reduced injury markers upon liver I/R injury in fasted mice. Mice (5-8 animals per group) were fasted for the indicated times prior to induction of 75 minutes of warm ischemia to the liver. Serum concentration of the liver-specific enzyme alanine aminotransferase (ALAT), indicative of liver damage, was measured at the indicated times following reperfusion. *$P < 0.05$, **$P < 0.01$ vs. the *ad libitum* fed group at the same time point. (B) Quantification of hemorrhagic necrosis. Left: Liver necrosis was scored blindly on a scale from 0 to 4, with 4 representing 100% of the area covered by hemorrhagic necrosis. The data are expressed as means ± SEM. **$P < 0.01$ vs. the *ad libitum* fed group. Right: Representative Hematoxylin and Eosin-stained liver sections from mice 24 hours after reperfusion. Note the large areas of hemorrhagic necrosis (in red) in the mouse fed *ad libitum* prior to I/R injury and its relative absence in 3-day fasted mice. Magnification 100x.

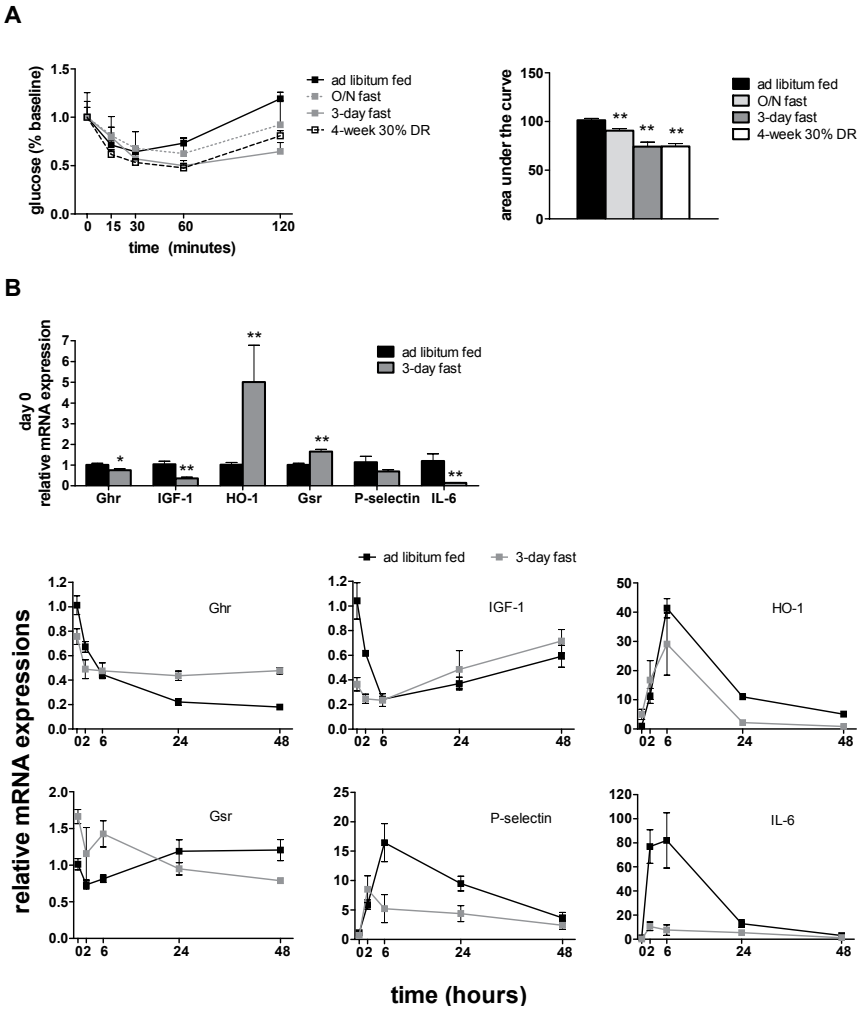
The mechanisms underlying the benefits of long-term DR, including resistance to I/R injury and extended longevity, remain unclear. Attenuation of oxidative stress, up-regulation of stress proteins and reduced inflammation have emerged as potential mechanisms of resistance to heart and brain ischemia by long-term DR,[21-23] while improved insulin sensitivity, reduced insulin/insulin-like growth factor (IGF)-1 signaling, up-regulation of stress proteins, reduced mitochondrial free radical production and reduced inflammation are thought to contribute to extended longevity by long-term DR.[16, 31] We asked if dietary preconditioning by short-term DR (defined here as 4 weeks or less) and/or fasting could function via these candidate mechanisms. We first measured insulin sensitivity of animals fasted for 1 or 3 days or 30% restricted for 4

weeks relative to *ad libitum* fed mice by injecting a bolus of insulin into the intraperitoneal cavity and measuring changes in blood glucose over time. The area under each curve is inversely proportional to the group's ability to clear glucose in response to insulin challenge. Fasting and short-term DR resulted in improved insulin sensitivity relative to *ad libitum* fed mice, correlating with improved outcome following renal I/R injury among our experimental groups (Figure 5A). Fasting improved insulin sensitivity in a dose-dependent manner from 1 to 3 days, with 3 days leading to an equivalent area under the curve as 4 weeks of DR.

We next analyzed components of the insulin/IGF-1 signaling pathway and the inflammatory response on the transcriptional level. At baseline, levels of kidney growth hormone receptor (Ghr) mRNA were significantly reduced in fasted relative to *ad libitum* fed animals (Figure 5B). Transcription of IGF-1 is dependent on binding of growth hormone to its cognate receptor, Ghr. Similar to Ghr, IGF-1 mRNA was significantly reduced in the fasted group (Figure 5B). Markers of antioxidant protection, including the inducible form of heme oxygenase-1 (HO-1) and glutathione reductase (Gsr), were significantly elevated on the transcriptional level in fasted mice relative to *ad libitum* fed mice (Figure 5B). Following renal I/R injury, Ghr and IGF-1 mRNA levels fell below baseline in both groups. Interestingly, the decline was proportionately less in the fasted group than in the *ad libitum* fed group. Also, relative to baseline, the levels 48 hours after reperfusion were higher in the fasted group than in the *ad libitum* fed group. HO-1 was strongly induced in both groups following ischemia, but to significantly lower levels and with a more rapid return to baseline in the fasted group. Gsr mRNA levels fell in the fasted group and increased in the fed group over the 48-hour time course following reperfusion. Markers of inflammation including the pro-inflammatory cytokine interleukin-6 and the neutrophil-recruiting endothelial adhesion molecule P-selectin were up-regulated on the transcriptional level following I/R injury in both groups with similar kinetics, but to a significantly lower degree in the fasted group (Figure 5B).

We turned to a global approach to quantify the degree to which changes in gene expression because of fasting and short-term DR overlap with each other and with long-term DR. To this end, we compared kidney transcriptomes of animals preconditioned with 4 weeks 30% DR or 3 days fasting to a common group of *ad libitum* fed mice (n = 3 animals per group) using Affymetrix arrays with 45101 unique probe sets representing 36431 target genes. Six hundred and forty-two (fasted) and 161 (short-term DR) probe sets were significantly differentially regulated versus the *ad libitum* fed control group using the criteria of fold change > 1.5 with a *P*-value of < 0.001 (Figure 6A). Twenty-four probe sets were common to both (Table 1), representing 14% and 4% of the total significant DR and fasted group probe sets, respectively. Seventy-nine per cent of these changes occurred in the same direction, with a Pearson's correlation of 0.529 and a Spearman's rho of 0.464. Notable in this group of genes is a number involved in lipid metabolism (Lpl, Acsm3, Cyp51, Decr2 and Ces1), organelle/membrane trafficking (Ccdc91, Vsp8 and Bnip1) and protein turnover (Cndp2, Wdr40b, Rnf180 and Mmp13).

In light of this modest number of overlapping genes between dietary groups, we next



F**igure 5.** Insulin/IGF-1 signaling in dietary preconditioning. (A) Improved insulin sensitivity in preconditioned mice. Left: Whole blood glucose levels at the indicated time points following an intraperitoneal injection of insulin. Right: Area under the curve. The data are expressed as means ± SEM. * $P < 0.05$, ** $P < 0.01$ relative to the *ad libitum* fed group (B) Differential expression of markers of antioxidant protection, inflammation and GH/IGF-1 axis. Changes in steady state mRNA levels of the indicated genes at baseline (bar graph, top) and over a 2-day time course following reperfusion (line graphs, bottom) as determined by qRT-PCR. All data points are expressed relative to the *ad libitum* fed group at t = 0. Each data point represents the mean expression value from five animals. Ghr, growth hormone receptor; IGF-1, insulin-like growth factor-1; HO-1, heme oxygenase-1; Gsr, glutathione reductase; IL-6, interleukin-6. Top: *$P < 0.05$, **$P < 0.01$ vs. the *ad libitum* fed group.

asked which pathways were significantly affected by short-term DR and fasting, and how many were common to both. To this end, we looked for over-representation of predefined gene sets within each data set using GAzer[28] with a false discovery rate of $q < 0.05$. Within the Gene

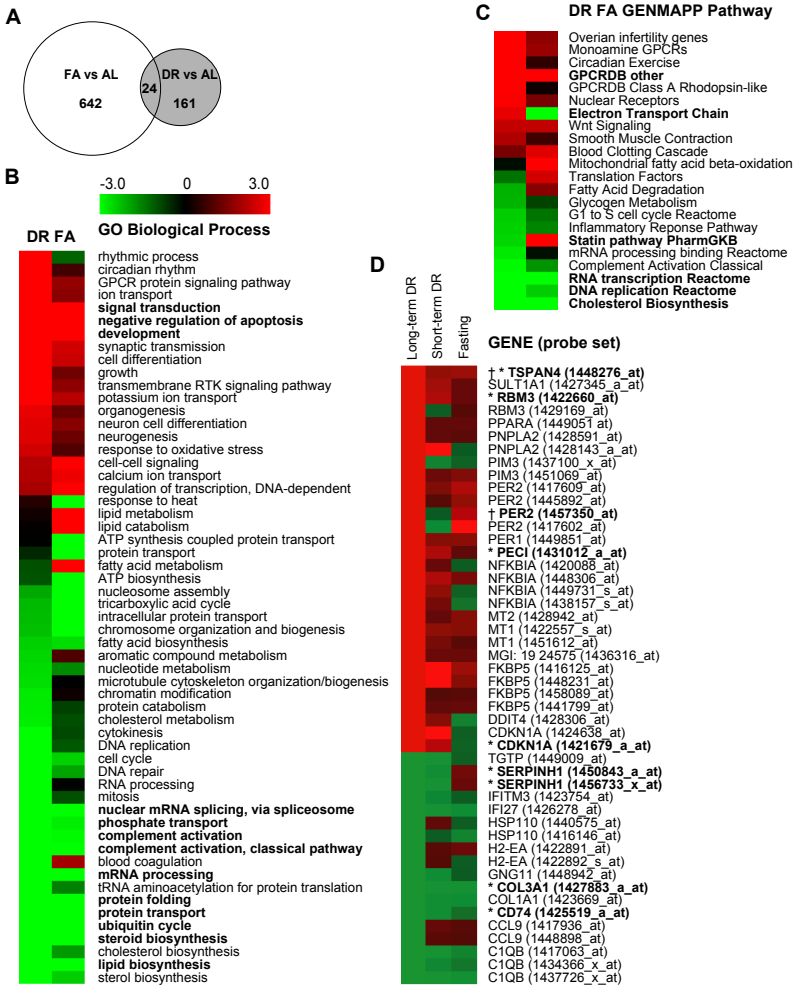


Figure 6. Global transcriptional changes upon short-term dietary restriction and fasting. (A) Venn diagram representing numbers and overlap of probe sets significantly differentially regulated in the kidney as a result of dietary preconditioning (4 weeks of 30% dietary restriction [DR] or 3 days of fasting [FA]) vs. *ad libitum* fed (AL) controls. Significance cutoffs were set by fold change > 1.5 and *P*-value < 0.001. (B) All Gene Ontology - Biological Processes and (C) GENMAPP Pathway gene sets overrepresented within either the short-term DR or fasted vs. *ad libitum* data sets were aligned according to *Z* score. Red, green and black indicate up-regulation, downregulation and/or no change, respectively, of that biological process because of the given dietary treatment relative to *ad libitum* feeding. Gene sets are ordered by *Z* score of the DR group. Note that all gene sets significantly over-represented in either treatment group (DR or FA) are included in the heat maps, and that those gene sets significantly over-represented in both treatment groups are in bold. (D) Similarity to long-term DR. Heat map of gene expression changes in a predefined set of 28 genes comprising a common transcriptional signature of DR across multiple mouse tissues.[29] Long-term DR probe sets are indicated either as up (red) or down (green) without regard to magnitude of expression; probe sets corresponding to short-term DR and fasted groups are colored as in (B). Significant genes from the short-term DR and fasted data sets as defined by fold change > 1.5 and *P*-value < 0.001 are in bold, with crosses and asterisks corresponding to short-term DR and fasting, respectively.

Table 1. Twenty-four genes significantly differentially regulated in both short-term DR and fasted (FA) groups with fold changes > 1.5 and P-values < 0.001. Expression level indicates the group geometric mean of the RMA nomalized Affymetrix data, ordered by the ad libitum (AL) fed group.

		Expression level			Fold change		
Gene	Symbol	AL	DR	FA	DR	FA	Putative function
CNDP dipeptidase 2 (metallopeptidase M20 family)	Cndp2	14 684	7766	3922	-1.9	-3.7	Proteolysis of nonspecific dipeptides in cytosol
Lipoprotein lipase	Lpl	10 727	6045	5504	-1.8	-1.9	Triacylglycerol hydrolysis, lipoprotein uptake
Acyl-CoA synthetase medium-chain family member 3	Acsm3	7964	3417	3306	-2.3	-2.4	Activation of fatty acids for anabolism or catabolism
Dopa decarboxylase	Ddc	2558	1241	6356	-2.1	2.5	Catecholamine biosynthesis
Solute carrier family 22 member 7	Slc22a7	2231	859	276	-2.6	-8.1	Bidirectional facilitative cGMP transporter
Flavin containing monooxygenase 2	Fmo2	2100	3792	3450	1.8	1.6	Oxidation of xenobiotics
Cytochrome P450, family 51	Cyp51	1731	1010	425	-1.7	-4.1	Key demethylase enzyme in all sterol biosynthesis
Coenzyme Q10 homolog B (S.cerevisiae)	Coq10b	974	2069	453	2.1	-2.2	Ubiqinone binding protein in yeast
Solute carrier family 6, member 6	Slc6a6	811	2177	2302	2.7	2.8	Taurine transporter
Vacuolar protein sorting 8 homolog (S. cerevisiae)	Vps8	698	437	170	-1.6	-4.1	Endosomal / lysosomal biogenesis
Tetraspanin 4	Tspan4	643	1186	1119	1.8	1.7	Cell surface transmembrane integrin signaling
2-4-Dienoyl-coenzyme A reductase 2, peroxisomal	Decr2	426	678	876	1.6	2.1	Peroxisomal oxidation of unsaturated fatty acids
RIKEN cDNA D630039A03 gene	D630039A03Rik	323	156	618	-2.1	1.9	Unknown
RIKEN cDNA 2310028N02 gene	2310028N02Rik	286	177	184	-1.6	-1.6	Transmembrane protein
Carboxylesterase 1	Ces1	249	87	74	-2.9	-3.4	Detoxification of xenobiotics, cholesterol metabolism
Coiled-coil domain containing 91	Ccdc91	245	136	130	-1.8	-1.9	Sorting of acid hydrolase to endosomes
BCL2 / adenovirus E1B interacting protein 1, NIP1	Bnip1	187	119	115	-1.6	-1.6	Anti-apoptotic factor / ER membrane fusion
Mannan-binding lectin serine peptidase 1	Masp1	185	303	118	1.6	-1.6	Complement activation and immune response
Aryl hydrocarbon receptor nuclear translocator-like	Arntl (Bmal1)	107	56	219	-1.9	2.0	Transcription factor required for circadian rhythmicity
WD repeat domain 40B	Wdr40b	80	45	48	-1.8	-1.7	Associated with DDB1 / Cullin 4 ubiquitin ligase
Matrix metallopeptidase 13	Mmp13	48	25	18	-1.9	-2.7	Breakdown of extracellular matrix
RIKEN cDNA 0610007P08 gene	0610007P08Rik	37	23	21	-1.6	-1.7	Possible DNA damage response
Ring finger protein 180	Rnf180	37	18	15	-2.1	-2.5	Membrane bound ubiquitin ligase
Similar to MOSC domain-containing protein 1	Loc100045982	30	69	642	2.3	21.2	Possible molybdenum cofactor biosynthesis

Ontology (GO) category of Biological Processes (GO-BP), 43 and 27 genes sets were significantly over-represented in the DR and fasted groups, respectively. Thirteen of these were common to both treatments, with *Z* scores indicating the same directionality in all 13 gene sets. In GENMAPP Pathway, 19 and 9 gene sets were significantly over-represented in the DR and fasted groups, respectively, with 4 of the 6 in common in the same direction. Heat maps of these enriched gene sets in both treatment groups ordered by *Z* score of the DR group are shown in Figures 6B-C. Similar overlaps were seen within other sets of predefined gene sets, including GO Molecular Functions (GO-MF; 40 DR and 25 fasted significant gene sets, 12 common to both, all in the same direction), GO Cellular Component (GO-CC; 18 DR and 7 fasted significant gene sets, 3 common to both, all in the same direction) and Interpro (41 DR and 49 fasted significant gene sets, 19 in common, all in the same direction). Thus, despite only modest overlap on the level of individual genes, 53 of the 161 and 117 significant gene sets in the short-term DR and fasted groups, respectively, were common to both treatments, with 51 of these in the same direction.

Among these pathways were many expected for nutrient/energy deprivation by either fasting or DR, including down-regulation of fatty acid, cholesterol and steroid biosynthesis. Pathways previously reported to be downregulated upon DR, such as DNA repair,[32] were also downregulated (although not significantly) in the fasted group. In addition, shared pathways consistent with increased resistance to I/R injury were significantly over-represented, including increased GO-BP negative regulation of apoptosis (up-regulated anti-apoptotic genes included angiopoietin-like 4 in both groups and Bcl2-like 1 in the fasted group), increased GO-MF glutathione transferase activity (increased Gstm1, Gstm3 in DR; increased Mgst-1, Gsta1, Gsta2, Gsta3, Gsto1, Gstt2, Gstm1 in FA) and decreased GO-BP complement activation. Of the 19 common significant pathways represented in Figure 6B, differences between dietary treatments were observed in only 2, Statin and Electron Transport Chain. In the Electron Transport Chain Pathway, for example, a number of genes encoding subunits of cytochrome *c* oxidase, NADH dehydrogenase and ATP synthase were significantly up-regulated in DR and downregulated in FA, accounting for the difference in directionality. Although ROS production and/or detoxification is thought to be a key player in the anti-aging effects of DR, the role of mitochondrial respiration itself remains controversial.[33] Further experiments will be required to test which if any of these pathways are involved in the beneficial effects of dietary preconditioning against I/R injury.

Finally, we asked if the effects of short-term DR and fasting on gene expression in the kidney were similar to what has been previously reported for long-term DR in other mouse tissues. For data regarding long-term DR, we turned to a meta-analysis covering ten mouse tissues subject to varying lengths (2 days to 2 years, average 6 months) and severity (10-75%, average 36%) of DR.[29] Twenty-eight genes that change significantly upon DR in at least five tissues were identified in this meta-analysis. Within this group of 28 genes/48 probe sets, 2 genes/2 probe sets (PER2, TSPAN4) from our DR data set and 8 genes/9 probe sets (CD74, COL3A1, TSPAN 4, FKBP5, RBM3, PECI, SERPINH1 and CDKN1A) from our fasted data set were

significantly differentially regulated in the kidney (fold change > 1.5, $P < 0.001$). This degree of enrichment makes it among the most significant of any predefined gene set tested ($P = 0.013$ and $2.353E^{-8}$ for DR and fasted, respectively, by Fisher's exact test). A heat map representing expression levels of probe sets corresponding to the 28 DR-associated genes is presented in Figure 6D.

Discussion

Timing of onset of benefits of DR

Voluntary adherence to a restricted diet is difficult for most people. Potential benefits of DR requiring long-term application, such as extended longevity, are thus considered moot in a clinical setting. However, extended longevity is only one of the potential benefits of DR (albeit a widely popularized one). DR also increases acute stress resistance in most organisms tested, including mammals. For example, DR lasting between 3 months and 1 year mitigates injury in rodent models of cardiac and cerebral ischemia.[21-23] What is currently not known is the length of restriction required for the onset of such benefits. Here, we show that as little as 2-4 weeks of 30% reduced daily feeding in mice led to significant protection against renal I/R injury. Interestingly, there was no increase in protection between 2 and 4 weeks of DR, suggesting that the maximal protection afforded by these short-term treatments was already reached by 2 weeks of DR. Whether or not longer periods of restriction will further increase protection remains to be experimentally determined.

In rodents, longevity extension by DR is roughly proportional to the amount of energy restriction (0-65%) up to the point of starvation.[1, 34] Beyond this point, restriction can lead to irreversible consequences and is eventually fatal. However, 100% restriction (water-only fasting) for shorter periods of time is possible without irreversible side-effects. We found that 3 days of water-only fasting led to protection against renal ischemic injury similar in magnitude to 2-4 weeks of 30% DR. Significant benefits were observed with respect to both survival and kidney function; however, the kinetics of onset and loss differed between these endpoints. Significant survival benefits occurred after a single overnight fast, while protection against renal dysfunction increased each fasting day for up to 3 days. Similarly, functional protection afforded by a 3-day fast was rapidly lost within hours of refeeding despite lingering survival benefits for up to several days. The reason for these apparent discrepancies in timing of onset and loss of benefits is currently not known. One possibility is that death and kidney dysfunction, although both initiated by renal I/R injury, have partially different causes. For example, mortality may be caused by reperfusion injury to distant organs such as the heart, lungs and brain[35] in addition to renal failure. Fasting may more rapidly affect the body's ability to withstand remote ischemic injury than acute renal ischemic injury. Another, non-mutually exclusive possibility is that different mechanisms of protection exist and that they are gained and lost at different rates. For example, transcriptional up-regulation of stress resistance genes,

including HO-1 and Gsr, may require several days of reduced insulin/IGF-1 signaling, but be rapidly lost upon the return of such signaling. Future experiments will be required to determine the molecular mechanisms underlying individual benefits and their timing of onset and loss as a function of different dietary regimens.

This is the first demonstration of the rapid onset of resistance to renal ischemic damage by short-term DR and fasting. However, it is consistent with a larger body of work demonstrating rapid onset of benefits of energy/nutrient deprivation, including both fasting and DR, in different organisms using different endpoints. For example, in fruit flies maximal effects on daily mortality rate are achieved within 1 to 3 days of DR.[15] Resistance to starvation stress is also increased by 4 days of DR and scales with the percent restriction.[8] In mice, 40% DR for 12-15 days is effective at preventing growth of tumor xenografts.[36] Dietary restriction initiated late in life rapidly shifts liver gene expression towards a long-term DR profile, beginning in as little as 2 weeks, and impacts longevity within 2 months.[37] Two weeks of DR also leads to feminization of liver gene expression in males,[38] an intriguing finding in light of the dramatically increased resistance of females to renal I/R injury relative to males in both humans and mice.[39] Shorter periods of more severe restriction have also proven efficacious in protecting against acute stress. For example, fasting for 3-4 days improves organ and animal survival in rodent models of orthotopic liver transplantation[40] and heterotopic heart transplantation.[41] Finally, 2 days of water-only fasting protects mice against the toxic effects of chemotherapeutic agents.[42] Although most of these studies were not designed to interrogate the time on a restricted diet required for the onset of maximal benefit, taken together with our own studies they suggest that benefits of nutrient/energy restriction measured by a variety of outcomes, including acute stress resistance, do not require long-term application.

Potential overlap between mechanisms of protection by fasting, short-term and long-term DR

One of the consequences of nutrient/energy deprivation is reduced insulin/IGF-1 signaling. Reduced signaling through these pathways increases stress resistance and extends longevity in part through activation of transcription factors leading to increased expression of stress resistance genes.[43] In rodents, improved insulin sensitivity is indicative of reduced baseline insulin signaling and is observed in both genetic and dietary models of extended longevity and increased stress resistance. Relative to *ad libitum* fed mice, we observed improved insulin sensitivity upon both fasting and short-term DR. We also observed reduced levels of Ghr and IGF-1 mRNAs in the kidney itself, as well as up-regulation of genes involved in antioxidant defense. These data are consistent with a model of increased stress resistance by both fasting and short-term DR involving reduced insulin/IGF signaling and concomitant up-regulation of cellular stress resistance mechanisms. As a consequence, oxidative injury, cell death and inflammation are all reduced subsequent to ischemic injury.

In a variety of experimental models, reduced signaling through insulin/insulin-like peptides results in extended longevity and increased stress resistance.[17] This may seem at odds

with the well-documented pro-growth and pro-survival effects these factors have in other settings, for example in response to an ischemic insult to the brain, heart or kidney.[44-47] Here, we observed improved insulin sensitivity and reduced Ghr and IGF-1 mRNA in fasted mice, consistent with reduced signaling through these pathways at baseline prior to I/R injury. Interestingly, we also observed a relative increase in mRNA levels of Ghr and IGF-1 after injury in the fasted group. Although further experiments are required to determine actual levels of GH/IGF-1 signaling, the present data are consistent with increased signaling through these pathways after an ischemic insult, and better survival. Based on these data, we hypothesize that the benefits of reduced and increased insulin/IGF-1 signaling can be separated by time relative to an acute injury, and are thus not mutually exclusive. According to this hypothesis, reduced IGF-1 signaling as a result of DR or fasting prior to ischemic insult may have two beneficial effects: up-regulation of stress resistance resulting in less initial damage and improved IGF-1 sensitivity resulting in increased survival signaling after injury.

In lower organisms, the genetic requirements for lifespan extension and stress resistance are more extensively characterized than in mammals and appear to vary depending on the type of DR method employed. In worms, for example, the source of bacteria (liquid culture vs. solid agar plates) and the timing of onset of DR can both affect the genetic requirements for longevity extension.[48] Here, we used two distinct dietary interventions, fasting and short-term DR, to modulate resistance to ischemic injury and found similar beneficial effects with both. On the level of global changes in the kidney transcriptome, 31% of the significant short-term DR and 49% of the significant fasting annotated gene sets identified in GO-BP, GO-MF, GO-CC, GENMAPP Pathway and Interpro were common to both treatment groups. Of the 53 significant gene sets in common, 51 were changed in the same direction. Furthermore, gene expression markers identified previously in a meta-analysis of mostly long-term DR studies of multiple mouse organs[29] were significantly enriched in our kidney data set from both short-term DR and fasted animals. Taken together, these data are consistent with an overlapping transcriptional response to fasting, short-term DR and long-term DR in the kidney. However, they are also consistent with different genetic requirements for different DR regimens as in lower organisms. Future experiments will be required to determine which of these changes, if any, underlie the benefits of DR on acute stress resistance.

Finally, it will be of great future interest to elucidate the relationship between acute stress resistance and other benefits of DR such as extended longevity. Without any detailed understanding of the mechanism (or mechanisms) underlying either of these benefits, the relationship between them remains only correlative in nature. It is easy to envisage how improved stress resistance could extend longevity, for example by increasing the likelihood of surviving a heart attack or stroke on any given day. What remains unclear is if, or how, this relates to the reduction in the rate of aging achieved by DR.

Fasting in DR and acute stress resistance

The role of periods of fasting in the beneficial action of DR remains unclear.[49] DR regimens in

rodents involve extended periods of fasting between meals, as animals are hungry and consume their food allotment quickly when fed. The length of these periods is not widely reported and likely depends both on the percent restriction as well as the frequency of meals (typically once daily to thrice weekly). Intermittent fasting regimens with *ad libitum* feeding between periods of fasting (e.g. every other day fasting or 4 days fasting every 2 weeks) have beneficial effects in rodents similar to DR, including extended lifespan[50, 51] and increased stress resistance.[52] In some strains, intermittent fasting is associated with reduced total food intake and reduced body weight typical of DR,[53] while in others, it produces tangible health benefits in the absence of weight loss.[51, 52] This suggests that periods of fasting rather than reduced overall calorie intake *per se* may underlie some of the benefits of DR. On the other hand, altering the temporal pattern of feeding of a restricted diet from once to twice daily, while affecting circadian rhythmicity, has no significant impact on lifespan.[54] This suggests that overall nutrient/energy restriction is more important than timing of food intake at least for longevity extension by DR. Nevertheless, these experiments did not rule out the potential role of fasting between meals, as both groups still underwent extended periods of fasting relative to the *ad libitum* fed group.[55] Experimental separation of reduced nutrient/energy intake from periods of fasting in rodents remains challenging, and further experiments will be required to resolve the contribution of periods of fasting, if any, to the benefits of DR. Our data are consistent with a role of fasting in at least one of these benefits, acute stress resistance, during the initiation phase of DR.

The benefits of fasting have been known at least since the time of Hippocrates, but the practice is largely absent from Western medicine today. Instead, it is often associated with malnutrition, a risk factor for postoperative survival,[56] wound healing, infection and multiple organ failure.[57] One of the few modern clinical applications of fasting is to reduce the risk of pulmonary aspiration of regurgitated stomach contents prior to operations involving anesthesia. The origins in the 1960s of the standard "nil by mouth after midnight" preoperative fast are somewhat obscure,[58] and today even this relatively short fast is perceived as overcautious and possibly detrimental to patient subjective well-being and postoperative recovery.[59] Current more liberal guidelines allow consumption of solids up to 6 hours prior to surgery and recommend consumption of liquids, including carbohydrate-rich beverages, up to 2 hours prior to surgery.[60] Our data suggest that slightly longer periods of fasting, or short periods of DR, prior to surgery may be beneficial for an entirely different purpose: protection against certain types of acute organ stress. Fasting may thus represent a non-invasive, cost-free method of protecting against multiple types of acute stress, including surgical I/R injury unavoidably encountered in elective surgeries, including living-donor organ transplantation, cardiac surgery, vascular surgery and liver resection. It remains to be seen if the benefits of fasting and short-term DR and their rapid onset observed in rodents will translate to humans. However, the conservation of rapid onset of DR benefits between flies[15] and rodents, combined with the efficacy of DR in improving markers of healthspan in both nonhuman primates[11, 61] and humans[14] suggests that it might.

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References

1. Weindruch R, Walford RL, Fligiel S, et al. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J Nutr 1986; 116: 641-654
2. Masoro EJ. Subfield history: caloric restriction, slowing aging, and extending life. Sci Aging Knowledge Environ 2003; 2003: RE2
3. Bishop NA, Guarente L. Genetic links between diet and lifespan: shared mechanisms from yeast to humans. Nat Rev Genet 2007; 8: 835-844
4. McCay CM, Crowell MF, Maynard LA. The Effect of Retarded Growth Upon the Length of Life Span and Upon the Ultimate Body Size. J Nutr 1935; 10: 63-79
5. Comfort A. Effect of Delayed and Resumed Growth on the Longevity of a Fish (Lebistes Reticulatus, Peters) in Captivity. Gerontologia 1963; 49: 150-155
6. Klass MR. Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mech Ageing Dev 1977; 6: 413-429
7. Partridge L, Green A, Fowler K. Effects of egg-production and of exposure to males on female survival in Drosophila melanogaster J Insect Physiol 1987; 33: 745-749
8. Chippindale AK, Leroi AM, Kim SB, et al. Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction. J Evol Biol 1993; 6: 171-193
9. Jiang JC, Jaruga E, Repnevskaya MV, et al. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. FASEB J 2000; 14: 2135-2137
10. Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science 2000; 289: 2126-2128
11. Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009; 325: 201-204
12. Heilbronn LK, de Jonge L, Frisard MI, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA 2006; 295: 1539-1548
13. Weiss EP, Racette SB, Villareal DT, et al. Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr 2006; 84: 1033-1042
14. Fontana L, Klein S. Aging, adiposity, and calorie restriction. JAMA 2007; 297: 986-994
15. Mair W, Goymer P, Pletcher SD, et al. Demography of dietary restriction and death in Drosophila. Science 2003; 301: 1731-1733
16. Sinclair DA. Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev 2005; 126: 987-1002
17. Brown-Borg HM. Longevity in mice: is stress resistance a common factor? Age (Dordr) 2006; 28: 145-162
18. Sun D, Muthukumar AR, Lawrence RA, et al. Effects of calorie restriction on polymicrobial peritonitis induced by cecum ligation and puncture in young C57BL/6 mice. Clin Diagn Lab Immunol 2001; 8: 1003-1011
19. Richardson A, Liu F, Adamo ML, et al. The role of insulin and insulin-like growth factor-I in mammalian ageing. Best Pract Res Clin Endocrinol Metab 2004; 18: 393-406
20. Friedewald JJ, Rabb H. Inflammatory cells in ischemic acute renal failure. Kidney Int 2004; 66: 486-491
21. Yu ZF, Mattson MP. Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. J Neurosci Res 1999; 57: 830-839
22. Chandrasekar B, Nelson JF, Colston JT, et al. Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2001; 280: H2094-2102

23. Ahmet I, Wan R, Mattson MP, et al. Cardioprotection by intermittent fasting in rats. Circulation 2005; 112: 3115-3121
24. Leemans JC, Stokman G, Claessen N, et al. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin Invest 2005; 115: 2894-2903
25. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29: e45
26. Irizarry RA, Bolstad BM, Collin F, et al. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 2003; 31: e15
27. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004; 3: Article3
28. Kim SB, Yang S, Kim SK, et al. GAzer: gene set analyzer. Bioinformatics 2007; 23: 1697-1699
29. Swindell WR. Comparative analysis of microarray data identifies common responses to caloric restriction among mouse tissues. Mech Ageing Dev 2008; 129: 138-153
30. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136
31. Gredilla R, Barja G. Minireview: the role of oxidative stress in relation to caloric restriction and longevity. Endocrinology 2005; 146: 3713-3717
32. Stuart JA, Karahalil B, Hogue BA, et al. Mitochondrial and nuclear DNA base excision repair are affected differently by caloric restriction. FASEB J 2004; 18: 595-597
33. Kaeberlein M, Hu D, Kerr EO, et al. Increased life span due to calorie restriction in respiratory-deficient yeast. PLoS Genet 2005; 1: e69
34. Merry BJ. Molecular mechanisms linking calorie restriction and longevity. Int J Biochem Cell Biol 2002; 34: 1340-1354
35. Kelly KJ. Distant effects of experimental renal ischemia/reperfusion injury. J Am Soc Nephrol 2003; 14: 1549-1558
36. Kalaany NY, Sabatini DM. Tumours with PI3K activation are resistant to dietary restriction. Nature 2009; 458: 725-731
37. Dhahbi JM, Kim HJ, Mote PL, et al. Temporal linkage between the phenotypic and genomic responses to caloric restriction. Proc Natl Acad Sci U S A 2004; 101: 5524-5529
38. Estep PW, 3rd, Warner JB, Bulyk ML. Short-term calorie restriction in male mice feminizes gene expression and alters key regulators of conserved aging regulatory pathways. PLoS One 2009; 4: e5242
39. Kher A, Meldrum KK, Wang M, et al. Cellular and molecular mechanisms of sex differences in renal ischemia-reperfusion injury. Cardiovasc Res 2005; 67: 594-603
40. Sumimoto R, Southard JH, Belzer FO. Livers from fasted rats acquire resistance to warm and cold ischemia injury. Transplantation 1993; 55: 728-732
41. Nishihara V, Sumimoto R, Fukuda Y, et al. Inhibition of warm ischemic injury to rat liver, pancreas, and heart grafts by controlling the nutritional status of both donor and recipient. Surg Today 1997; 27: 645-650
42. Raffaghello L, Lee C, Safdie FM, et al. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. Proc Natl Acad Sci U S A 2008; 105: 8215-8220
43. Tatar M, Bartke A, Antebi A. The endocrine regulation of aging by insulin-like signals. Science 2003; 299: 1346-1351
44. Miller SB, Martin DR, Kissane J, et al. Insulin-like growth factor I accelerates recovery from ischemic acute tubular necrosis in the rat. Proc Natl Acad Sci U S A 1992; 89: 11876-11880
45. Guan J, Bennet L, Gluckman PD, et al. Insulin-like growth factor-1 and post-ischemic brain injury. Prog Neurobiol 2003; 70: 443-462

46. Takeda R, Nishimatsu H, Suzuki E, et al. Ghrelin improves renal function in mice with ischemic acute renal failure. J Am Soc Nephrol 2006; 17: 113-121
47. Suleiman MS, Singh RJ, Stewart CE. Apoptosis and the cardiac action of insulin-like growth factor I. Pharmacol Ther 2007; 114: 278-294
48. Greer EL, Dowlatshahi D, Banko MR, et al. An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol 2007; 17: 1646-1656
49. Mattson MP. The need for controlled studies of the effects of meal frequency on health. Lancet 2005; 365: 1978-1980
50. Goodrick CL, Ingram DK, Reynolds MA, et al. Effects of intermittent feeding upon growth and life span in rats. Gerontology 1982; 28: 233-241
51. Sogawa H, Kubo C. Influence of short-term repeated fasting on the longevity of female (NZB x NZW)F1 mice. Mech Ageing Dev 2000; 115: 61-71
52. Anson RM, Guo Z, de Cabo R, et al. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proc Natl Acad Sci U S A 2003; 100: 6216-6220
53. Varady KA, Hellerstein MK. Alternate-day fasting and chronic disease prevention: a review of human and animal trials. Am J Clin Nutr 2007; 86: 7-13
54. Masoro EJ, Shimokawa I, Higami Y, et al. Temporal pattern of food intake not a factor in the retardation of aging processes by dietary restriction. J Gerontol A Biol Sci Med Sci 1995; 50A: B48-53
55. Masoro EJ. Caloric intake versus temporal pattern of food intake. Aging Clin Exp Res 2004; 16: 423-424
56. Studley HO. Percentage of weight loss: a basic indicator of surgical risk in patients with chronic peptic ulcer. JAMA 1936; 106: 458-460
57. Chung A. Perioperative nutrition support. Nutrition 2002; 18: 207-208
58. Maltby JR. Fasting from midnight--the history behind the dogma. Best Pract Res Clin Anaesthesiol 2006; 20: 363-378
59. Diks J, van Hoorn DE, Nijveldt RJ, et al. Preoperative fasting: an outdated concept? JPEN J Parenter Enteral Nutr 2005; 29: 298-304
60. Soreide E, Eriksson LI, Hirlekar G, et al. Pre-operative fasting guidelines: an update. Acta Anaestsiol Scand 2005; 49: 1041-1047
61. Colman RJ, Beasley TM, Allison DB, et al. Attenuation of sarcopenia by dietary restriction in rhesus monkeys. J Gerontol A Biol Sci Med Sci 2008; 63: 556-559

Chapter 4

Altered mitochondrial functioning induced by preoperative fasting may underlie protection against renal ischemia/reperfusion injury

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Submitted

Chapter 5

Glucose supplementation does not interfere with fasting-induced protection against renal ischemia/reperfusion injury in mice

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Abstract

Background: Preoperative fasting induces robust protection against renal ischemia/reperfusion (I/R) injury in mice, but is considered overcautious and possibly detrimental for postoperative recovery in humans. Furthermore, fasting seems to conflict with reported benefits of preoperative nutritional enhancement with carbohydrate-rich drinks. Here, we investigated whether preoperative ingestion of a glucose solution interferes with fasting-induced protection against renal I/R injury.

Methods: Mice were randomized into the following groups: fasted for three days with access to water (fasted), or a glucose solution (fasted + glc), or fed *ad libitum* with water (fed), or a glucose solution (fed + glc). After induction of bilateral renal I/R injury, all animals had free access to food and water. Calorie intake, body weight, insulin sensitivity, kidney function and animal survival were determined.

Results: Fed + glc mice had a comparable daily calorie intake as fed mice, but 50% of those calories were obtained from the glucose solution. Fasted + glc mice had a daily calorie intake of approximately 75% of the intake of both fed groups. This largely prevented the substantial body weight loss seen in fasted animals. Preoperative insulin sensitivity was significantly improved in fasted + glc mice versus fed mice. After I/R injury, kidney function and animal survival were superior in both fasted groups.

Conclusion: The benefits of fasting and preoperative nutritional enhancement with carbohydrates are not mutually exclusive, and may be a clinically feasible regimen to protect against renal I/R injury.

Introduction

In clinical kidney transplantation, organ recovery and cold storage of the renal graft result in the initiation of renal ischemia/reperfusion (I/R) injury.[1] On transplantation in the recipient, reperfusion of the graft promotes the generation of reactive oxygen species,[2] and the activation of an inflammatory response,[3] resulting in profound graft injury after transplantation. Renal I/R injury is a risk factor for the development of delayed graft function,[4] primary non-function,[5] acute rejection[6] and chronic allograft nephropathy.[7] I/R injury is also unavoidably induced in elective surgeries including cardiac[8] and vascular surgery.[9] Treatment of I/R injury is still unsatisfactory. Therefore, the development of new therapeutic interventions to reduce or prevent I/R injury remains warranted.

We have reported recently that preoperative fasting from solid food strongly improves postoperative kidney function and animal survival following renal I/R injury. Three days of fasting induced a significant reduction in oxidative damage, inflammation, and cell death in the post-ischemic kidneys from preoperatively fasted mice.[10]

The beneficial effects of preoperative fasting against renal I/R injury are in apparent conflict with reported benefits of a different nutritional intervention: preoperative carbohydrate loading. In the clinical setting it has been shown that the use of liquid carbohydrate-rich drinks up to 2 hours before surgery does not increase the risk for pulmonary aspiration of regurgitated stomach contents during anesthesia.[11, 12] Moreover, preoperative use of carbohydrate-rich drinks improves preoperative patient subjective well-being.[12] After surgery, it decreases the catabolic response to surgery, including postoperative insulin resistance.[13, 14] It improves postoperative recovery,[15] and reduces postoperative hospital stay.[16]

The purpose of the present study was to investigate whether the combination of preoperative fasting from solid food and concomitant administration of a carbohydrate-rich drink interferes with protection against renal I/R injury in mice. To do this, we allowed mice free access to a glucose solution during a 3-day preoperative fasting regimen, and investigated its effect on fasting-induced protection against renal I/R injury. Here, we report that the consumption of an oral glucose solution does not interfere with fasting-induced protection against renal I/R injury.

Material and Methods

Animals

Male C57BL/6 mice (~25 g) were obtained from Harlan (Horst, the Netherlands). On arrival, mice were housed at random under standard conditions in individually ventilated cages (n = 3-4 mice per cage) with free access to tap water (pH = 2.4-2.7) and food (Special Diet Services, Witham, UK). At the start of the experiment, all mice were transferred to clean cages at 5:00 pm. Fed and fed + glc mice were allowed free access to food and water or a glucose solution for 3

consecutive days. Fasted and fasted + glc mice had free access to water or a glucose solution, but were withheld from food for 3 days. No deaths occurred during this fasting regimen. In a separate experiment, mice were fed 70% of the daily intake of *ad libitum* fed mice for 3 days (30% food restricted group). The experimental protocol was approved by the Animal Experiments Committee under the Dutch National Experiments on Animals Act, and it complied with Directive 86/609/EC (1986) of the Council of Europe.

Surgical procedure

Bilateral renal I/R injury was induced in fasted, fasted + glc, fed, fed + glc, and 30% food restricted mice as described.[10]

Kidney function

Kidney function was determined in fasted, fasted + glc, fed, fed + glc, and 30% food restricted mice as described.[10]

Dietary intake

At the start of the experiment a 60% (wt/vol) glucose solution was prepared (D (+) glucose, Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). This solution contained 2.2 calories per milliliter. The daily intake of the glucose solution or the water was measured by weighing the drinking bottles. The measurements were corrected for leakage by recording weight change of 4 control bottles (2 filled with the glucose solution, 2 filled with water). The daily food intake was measured by weighing the food. The average calorie intake per mouse per day was determined until the day of surgery by calculating the amount of calories ingested by the animals from the food and the glucose solution.

Insulin sensitivity

Insulin sensitivity was determined in separate groups of fasted, fasted + glc, fed, and fed + glc mice as described.[10] These mice were not subjected to renal I/R injury.

Statistical analysis

The data are expressed as means ± SEM. Differences between the groups were compared by Mann-Whitney U tests (SPSSv15). Differences in survival rates were analyzed by Log-rank tests (SPSSv15). The area under the curve was calculated using GraphPad Prism 5.0. Differences between groups were considered significant at $P \leq 0.05$.

Results

Effect of glucose supplementation on food, liquid and calorie intake

We determined whether the intake of an oral glucose solution has an effect on the eating and

drinking behavior of fed, fed + glc, fasted and fasted + glc mice before subjecting them to bilateral renal I/R injury. Fed mice drank 3.5 ± 0.3 mL water (Figure 1A), and consumed 4.0 ± 0.3 gram of food per day (Figure 1B), which was equivalent to a calorie intake of 14.4 ± 1.1 kcal per day (Figure 1C). Fed + glc mice consumed half the amount of food as the fed animals (2.0 ± 0.1 gram vs. food intake of fed mice, $P = 0.050$; Figure 1B). These mice drank 3.5 ± 0.4 mL per day of the glucose solution (Figure 1A) and received 50% of their calories from the glucose solution. This resulted in a comparable daily calorie intake (14.7 ± 0.9 kcal per day) as the fed mice (Figure 1C). During the 3-day fasting regimen, fasted mice had no access to calories and drank only 0.4 ± 0.1 mL of water per day ($P = 0.050$ vs. fed mice). In contrast, fasted + glc mice drank 1.5 times more than the fed + glc mice (5.2 ± 0.2 mL per day, $P = 0.050$ vs. fed + glc mice; Figure 1A). These mice obtained a daily calorie intake from the glucose solution of approximately 75% of the intake of both fed groups (11.1 ± 0.5 kcal per day; Figure 1C).

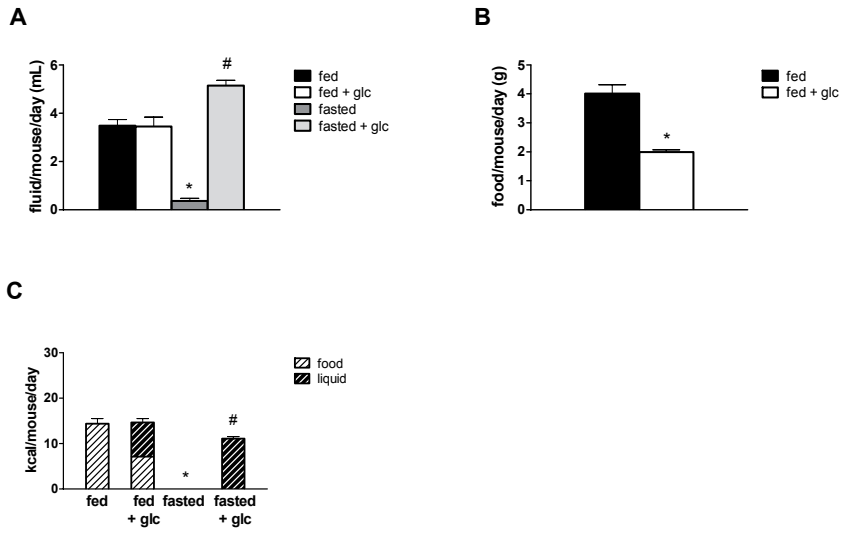

Figure 1. The effect of oral glucose supplementation on food, liquid and calorie intake. (A) Fluid intake per mouse per day. Fasted + glc mice compensated for the calorie loss by increasing their fluid intake during the three day fasting period. (B) Food intake per mouse per day. Administration of the glucose solution reduced the solid food intake of fed + glc mice as compared with fed mice. (C) Kilocalorie intake determined per mouse per day. Each day, fasted + glc mice obtained ~75% of the calories of both fed groups from the glucose solution. Graphs represent the average intake per mouse per group over three days (n = 8 per group). Error bars indicate SEM. $^{*}P \leq 0.05$ vs. fed mice; $^{\#}P < 0.05$ vs. fed + glc mice.

Effect of preoperative glucose supplementation on perioperative weight

Next, we investigated the effect of preoperative glucose supplementation on perioperative body weight in preoperatively fed mice and 3-day fasted mice. After three days of fasting, fasted mice had lost 26% of their body weight, whereas fasted + glc mice only lost 8% of their

body weight (P = 0.001 vs. fasted mice; Figure 2A). After surgery, fasted mice started eating rapidly. This resulted in an 8% increase in body weight on postoperative day (POD) 2 (P = 0.001 vs. the baseline body weight). Fasted + glc mice showed a similar response as both fed groups. On POD 2, these animals had lost body weight (fed: 13%; fed + glc: 15%; fasted + glc: 9%) as compared with their body weights on the day of surgery (P = 0.003, P = 0.002, and P = 0.007, respectively; Figure 2B).

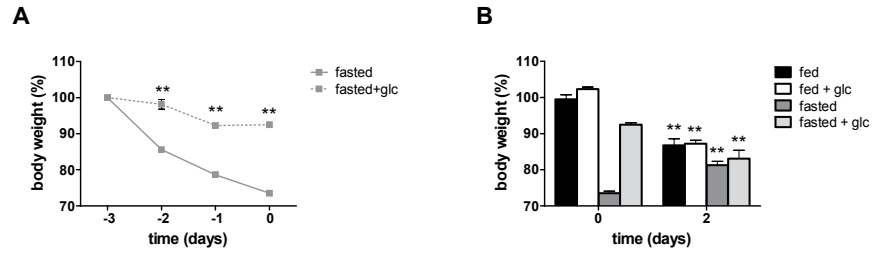

Figure 2. Perioperative body weight. (A) Administration of an oral glucose solution during the 3-day fasting regimen prevented the substantial body weight loss seen in fasted mice. The data are expressed as means ± SEM (n = 8 per group). **P < 0.01 vs. fasted mice at the indicated time points. (B) Two days after surgery, the body weight of fasted mice was increased versus their body weight at baseline, whereas it decreased in fasted + glc mice, fed mice, and fed + glc mice. The data are expressed as means ± SEM (n = 8 per group). **P < 0.01 vs. the same group at baseline (t = day 0).

Effect of preoperative glucose supplementation on kidney function and animal survival

Three days of fasting before induction of renal I/R injury improves postoperative kidney function and animal survival.[10] Here, we investigated the effect of oral glucose supplementation on postoperative kidney function and animal survival in preoperatively fed mice and 3-day fasted mice. Both fasted mice (66.6 ± 8.1 mmol/L) and fasted + glc mice (43.8 ± 10.5 mmol/L) had significantly lower serum urea concentrations on POD 2 than fed mice (101.9 ± 3.4 mmol/L, P = 0.008 vs. fasted mice) and fed + glc mice (112.7 ± 2.1 mmol/L, P = 0.007 vs. fasted + glc mice; Figure 3A). Renal I/R injury reduced the survival rate of fed + glc mice (0%) and fed mice (25%) on POD 7 (Figure 3B). Glucose supplementation did not abolish the protective effect induced by preoperative fasting, as fasted + glc mice had a survival rate equal to fasted mice (100%, P = 0.003 vs. fed mice and P = 0.000 vs. fed + glc mice; Figure 3B).

Effect of fasting with or without additional glucose supplementation on insulin sensitivity

Preoperative intake of a carbohydrate-rich drink improves insulin sensitivity at the onset of surgery,[17] and might be related to reduced development of insulin resistance after surgery.[18] We investigated the effect of oral glucose supplementation on insulin sensitivity in *ad libitum* fed mice and 3-day fasted mice with an insulin sensitivity test. At baseline, the blood glucose levels of fed mice and fed + glc mice were 10.4 ± 0.2 mmol/L and 9.4 ± 0.2 mmol/L, respectively (Figure 4A). Three days of fasting significantly lowered the baseline blood glucose

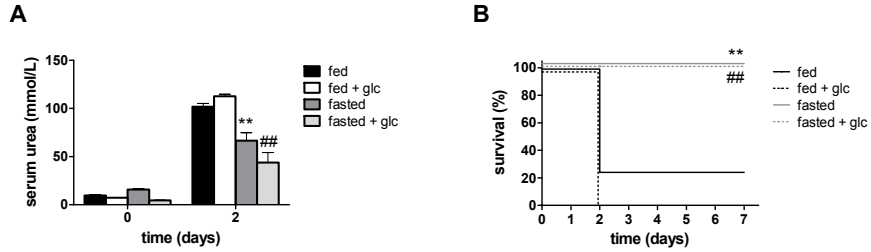

Figure 3. The effect of preoperative glucose supplementation on kidney function and animal survival. (A) Kidney function. After renal I/R injury, kidney function was significantly better in fasted mice and fasted + glc mice as compared with both fed groups. The data are expressed as means ± SEM (n = 8 per group). $^{**}P < 0.01$ vs. fed mice; $^{\#\#}P < 0.01$ vs. fed + glc mice. (B) Kaplan Meier survival curve. The survival rates of fasted mice and fasted + glc mice were significantly better than those of both fed groups (n = 8 per group). $^{**}P < 0.01$ vs. fed mice; $^{\#\#}P < 0.01$ vs. fed + glc mice.

levels of fasted mice (6.1 ± 0.3 mmol/L) as compared to fed mice. Glucose supplementation during fasting ameliorated this decrease and resulted in blood glucose levels comparable to those of *ad libitum* fed mice (11.1 ± 0.4 mmol/L). Two hours after an intraperitoneal injection with insulin, the blood glucose levels of fed mice and fed + glc mice raised above baseline values. In both fasted groups these values remained lower (Figure 4A). The area under the curve of the blood glucose levels is inversely proportional to glucose clearance in response to insulin challenge. Because the area under the curve was significantly lower in fasted + glc mice (935 ± 31) as compared to fed mice (1097 ± 10, $P = 0.001$; Figure 4B), this indicated that preoperative insulin sensitivity was significantly improved in fasted + glc mice.

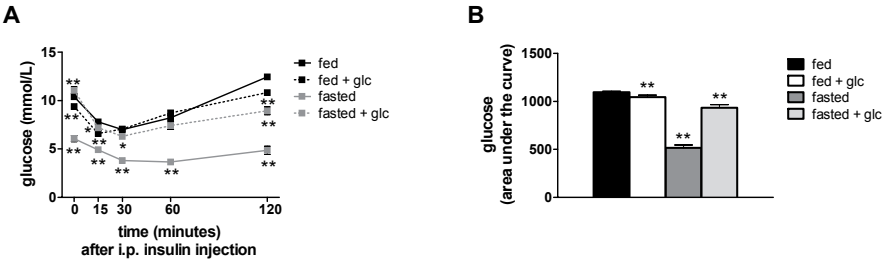

Figure 4. Insulin sensitivity test. (A) After an intraperitoneal injection with a bolus of insulin, the blood glucose levels of fasted mice and fasted + glc mice remained lower than those of both fed groups. The data are expressed as means ± SEM (n = 8-9 per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. fed mice. (B) Area under the curve. Preoperative insulin sensitivity was significantly improved in fasted + glc mice vs. fed mice. The data are expressed as means ± SEM (n = 8-9 per group). $^{**}P < 0.01$ vs. fed mice.

Effect of a 3-day reduction in food intake on renal I/R injury

During the fasting regimen, fasted + glc mice obtained approximately 75% of their normal

calorie intake from the glucose solution. Therefore, we determined whether a 3-day reduction in calorie intake by approximately 25% also protects against renal I/R injury. For three days, mice received only 70% of their normal amount of daily food before they were subjected to bilateral renal I/R injury. During the three days of restriction, these mice lost 8% body weight, which was similar to the reduction in body weight of the fasted + glc mice (Figure 5A). However, 30% food restriction did not induce protection against renal I/R injury. The survival rate of the 30% food restricted group was 33%, whereas in the fasted + glc group it was 100% ($P = 0.011$; Figure 5B). On POD 2, the kidney function was significantly worse in the food restricted mice (119.7.3 ± 17.7 vs. 43.8 ± 10.5 mmol/L in fasted + glc mice, $P = 0.011$; Figure 5C).

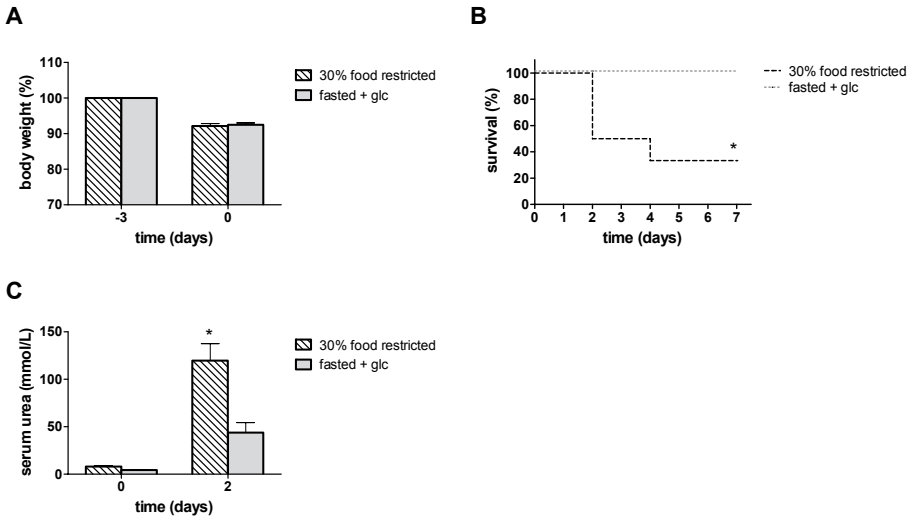

Figure 5. Three days 30% reduction in food intake does not protect against renal I/R injury. (A) Perioperative body weights. Mice restricted to 70% of the normal daily calorie intake of fed mice had a similar reduction in preoperative body weight as compared with the fasted + glc group. The data are expressed as means ± SEM (n = 6-8 per group). (B) Kaplan Meier survival curve. Three days of food restriction had no effect on postoperative animal survival. Survival rate of the 30% food restricted group was 33% versus 100% in the fasted + glc group (n = 6-8 per group). *$P < 0.05$ vs. fasted + glc mice. (C) Kidney function. Following renal I/R, kidney function was significantly worse in the 30% food restricted group versus the fasted + glc group. The data are expressed as means ± SEM (n = 6-8 per group). *$P < 0.05$ vs. fasted + glc mice.

Discussion

The robust protection afforded by preoperative fasting on renal I/R injury, we described previously[10] is in apparent conflict with the reported benefits of preoperative administration of carbohydrate-rich drinks on patient subjective well-being[15] and postoperative recovery.[12] In the present study, we determined whether the combination of fasting from solid food and

administration of a carbohydrate-rich drink interferes with the protective effect of preoperative fasting against renal I/R injury in mice. We show that consumption of an oral glucose solution during the preoperative fasting regimen has no significant effect on postoperative kidney function or animal survival. Although fasted + glc mice lose some body weight, ingestion of a glucose solution during the preoperative fasting regimen prevents the excessive weight loss seen in water-only fasted animals. In addition, it significantly improves preoperative insulin sensitivity as compared with *ad libitum* feeding.

Preoperative fasting has been associated with negative postoperative outcomes including poor wound healing, increased risk of infection and multiple organ failure.[19] One of the few clinical applications of preoperative fasting is to reduce the risk of pulmonary aspiration of regurgitated stomach contents during anesthesia. Although the origins in the 1960s of the standard "nil by mouth after midnight" fasting policy is somewhat obscure,[20] today even this relatively short fasting period is perceived as overcautious and possibly detrimental for patient subjective well-being and postoperative recovery.[21] However, recently we have shown that slightly longer fasting periods may be beneficial for an entirely different purpose: protection against acute oxidative stress induced by renal I/R injury.[10]

Currently more liberal fasting guidelines allow consumption of solids up to 6 hours before surgery, and recommend consumption of liquids, including carbohydrate-rich drinks, up to 2 hours prior to surgery.[22] We show that the benefits of preoperative fasting do not seem to be mutually exclusive with the benefits of preoperative carbohydrate supplementation. This may be due in part to the different endpoints addressed by these two different nutritional interventions. Whereas preoperative fasting may activate endogenous stress resistance, preoperative carbohydrate consumption may improve measures of patient subjective well-being such as thirst, hunger, irritability and headache associated with preoperative fasting.[23] The fact that consumption of a glucose solution does not significantly interfere with the benefits of fasting on postoperative kidney function and animal survival suggests that the lack of glucose, and possibly other carbohydrates is not the sole trigger underlying the benefits of preoperative fasting.

The benefits of fasting before general anesthesia remain controversial. Although it is well known that overnight fasting reduces the risk of pulmonary aspiration during anesthesia,[20] other benefits of preoperative fasting in humans are not known. However, in rat transplant models, fasting of the donor for 3 to 4 days improves the tolerance of the graft to warm and cold ischemia.[24, 25] In addition, 1 to 3 days of preoperative fasting protects against renal I/R injury in rats[26] and mice.[10] Depite these findings, others have reported adverse effects of preoperative fasting. In isolated rat liver perfusion models, preoperative fasting increases the hepatic injury after I/R.[27, 28] In humans, preoperative overnight fasting reduces subjective well-being.[12, 29] Studies in healthy volunteers showed that fasting for 48 or 60 hours, but not for 12 hours, induces insulin resistance.[30-32] In addition, it has been shown that overnight fasting can induce insulin resistance. However, in this setting, insulin resistance develops as a result of preoperative fasting, and adds to the catabolic response to surgery.[33]

Development of postoperative insulin resistance is considered to be detrimental, because it is associated with increased mortality and morbidity,[34] prolonged hospital stay,[35] postoperative infections,[36] and decreased wound healing.[37] Preoperative carbohydrate administration by an intravenous infusion of a glucose solution or by the oral intake of drinks containing a carbohydrate mixture as the only energy source, can reduce the degree of postoperative insulin resistance.[13, 14, 18, 33] In addition, Svanfeldt *et al.*[17] reported that the intake of carbohydrate-rich drinks before and after an overnight fasting period enhances preoperative insulin sensitivity in humans. They suggest that the reduction in postoperative insulin resistance[18] may, at least in part, be mediated by improved preoperative insulin sensitivity. In our study, the baseline blood glucose levels in water-only fasted mice were significantly lower than in fasted mice that received a glucose solution during fasting. However, the baseline blood glucose levels of the fasted + glc mice were not different from that of the fed animals. Nevertheless, preoperative insulin sensitivity of fasted + glc mice was significantly better than that of fed mice. This strongly suggests that the insulin sensitivity of water-only fasted mice is also improved, although we cannot formally prove this because of the lower baseline blood glucose levels of these animals.

In this study we showed that preoperative fasted + glc mice obtained approximately 75% of the normal calorie intake of fed mice by ingestion of the glucose solution. Although the intake of calories resulted in substantially less body weight loss during the 3-day fasting regimen, it had no significant effect on the protection against renal I/R injury. When mice received only 70% of their normal amount of daily food for three consecutive days, a similar reduction in preoperative body weight was observed. However, this did not protect against renal I/R injury. These findings suggest that protection against renal I/R injury is not only directly related to an absolute reduction in calorie intake *per se*, but also to the absence or surplus of certain food components. Further experiments are required to investigate which specific food components induce the protection by preoperative fasting.

The question remains what the mechanism of protection by preoperative fasting with/or without additional glucose supplementation is. Preoperative fasting with glucose supplementation improved postoperative kidney function and animal survival, and was associated with improved preoperative insulin sensitivity. Improved insulin sensitivity is correlated with reduced baseline insulin/insulin-like growth factor-1 (IGF-1) signaling.[38] We have shown previously that preoperative fasting increases the expression of antioxidant enzymes, and reduces the expression of inflammatory markers before induction of renal I/R injury. Accordingly, preoperative fasting results in less oxidative injury, cell death and inflammation after surgery.[10] Our findings suggest that the improved insulin sensitivity and subsequent reduction in insulin/IGF-1 signaling before renal I/R increases stress resistance through up-regulation of antioxidant and cytoprotective enzymes.

Sirtuin1 (SIRT1) is a deacetylase induced by food restriction.[39] In response to oxidative stress, SIRT1 regulates forkhead box O (FOXO), a family of transcription factors which become activated when insulin/IGF-1 signaling is reduced.[40] Activation of FOXO3a by SIRT1 increases resistance to oxidative stress and reduces cell death.[40] Overexpression of FOXO1 in mouse

livers improves insulin sensitivity.[41] This suggests that a possible mechanism underlying the protective response by preoperative fasting might involve the activation of SIRT1 and the FOXO family of transcription factors. Future experiments will be required to determine whether any of these changes underlie the benefits of preoperative fasting

In the clinical setting, we have shown recently that a preoperative reduction in food intake is not only feasible in live kidney donors,[42] but also induces beneficial changes in the acute phase response after organ retrieval.[43] Thus, although the data obtained in mice cannot be translated to humans directly, our clinical data indicate that preoperative food restriction in humans is feasible and beneficial. Carbohydrate loading may alleviate the unfavorable effects of preoperative food restriction on patient well-being, and make preoperative food restriction in surgical patients more readily applicable.

In conclusion, preoperative fasting may represent a non-invasive method to protect against renal I/R injury. The benefits of preoperative fasting are not adversely affected by the ingestion of a glucose solution during fasting. This suggests that the benefits of preoperative fasting are not necessarily incompatible with the current practice of preoperative carbohydrate loading. Combining carbohydrate-rich beverages with preoperative fasting may be a clinically feasible regimen to protect against renal I/R injury without the adverse effects of fasting.

Acknowledgements

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References

1. Hosgood SA, Bagul A, Yang B, et al. The relative effects of warm and cold ischemic injury in an experimental model of nonheartbeating donor kidneys. Transplantation 2008; 85: 88-92
2. Paller MS, Hoidal JR, Ferris TF. Oxygen free radicals in ischemic acute renal failure in the rat. J Clin Invest 1984; 74: 1156-1164
3. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? Kidney Int 2004; 66: 480-485
4. Sola R, Alarcon A, Jimenez C, et al. The influence of delayed graft function. Nephrol Dial Transplant 2004; 19 Suppl 3: iii32-37
5. Asaka M, Imumura H, Sato K, et al. Ischemia–reperfusion injury as the leading cause of primary non-function in renal transplantation using donors with prolonged warm ischemic time. Clinical Transplantation 2008; 22: 19-24
6. Troppmann C, Gillingham KJ, Benedetti E, et al. Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. The multivariate analysis. Transplantation 1995; 59: 962-968
7. Fletcher JT, Nankivell BJ, Alexander SI. Chronic allograft nephropathy. Pediatr Nephrol 2009; 24: 1465-1471
8. Beyersdorf F. The use of controlled reperfusion strategies in cardiac surgery to minimize ischaemia/reperfusion damage. Cardiovasc Res 2009; 83: 262-268
9. Raby KE, Brull SJ, Timimi F, et al. The effect of heart rate control on myocardial ischemia among high-risk patients after vascular surgery. Anesth Analg 1999; 88: 477-482
10. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010; 9: 40-53
11. Nygren J, Thorell A, Jacobsson H, et al. Preoperative gastric emptying. Effects of anxiety and oral carbohydrate administration. Ann Surg 1995; 222: 728-734
12. Hausel J, Nygren J, Lagerkranser M, et al. A carbohydrate-rich drink reduces preoperative discomfort in elective surgery patients. Anesth Analg 2001; 93: 1344-1350
13. Faria MS, de Aguilar-Nascimento JE, Pimenta OS, et al. Preoperative fasting of 2 hours minimizes insulin resistance and organic response to trauma after video-cholecystectomy: a randomized, controlled, clinical trial. World J Surg 2009; 33: 1158-1164
14. Soop M, Nygren J, Myrenfors P, et al. Preoperative oral carbohydrate treatment attenuates immediate postoperative insulin resistance. Am J Physiol Endocrinol Metab 2001; 280: E576-583
15. Hausel J, Nygren J, Thorell A, et al. Randomized clinical trial of the effects of oral preoperative carbohydrates on postoperative nausea and vomiting after laparoscopic cholecystectomy. Br J Surg 2005; 92: 415-421
16. Noblett SE, Watson DS, Huong H, et al. Pre-operative oral carbohydrate loading in colorectal surgery: a randomized controlled trial. Colorectal Dis 2006; 8: 563-569
17. Svanfeldt M, Thorell A, Hausel J, et al. Effect of "preoperative" oral carbohydrate treatment on insulin action-a randomised cross-over unblinded study in healthy subjects. Clin Nutr 2005; 24: 815-821
18. Nygren J, Soop M, Thorell A, et al. Preoperative oral carbohydrate administration reduces postoperative insulin resistance. Clin Nutr 1998; 17: 65-71
19. Chung A. Perioperative nutrition support. Nutrition 2002; 18: 207-208
20. Maltby JR. Fasting from midnight--the history behind the dogma. Best Pract Res Clin Anaesthesiol 2006; 20: 363-378
21. Diks J, van Hoorn DE, Nijveldt RJ, et al. Preoperative fasting: an outdated concept? JPEN J Parenter Enteral Nutr 2005; 29: 298-304
22. Soreide E, Eriksson LI, Hirlekar G, et al. Pre-operative fasting guidelines: an update. Acta Anaesthesiol Scand 2005; 49: 1041-1047
23. Crenshaw JT, Winslow EH. Preoperative fasting: old habits die hard. Am J Nurs 2002; 102: 36-44; quiz 45

24. Sumimoto R, Southard JH, Belzer FO. Livers from fasted rats acquire resistance to warm and cold ischemia injury. Transplantation 1993; 55: 728-732
25. Nishihara V, Sumimoto R, Fukuda Y, et al. Inhibition of warm ischemic injury to rat liver, pancreas, and heart grafts by controlling the nutritional status of both donor and recipient. Surg Today 1997; 27: 645-650
26. Wystrychowski A, Wystrychowski W, Krol P, et al. Pentoxifylline augments fasting-induced nephroprotection in acute ischemic kidney injury in rats. Transplant Proc 2009; 41: 2992-2993
27. Stadler M, Nuyens V, Seidel L, et al. Effect of nutritional status on oxidative stress in an ex vivo perfused rat liver. Anesthesiology 2005; 103: 978-986
28. Tanigawa K, Kim YM, Lancaster JR, Jr., et al. Fasting augments lipid peroxidation during reperfusion after ischemia in the perfused rat liver. Crit Care Med 1999; 27: 401-406
29. Helminen H, Viitanen H, Sajanti J. Effect of preoperative intravenous carbohydrate loading on preoperative discomfort in elective surgery patients. Eur J Anaesthesiol 2009; 26: 123-127
30. van der Crabben SN, Allick G, Ackermans MT, et al. Prolonged fasting induces peripheral insulin resistance, which is not ameliorated by high-dose salicylate. J Clin Endocrinol Metab 2008; 93: 638-641
31. Newman WP, Brodows RG. Insulin action during acute starvation: evidence for selective insulin resistance in normal man. Metabolism 1983; 32: 590-596
32. Bjorkman O, Eriksson LS. Splanchnic glucose metabolism during leg exercise in 60-hour-fasted human subjects. Am J Physiol 1983; 245: E443-448
33. Ljungqvist O, Thorell A, Gutniak M, et al. Glucose infusion instead of preoperative fasting reduces postoperative insulin resistance. J Am Coll Surg 1994; 178: 329-336
34. van den Berghe G, Wouters P, Weekers F, et al. Intensive insulin therapy in the critically ill patients. N Engl J Med 2001; 345: 1359-1367
35. Thorell A, Nygren J, Hirshman MF, et al. Surgery-induced insulin resistance in human patients: relation to glucose transport and utilization. Am J Physiol 1999; 276: E754-761
36. Thomas MC, Mathew TH, Russ GR, et al. Early peri-operative glycaemic control and allograft rejection in patients with diabetes mellitus: a pilot study. Transplantation 2001; 72: 1321-1324
37. Pierre EJ, Barrow RE, Hawkins HK, et al. Effects of insulin on wound healing. J Trauma 1998; 44: 342-345
38. Coschigano KT, Holland AN, Riders ME, et al. Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span. Endocrinology 2003; 144: 3799-3810
39. Cohen HY, Miller C, Bitterman KJ, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004; 305: 390-392
40. Brunet A, Sweeney LB, Sturgill JF, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004; 303: 2011-2015
41. Matsumoto M, Han S, Kitamura T, et al. Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest 2006; 116: 2464-2472
42. van Ginhoven TM, de Bruin RW, Timmermans M, et al. Pre-operative dietary restriction is feasible in live-kidney donors. Clin Transplant 2011; 25(3):486-94
43. van Ginhoven TM, Dik WA, Mitchell JR, et al. Dietary restriction modifies certain aspects of the postoperative acute phase response. J Surg Res epub

Chapter 6

Protein, tryptophan and leucine-free diets induce protection against renal ischemia/reperfusion injury in mice

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Chapter 7

The use of preoperative nutritional interventions to protect against hepatic ischemia/reperfusion injury

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Abstract

Preoperative fasting was introduced in the 19th century to reduce the risk of aspiration pneumonia while patients were under general anesthesia. During the last decades, the value of preoperative fasting has been questioned, and more liberal guidelines have been proposed, such as the use of preoperative carbohydrate-rich drinks. Here, we review both old and new evidence supporting the view that fasting slightly longer than overnight is beneficial for an entirely different purpose: protection against certain types of stress, such as ischemia/reperfusion injury. We provide a framework to explain these benefits as well as future applications and alternatives that could be used to induce the protection afforded by nutritional interventions.

Introduction

Perioperative nutrition is a recurrent issue in experimental and clinical research related to the safety of anesthesia on one hand and the metabolic response to surgical trauma on the other hand. Studley[1] in 1936 was the first to report a negative correlation between (excessive) preoperative weight loss and surgical outcome following major abdominal or thoracic surgery. Although it is difficult to draw firm conclusions from this study because there was no control group that failed to lose weight or actually gained weight prior to surgery, in the clinical setting, malnutrition has been proved to indeed be a risk factor for surgical complications.[2-4]

The appreciation that a large number of hospitalized patients suffer from undernutrition has further fueled the attention paid to preoperative and postoperative feeding. Preoperative and postoperative nutritional support not only may reduce the complications of surgery, as shown in a randomized clinical trial by Beattie *et al.*,[5] but also may speed up the postoperative recovery.[6-8]

As early as 1858, John Snow wrote that the best time for an operation is before breakfast (thus after a night of fasting), arguing that "the possibility of vomiting constitutes an unpleasantness and inconvenience which is desirable to avoid." Anesthesia relaxes the gag reflex and increases the chance for either pulmonary aspiration or aspiration pneumonia. To reduce the risk of pulmonary aspiration, patients at the end of the 18th century and in the early 19th century were mainly allowed only a cup of tea up to a few hours before surgery.[9] With little evidence to prove its usefulness, this practice has evolved to become the accepted "nil by mouth from midnight" regimen, which has been widely used in the 20th century. During the last decades, the value of preoperative fasting has been questioned, and it has been shown recently that the intake of clear fluids up to 2 hours before surgery does not increase the gastric residual volume or risk of aspiration in comparison with overnight fasting.[10] Nowadays, a 6-hour fast from solid foods and a 2-hour fast from clear liquids prior to surgery are accepted as safe for healthy individuals.[10] Along with the increasing understanding that patients require an optimal nutritional status before surgery, it has been shown that surgery induces resistance to the actions of insulin, which may be ameliorated by the preoperative administration of carbohydrates. Randomized studies in which glucose was administered either as an infusion or as a carbohydrate-rich drink taken 2 to 3 hours before surgery found that glucose reduced postoperative insulin resistance and increased the subjective well-being of patients before and after surgery.[11-16]

Although the literature is replete with studies showing the adverse effects of the fasted state for surgical patients,[17-19] there are a number of older studies as well as emerging new data in the field indicating that different types of dietary restriction (DR; i.e., reduced food intake without malnutrition) in well-nourished patients may in fact be beneficial as a way of protecting against acute organ stress. DR can be performed by means of different regimens such as calorie restriction (CR; i.e., reduced daily calorie intake), fasting (no food intake), and alternate day fasting (ADF), which are associated in the experimental literature with lifespan

extension and increased stress resistance in a wide range of organisms.[20-23] In this review, we provide an overview of the studies lending support to the beneficial effects of DR in the context of increased resistance to surgical stress in the liver. More generally, we provide a perspective that attempts to explain how various types of DR, including CR, fasting, and ADF, up-regulate endogenous cell resistance mechanisms and how these benefits may be further explored in liver transplantation and surgery.

Fasting protects against hepatic ischemia/reperfusion injury

Ischemia/reperfusion (I/R) injury is unavoidable during liver transplantation and is commonly induced during liver resections when vascular occlusion techniques, such as the Pringle maneuver, are used. The effects of fasting on I/R injury were investigated in the 1990s, because it was suggested that donor nutritional status may affect the outcome after liver transplantation and that starvation of donors, due to prolonged stays in the intensive care unit, may adversely affect the transplanted liver.[24]

Aiming to investigate the effects of the duration of donor fasting on the outcome after orthotopic liver transplantation (OLT) in a rat model, Sumimoto *et al.*[25] found to their surprise that livers from fasted donors were more viable than livers from fed donors. After 45 minutes of warm ischemia, 50% of the recipients survived when the liver was obtained from a fed donor, whereas 80% survived when the liver was obtained from a 3-day-fasted donor. Increasing the warm ischemia time to 60 minutes resulted in 100% mortality in the fed donor group. In contrast, when the donor was fasted for 3 days, 89% of the transplanted animals survived for 7 days. Livers that were cold-stored for 30 hours were 50% viable, and fasting for 1 to 3 days did not affect this outcome. However, when the donor was fasted for 4 days, 100% survival was obtained. After 44 hours of cold preservation, only 29% of the recipients survived for 7 days. When the donor was fasted for 4 days, survival increased to 83%. In addition, liver function, bile production, and serum aminotransferases were better in livers from the fasted donors than in livers from the surviving fed rats (Table 1).

The glycogen content of the liver has been studied as a possible factor determining the outcome after OLT. As glycogen provides energy to maintain cellular function, glycogen was expected to reduce ischemic preservation injury. The results of these experiments were somewhat paradoxical because both fasting (glycogen depletion) and feeding plus oral glucose supplementation (glycogen restoration) were beneficial for survival after the transplantation of cold-stored liver grafts. Glucose supplementation in rats, prior to the harvesting of the liver, could result in newly synthesized glycogen, which could lead to reduced hepatocellular damage.[26] Because this study also found that fasting and fasting plus oral glucose supplementation had similar beneficial effects, an alternative explanation is that the glucose-supplemented rats obtained their calories from glucose and consumed less chow and thus were restricted in calorie intake from food. However, Sumimoto *et al.*[27] reported that,

Table 1. Studies reporting beneficial effects of donor fasting prior to OLT.

Reference	Animal	OLT model	Intervention	Outcome compared to an *ad libitum* fed control group
Sumimoto *et al.*[25] (1993)	Brown Norway rat	- warm ischemia (45/60 minutes) - cold ischemia (30/44 hours)	- 24, 48, 72 or 96 hours of water-only fasting	- Fasting induced higher recipient survival rates after cold and warm ischemia and lower ALT and AST serum levels.
Sun *et al.*[28] (2001)	Wistar rat	- cold ischemia (24 hours)	- 96 hours of water-only fasting	- Fasting induced higher 14-day survival rates in the recipient (0% vs. 90%). - Fasting reduced the number of apoptotic SLCs in comparison with the *ad libitum* fed donors. - Fasting reduced the LDH serum levels 6 hours after transplantation.
Sankary *et al.*[29] (1995)	Lewis rat	- cold ischemia (8 or 12 hours)	- 48 hours of water-only fasting	- Fasting resulted in significantly higher recipient survival rates after 12 hours of ischemia (0% vs. 83%). - Fasting resulted in a significant decrease in peripheral blood TNF-α levels after reperfusion.
Takahashi *et al.*[31] (1998)	Brown Norway rat	- cold ischemia (48, 60, 72 or 96 hours)	- 24, 48, 72, or 96 hours of water-only fasting	- Ninety-six hours of fasting resulted in significantly higher recipient survival rates after 48 and 72 hours of ischemia. - HSP60 and HSP70 showed increased expression after 4 days of fasting.
Uchida *et al.*[33] (2000)	Lewis rat	- cold ischemia (24 hours)	- 48 hours of water-only fasting	- Fasting induced higher 7-day survival rates in the recipient (0% vs. 87.5%. - Fasting induced expression of HO-1 in Kupffer cells. - The tissue GSH content was less reduced in livers from fasted donors.
Sumimoto *et al.*[27] (1996)	Brown Norway rat	- cold ischemia (30-44 hours)	- 96 hours of water-only fasting with or without oral glucose water	- Fasting induced higher recipient survival rates after cold ischemia, whereas glucose supplementation lowered survival.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, glutathione; HO-1, heme oxygenase-1; HSP, heat shock protein; LDH, lactate dehydrogenase; OLT, orthotopic liver transplantation; SLC, sinusoidal lining cell; TNF-a, tumor necrosis factor-alpha

in a rat model, 4 days of donor fasting resulted in the highest survival rate of the recipient, whereas the fasted group with glucose supplementation had high glycogen levels but the worst survival rates.

In 1995, Sankary *et al.*[28] showed that recipient survival after OLT in a rat model after 12 hours of cold ischemia was significantly higher with donors that were fasted for 48 hours versus *ad libitum* fed donors. In addition, they showed that fasting was associated with significantly lower tumor necrosis factor alpha serum levels after transplantation, which suggested lower Kupffer cell activation.

When cells are exposed to stress, the expression of heat shock proteins (HSPs) is transcriptionally up-regulated.[29] HSPs are cytoprotective molecular chaperones that aid in protein folding, refolding, and degradation. In 1998, Takahashi *et al.*[30] showed that HSP60 and HSP70 were up-regulated in livers from fasted rats after 72 hours of cold storage. These fasted livers were significantly more viable than normal fed livers after cold storage. Recent data suggest that HSP70 is hepato-protective during hepatic I/R injury, and this suggests that up-regulation of HSPs is (partially) responsible for the protective effect of fasting.[31]

Using a similar model, Uchida *et al.*[32] showed in 2000 that 4 days of fasting significantly increased the levels of heme oxygenase in the liver after 24 hours of cold storage. As heme oxygenase-1 (HO-1) is an inducible stress protein that confers cytoprotection against oxidative stress *in vitro* and *in vivo* and up-regulation of HO-1 has been shown to confer protection against hepatic cold preservation injury, it is likely also involved in the protective effect of fasting.[33] In contrast, fasting for 36 hours in male rats reduced the levels of catalase and copper-zinc superoxide dismutase (antioxidant enzymes), whereas the activity of glutathione peroxidase remained the same.[34]

The effect of donor fasting was also studied in a large animal model. Donor pigs were divided into 3 groups: group 1 was fasted for 7 days and received an intravenous administration of saline, group 2 was fed *ad libitum*, and group 3 was fasted for 7 days but was given 20% glucose intravenously. The mean survival time after OLT in the last group (group 3) was 37.2 days, which was significantly longer than the periods of 5.8 ± 0.7 and 9.8 ± 2.0 days in groups 1 and 2, respectively.[35] In another study, 5 days of fasting resulted in deteriorated adenosine triphosphate synthesis and less sinusoidal lining cell viability in comparison with 1 day of fasting. The survival in the 1-day-fasted group was 75% (1/4, death due to a technical error), in contrast to 25% in the 5-day-fasted group.[36] Both studies indicate that extended periods of fasting in pigs do not protect against I/R injury in an OLT model.

Several studies used isolated perfusion models of the liver to examine the effect of nutritional interventions on I/R injury after cold and/or warm storage. The results of these studies differ from those of the orthotopic transplant models. With isolated liver perfusion, fasted livers release more aminotransferases in the perfusate than livers of fed animals.[37-40] These studies suggest that isolated liver perfusion is not a suitable model to reveal the beneficial effects of fasting observed in *in vivo* studies.

The previous results were obtained using animals with healthy livers. Fatty livers are

known to be more sensitive to the deleterious effects of I/R injury, and livers with more than 60% steatosis are currently regarded as unsuitable for transplantation.[41] Because the incidence of obesity and the concomitant incidence of steatosis are rapidly increasing,[42, 43] this leads to the loss of potential donors. In 1999, Caraceni *et al.*[44] used fed and fasted rats with normal or fatty livers (induced by a choline-deficient diet) that underwent 1 hour of warm hepatic ischemia followed by reperfusion. Although survival was similar in fasted and fed rats with normal livers (90% vs. 100%), 18 hours of water-only fasting dramatically reduced the survival in rats with fatty livers (14% vs. 64%). The duration of the fasting period could be a determining factor. In rats with steatosis induced by a choline-deficient diet for 28 days, 2 or 4 days of fasting had no effect on the severity of steatosis but afforded a time-dependent increase in the protection against warm and cold preservation injury and I/R injury.[45]

In summary, fasting for 1 to 4 days protects livers from cold preservation injury and results in higher survival rates after OLT in animal studies. Proposed mechanisms include up-regulation of cytoprotective molecules such as HSPs and HO-1. The depletion or augmentation of donor glycogen stores does not affect the outcome. The beneficial effects of preoperative fasting were not found when isolated liver perfusion models were used.

A framework to understand the beneficial effect of fasting

Why did former studies showing beneficial effects of fasting, with their enormous potential to influence preoperative nutritional care, have so little measurable impact in the past decade and a half since they were first published? First, these studies were published at a time when a shiftin preoperative nutritional care was underway, namely, the replacement of strict preoperative overnight fasting guidelines with more liberal ones and later with liquid carbohydrate-rich beverages specifically used to avoid the catabolic state associated with fasting and to improve subjective perioperative well-being.[11-16] Second, a number of previous and subsequent studies demonstrated detrimental effects of fasting and/or malnutrition in various different experimental systems (e.g. isolated liver perfusion), leaving the overall picture cloudy and controversial.[13, 46, 47] Finally, these studies lacked a mechanistic framework by which to understand the results and make predictions about what would and would not work and why, and this is essential in order to have an impact on clinical practice. In this section, we aim to provide a framework for understanding these results.

The effects of long-term DR regimens have been widely studied and provide mechanistic insights with which the effect of DR on acute stress resistance may be explained. DR is the most robust, noninvasive intervention that increases lifespan and reduces the rate of aging.[48] This life-extending action has been found to occur in both genders of many different rat and mouse strains as well as hamsters and non-mammalian species such as fish, flies, and water fleas.[48-53] Long-term DR lowers steady-state levels of oxidative stress, decreases mitochondrial electron and proton leak in mammalian cells, attenuates damage resulting from intracellular

oxidative stress,[22, 54-56] reduces susceptibility to chronic diseases, and retards age-associated functional decline.[21, 57] DR also augments antioxidant defense systems and increases stress resistance to both oxidative and non-oxidative challenges in models of extended longevity. Hormesis is a common biological phenomenon in which exposure to a low-intensity stressor induces a general adaptive response that has net beneficial effects on the cellular and/or organismal level, including protection against subsequent higher dose exposures as well as different types of stress.[58-60] DR has been proposed to act as a mild stressor that extends longevity through hormetic mechanisms.[61, 62] Interestingly, ischemic preconditioning, a procedure used to protect against ischemic insult that entails brief periods of ischemia prior to a longer ischemia time, is also thought to function via hormesis.[63]

DR may be performed by various regimens, such as CR, fasting, and ADF. CR refers to an intervention in which the number of total daily calories provided to an animal or organism is limited to a certain percentage of the animals normal daily intake. ADF regimens involve alternating "feast days" on which food is consumed *ad libitum* and "fast days" on which food is withheld. A key difference in the ADF approach is that the overall calorie intake does not need to be limited. The alternating days of fasting are sufficient to act as low-dose stressors inducing a hormetic response,[64] which can also extend lifespan and protect multiple organ systems against diseases in rodents.[65, 66] All regimens can be applied for longer (lifetime years) or shorter (months or days) time periods (Figure 1). Although long-term regimens induce many beneficial effects, 4 weeks of CR is able to induce many of the genomic expression changes seen after long-term CR. Short-term CR induced all of the changes seen after long-term CR in xenobiotic metabolism and stress response/chaperone protein gene expression. It also reproduced 67% of the effects of long-term CR on inflammatory response gene expression. These results suggest that the effects seen after long-term CR are induced rapidly and that short-term and long-term CR may act via a common protective mechanism.[67]

Although the mechanisms responsible for the up-regulation of defense systems during both long-term and short-term DR are not well understood, we are now able to see a mechanistic framework to explain the early studies on nutritional interventions in liver donors. The data and insights discussed next reveal new areas of applications that may therapeutically benefit from the changes triggered by the low-grade stress induced by DR as predicted by the hormesis hypothesis.

Protection by short-term DR extends to other organs and is not limited to ischemia/reperfusion injury

Studies have been reported in which DR has been used in the context of enhanced stress resistance to prevent or reduce injury in clinically relevant situations, such as I/R injury. As described next, different organ systems such as the brain, heart, liver, and retina have been shown to enjoy protection by various forms of clinically applicable DR regimens.

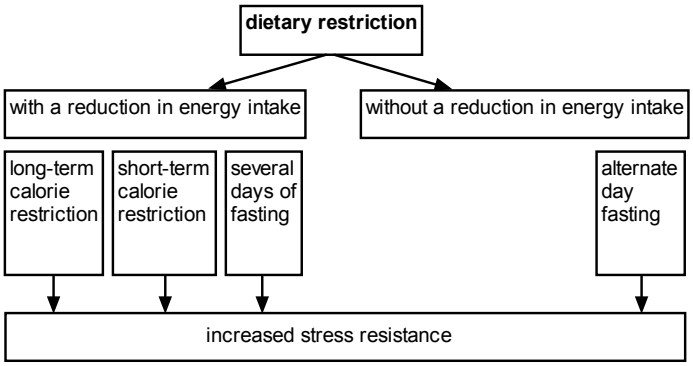

Figure 1. Overview of various forms of dietary restriction that are capable of inducing increased stress resistance. Calorie restriction refers to an intervention in which the total number of daily calories provided to an animal or organism is limited to a certain percentage of the animals *ad libitum* daily intake. Alternate day fasting regimens involve alternating "feast days" on which food is consumed *ad libitum* and "fast days" on which food is withheld.

Broad protection against ischemia/reperfusion injury

DR has recently been shown to facilitate the functional recovery of ischemically damaged neurons in the brain. The performance of DR rats in spatial tasks after an ischemic insult, such as the radial arm maze, was significantly better than that of *ad libitum* fed rats.[68] Furthermore, DR prior to cerebral I/R injury resulted in a highly significant decrease in infarct volume in comparison with the *ad libitum* fed group. Immunoblot analysis showed that levels of HSP70 were greatly increased in the neuronal tissue of DR mice compared with *ad libitum* fed controls.[69] In the heart, DR attenuated the post-ischemic inflammatory response of rats subjected to 15 minutes of partial cardiac I/R injury in comparison with *ad libitum* fed animals. This was shown by a reduced activation of nuclear factor kappa beta and a faster return to baseline of antioxidant enzymes.[70] Similar benefits were found in the retinas of rats subjected to DR. DR was neuroprotective in the retina following ischemia, and this was associated with increased levels of HSP70.[71]

Unfortunately, the onset of heart attack and cerebral ischemia is unpredictable and thus not readily amenable to planned nutritional interventions. However, Plunet *et al.*[72] showed that DR may also be effective when applied after the injury. After surgical induction of cervical spinal injury, rats that were on DR showed a 50% reduction in lesion volume and improved regeneration and behavioral recovery.

DR protects the liver against various toxic insults

DR for 3 weeks protected rats against a lethal dose of the hepatotoxic compound thioacetamide (TA). DR rats showed 70% survival vs. 10% survival in *ad libitum* fed rats. Paradoxically, DR and *ad libitum* fed animals showed similar hepatocellular injury, and the survival benefit was due to stimulation of tissue repair in the DR group resulting in the arrest of progressive

injury and enhanced regeneration.[73] Expression of hepatocyte growth factor was consistently higher in the livers of DR rats after the administration of TA. Epidermal growth factor receptor expression was higher in DR rats before TA administration and remained higher until 48 hours after TA intoxication. DR induced a 2-fold increase in hepatic inducible nitric oxide synthase activity, which was consistent with early cell division in DR rats after TA challenge. These data suggest that the augmented liver tissue repair after TA-induced hepatotoxicity in DR rats was due to faster and higher expression of growth stimulatory cytokines and growth factors.[74] Protection from acetaminophen hepatotoxicity was found in mice that had been exposed to DR for 8 months, as shown by negligible increases in serum alanine aminotransferase and lactate dehydrogenase in the DR group and high levels of alanine aminotransferase and lactate dehydrogenase in the *ad libitum* fed controls.[75]

Discussion and future perspectives

Although animal studies suggest potential uses for DR in the clinic, there are several drawbacks that need attention. Randomized clinical trials have shown that preoperative carbohydrate-rich drinks contribute to better insulin sensitivity and increased patient well-being. However, clinical studies on the effects of preoperative DR are currently lacking. Recently, a study was published in which human subjects adhered to a DR diet for 3 months, which led to a significant increase in verbal memory scores in comparison with the *ad libitum* fed group.[76] In addition, the Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy (CALERIE) trial reported a reduced risk for cardiovascular events in healthy non-obese individuals[77] and improved insulin sensitivity in non-obese humans adhering to a DR diet.[78] Furthermore, overweight patients with asthma subjected to DR revealed improved well-being and reduced levels of circulating tumor necrosis factor alpha, brain-derived neurotrophic factor, and ceramides.[79] These studies indicate that DR in humans is feasible and capable of exerting beneficial effects. However, more clinical studies are needed to develop DR regimens (length, reduction, and timing) for different pathological conditions.

Second, animal studies have shown that DR protects organs against various forms of stress. It is not known whether surgical patients benefit more from preoperative feeding or from the beneficial effects of DR on an organ-specific level. Recently, it was shown that 2 days of fasting was able to confer protection against the adverse side effects of a high dose of the chemotherapeutic agent etoposide in mice.[80, 81] Etoposide displays a generalized toxicity profile ranging from myelosuppression to liver and neurological damage. This suggests that DR acts on an organismal level rather than on a single organ-specific level.

Third, protein restriction without a reduction in calories has been shown to increase maximum longevity in rats and mice as well.[82] Although the magnitude of these increases is around 30% to 40% of that of DR, neither carbohydrate[83] nor lipid restriction[84, 85] exerted these effects. Restriction of proteins could therefore be another way to induce the effects seen

after DR. These data also suggest that the beneficial effects of preoperative carbohydrate-rich drinks and DR may not be mutually exclusive.

Finally, the use of DR mimetics may be a way to overcome the problems associated with DR in surgical patients. A DR mimetic can be loosely defined as any pharmacological intervention that produces beneficial effects of DR without causing or requiring a significant reduction in calorie intake. One compound that has received considerable attention as a DR mimetic is resveratrol, a naturally occurring polyphenol found in red wine. Resveratrol induces genomic changes that resemble many of the genetic alterations induced by DR[86] and, at doses that can be readily achieved in humans, mimics aspects of DR, including increased resistance to oxidative stress.[87, 88] In addition, resveratrol treatment decreases liver injury induced by I/R injury by significantly increasing glutathione reductase, copper-zinc superoxide dismutase, and catalase activities.[89]

Conclusions

Together, these results support an emerging view that the increased resistance to stress that is associated with longevity in animals on long-term DR may be tapped for short-term benefits. These range from neuroprotection and resistance to the adverse effects of chemotherapy to protection against preservation and I/R injury in organ allografts, cardiothoracic surgery, and liver resection. Although these data are robust and convincing, more research is needed to identify the appropriate diet for each condition. The notion that protein restriction and not DR *per se* can induce similar effects may offer new avenues for combining preoperative nutrition (carbohydrate-rich beverages) with restriction of proteins and thereby protecting the target organ without compromising patient well-being. Furthermore, new drugs are able to mimic the protective effects of DR and must be studied more extensively with respect to I/R injury of the liver.

References

1. Studley HO. Percentage of weight loss. Jour. A.M.A. 1936; 458-460
2. Sungurtekin H, Sungurtekin U, Balci C, et al. The influence of nutritional status on complications after major intraabdominal surgery. J Am Coll Nutr 2004; 23: 227-232
3. Hill GL, Blackett RL, Pickford I, et al. Malnutrition in surgical patients. An unrecognised problem. Lancet 1977; 1: 689-692
4. Warnold I, Lundholm K. Clinical significance of preoperative nutritional status in 215 noncancer patients. Ann Surg 1984; 199: 299-305
5. Beattie AH, Prach AT, Baxter JP, et al. A randomised controlled trial evaluating the use of enteral nutritional supplements postoperatively in malnourished surgical patients. Gut 2000; 46: 813-818
6. Shaw-Stiffel TA, Zarny LA, Pleban WE, et al. Effect of nutrition status and other factors on length of hospital stay after major gastrointestinal surgery. Nutrition 1993; 9: 140-145
7. Schiesser M, Muller S, Kirchhoff P, et al. Assessment of a novel screening score for nutritional risk in predicting complications in gastro-intestinal surgery. Clin Nutr 2008; 27: 565-570
8. Fan ST, Lo CM, Lai EC, et al. Perioperative nutritional support in patients undergoing hepatectomy for hepatocellular carcinoma. N Engl J Med 1994; 331: 1547-1552
9. Maltby JR. Fasting from midnight--the history behind the dogma. Best Pract Res Clin Anaesthesiol 2006; 20: 363-378
10. Brady M, Kinn S, Stuart P. Preoperative fasting for adults to prevent perioperative complications. Cochrane Database Syst Rev 2003; CD004423
11. Hausel J, Nygren J, Lagerkranser M, et al. A carbohydrate-rich drink reduces preoperative discomfort in elective surgery patients. Anesth Analg 2001; 93: 1344-1350
12. Svanfeldt M, Thorell A, Hausel J, et al. Effect of "preoperative" oral carbohydrate treatment on insulin action--a randomised cross-over unblinded study in healthy subjects. Clin Nutr 2005; 24: 815-821
13. Nettelbladt CG, Alibergovic A, Ljungqvist O. Pre-stress carbohydrate solution prevents fatal outcome after hemorrhage in 24-hour food-deprived rats. Nutrition 1996; 12: 696-699
14. Nygren JO, Thorell A, Soop M, et al. Perioperative insulin and glucose infusion maintains normal insulin sensitivity after surgery. Am J Physiol 1998; 275: E140-148
15. Soop M, Nygren J, Myrenfors P, et al. Preoperative oral carbohydrate treatment attenuates immediate postoperative insulin resistance. Am J Physiol Endocrinol Metab 2001; 280: E576-583
16. Bisgaard T, Kristiansen VB, Hjortso NC, et al. Randomized clinical trial comparing an oral carbohydrate beverage with placebo before laparoscopic cholecystectomy. Br J Surg 2004; 91: 151-158
17. Diks J, van Hoorn DE, Nijveldt RJ, et al. Preoperative fasting: an outdated concept? JPEN J Parenter Enteral Nutr 2005; 29: 298-304
18. Garretsen MK, Melis GC, Richir MC, et al. [Perioperative nutrition] Perioperatieve voeding. Ned Tijdschr Geneeskd 2006; 150: 2745-2749
19. Nygren J. The metabolic effects of fasting and surgery. Best Pract Res Clin Anaesthesiol 2006; 20: 429-438
20. Weindruch R, Walford RL. Dietary restriction in mice beginning at 1 year of age: effect on life-span and spontaneous cancer incidence. Science 1982; 215: 1415-1418
21. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science 1996; 273: 59-63
22. Ramsey JJ, Harper ME, Weindruch R. Restriction of energy intake, energy expenditure, and aging. Free Radic Biol Med 2000; 29: 946-968
23. Goodrick CL, Ingram DK, Reynolds MA, et al. Effects of intermittent feeding upon growth, activity, and lifespan in rats allowed voluntary exercise. Exp Aging Res 1983; 9: 203-209
24. Pruim J, van Woerden WF, Knol E, et al. Donor data in liver grafts with primary non-function--a preliminary analysis by the European Liver Registry. Transplant Proc 1989; 21: 2383-2384

25. Sumimoto R, Southard JH, Belzer FO. Livers from fasted rats acquire resistance to warm and cold ischemia injury. Transplantation 1993; 55: 728-732
26. Lindell SL, Hansen T, Rankin M, et al. Donor nutritional status--a determinant of liver preservation injury. Transplantation 1996; 61: 239-247
27. Sumimoto R, Fukuda Y, Nishihara M, et al. Liver glycogen in fasted rat livers does not improve outcome of liver transplantation. Transpl Int 1996; 9: 541-545
28. Sankary H, Chong A, Foster P, et al. Improved viability of hepatic allografts from fasted donors is associated with decreased peripheral TNF activity. J Surg Res 1995; 58: 337-343
29. Westerheide SD, Morimoto RI. Heat shock response modulators as therapeutic tools for diseases of protein conformation. J Biol Chem 2005; 280: 33097-33100
30. Takahashi Y, Tamaki T, Tanaka M, et al. Efficacy of heat-shock proteins induced by severe fasting to protect rat livers preserved for 72 hours from cold ischemia/reperfusion injury. Transplant Proc 1998; 30: 3700-3702
31. Kuboki S, Schuster R, Blanchard J, et al. Role of heat shock protein 70 in hepatic ischemia-reperfusion injury in mice. Am J Physiol Gastrointest Liver Physiol 2007; 292: G1141-1149
32. Uchida Y, Tamaki T, Tanaka M, et al. De novo protein synthesis induced by donor nutritional depletion ameliorates cold ischemia and reperfusion injury in rat liver. Transplant Proc 2000; 32: 1657-1659
33. Kato H, Amersi F, Buelow R, et al. Heme oxygenase-1 overexpression protects rat livers from ischemia/reperfusion injury with extended cold preservation. Am J Transplant 2001; 1: 121-128
34. Marczuk-Krynicka D, Hryniewiecki T, Piatek J, et al. The effect of brief food withdrawal on the level of free radicals and other parameters of oxidative status in the liver. Med Sci Monit 2003; 9: BR131-135
35. Sadamori H, Tanaka N, Yagi T, et al. The effects of nutritional repletion on donors for liver transplantation in pigs. Transplantation 1995; 60: 317-321
36. Fukumori T, Ohkohchi N, Tsukamoto S, et al. Long-term fasting of donors deteriorates mitochondrial adenosine triphosphate synthesis in liver grafts during cold preservation. Transplant Proc 1997; 29: 3360-3361
37. Boudjema K, Lindell SL, Southard JH, et al. The effects of fasting on the quality of liver preservation by simple cold storage. Transplantation 1990; 50: 943-948
38. Cywes R, Greig PD, Morgan GR, et al. Rapid donor liver nutritional enhancement in a large animal model. Hepatology 1992; 16: 1271-1279
39. Morgan GR, Sanabria JR, Clavien PA, et al. Correlation of donor nutritional status with sinusoidal lining cell viability and liver function in the rat. Transplantation 1991; 51: 1176-1183
40. Palombo JD, Hirschberg Y, Pomposelli JJ, et al. Decreased loss of liver adenosine triphosphate during hypothermic preservation in rats pretreated with glucose: implications for organ donor management. Gastroenterology 1988; 95: 1043-1049
41. Imber CJ, St Peter SD, Handa A, et al. Hepatic steatosis and its relationship to transplantation. Liver Transpl 2002; 8: 415-423
42. Blokstra A. Factsheet overgewicht, prevalentie en trend. RIVM 2003; report 260301/f1/2003:
43. Silverman JF, O'Brien KF, Long S, et al. Liver pathology in morbidly obese patients with and without diabetes. Am J Gastroenterol 1990; 85: 1349-1355
44. Caraceni P, Nardo B, Domenicali M, et al. Ischemia-reperfusion injury in rat fatty liver: role of nutritional status. Hepatology 1999; 29: 1139-1146
45. Shiino Y, Nakamura J, Okamoto T, et al. Improved quality of fatty liver allografts by starvation in rats. Transplant Proc 1998; 30: 3294-3295
46. Alibegovic A, Ljungqvist O. Pretreatment with glucose infusion prevents fatal outcome after hemorrhage in food deprived rats. Circ Shock 1993; 39: 1-6

47. Schumer W, Kuttner RE, Sugai T, et al. Hepatic glycolytic intermediates in fed and fasted rats after severe hemorrhage. J Trauma 1986; 26: 1009-1012
48. Civitarese AE, Carling S, Heilbronn LK, et al. Calorie Restriction Increases Muscle Mitochondrial Biogenesis in Healthy Humans. PLoS Med 2007; 4: e76
49. Velthuis-te Wierik EJ, van den Berg H, Schaafsma G, et al. Energy restriction, a useful intervention to retard human ageing? Results of a feasibility study. Eur J Clin Nutr 1994; 48: 138-148
50. Fontana L, Klein S. Aging, adiposity, and calorie restriction. Jama 2007; 297: 986-994
51. Meyer TE, Kovacs SJ, Ehsani AA, et al. Long-term caloric restriction ameliorates the decline in diastolic function in humans. J Am Coll Cardiol 2006; 47: 398-402
52. Weindruch R. The retardation of aging by caloric restriction: studies in rodents and primates. Toxicol Pathol 1996; 24: 742-745
53. Masoro EJ. Caloric restriction and aging: an update. Exp Gerontol 2000; 35: 299-305
54. Ayala V, Naudi A, Sanz A, et al. Dietary protein restriction decreases oxidative protein damage, peroxidizability index, and mitochondrial complex I content in rat liver. J Gerontol A Biol Sci Med Sci 2007; 62: 352-360
55. Lenaz G, D'Aurelio M, Merlo Pich M, et al. Mitochondrial bioenergetics in aging. Biochim Biophys Acta 2000; 1459: 397-404
56. Lopez-Torres M, Gredilla R, Sanz A, et al. Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. Free Radic Biol Med 2002; 32: 882-889
57. Weindruch R, Sohal RS. Seminars in medicine of the Beth Israel Deaconess Medical Center. Caloric intake and aging. N Engl J Med 1997; 337: 986-994
58. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136
59. Mattson MP. Hormesis defined. Ageing Res Rev 2008; 7: 1-7
60. Rattan SI, Sejersen H, Fernandes RA, et al. Stress-mediated hormetic modulation of aging, wound healing, and angiogenesis in human cells. Ann N Y Acad Sci 2007; 1119: 112-121
61. Turturro A, Hass BS, Hart RW. Does caloric restriction induce hormesis? Hum Exp Toxicol 2000; 19: 320-329
62. Sinclair DA. Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev 2005; 126: 987-1002
63. Arumugam TV, Gleichmann M, Tang SC, et al. Hormesis/preconditioning mechanisms, the nervous system and aging. Ageing Res Rev 2006; 5: 165-178
64. Mattson MP, Wan R. Beneficial effects of intermittent fasting and caloric restriction on the cardiovascular and cerebrovascular systems. J Nutr Biochem 2005; 16: 129-137
65. Goodrick CL, Ingram DK, Reynolds MA, et al. Effects of intermittent feeding upon growth and life span in rats. Gerontology 1982; 28: 233-241
66. Anson RM, Guo Z, de Cabo R, et al. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proc Natl Acad Sci U S A 2003; 100: 6216-6220
67. Cao SX, Dhahbi JM, Mote PL, et al. Genomic profiling of short- and long-term caloric restriction effects in the liver of aging mice. Proc Natl Acad Sci U S A 2001; 98: 10630-10635
68. Roberge MC, Hotte-Bernard J, Messier C, et al. Food restriction attenuates ischemia-induced spatial learning and memory deficits despite extensive CA1 ischemic injury. Behav Brain Res 2008; 187: 123-132
69. Yu ZF, Mattson MP. Dietary restriction and 2-deoxyglucose administration reduce focal ischemic brain damage and improve behavioral outcome: evidence for a preconditioning mechanism. J Neurosci Res 1999; 57: 830-839
70. Chandrasekar B, Nelson JF, Colston JT, et al. Calorie restriction attenuates inflammatory responses to myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 2001; 280: H2094-2102

71. Kim KY, Ju WK, Neufeld AH. Neuronal susceptibility to damage: comparison of the retinas of young, old and old/caloric restricted rats before and after transient ischemia. Neurobiol Aging 2004; 25: 491-500
72. Plunet WT, Streijger F, Lam CK, et al. Dietary restriction started after spinal cord injury improves functional recovery. Exp Neurol 2008; 213: 28-35
73. Apte UM, Limaye PB, Desaiah D, et al. Mechanisms of increased liver tissue repair and survival in diet-restricted rats treated with equitoxic doses of thioacetamide. Toxicol Sci 2003; 72: 272-282
74. Apte UM, Limaye PB, Ramaiah SK, et al. Upregulated promitogenic signaling via cytokines and growth factors: potential mechanism of robust liver tissue repair in calorie-restricted rats upon toxic challenge. Toxicol Sci 2002; 69: 448-459
75. Harper JM, Salmon AB, Chang Y, et al. Stress resistance and aging: influence of genes and nutrition. Mech Ageing Dev 2006; 127: 687-694
76. Witte AV, Fobker M, Gellner R, et al. Caloric restriction improves memory in elderly humans. Proc Natl Acad Sci U S A 2009; 106: 1255-1260
77. Lefevre M, Redman LM, Heilbronn LK, et al. Caloric restriction alone and with exercise improves CVD risk in healthy non-obese individuals. Atherosclerosis 2008; Mar;203(1):206-13.:
78. Weiss EP, Racette SB, Villareal DT, et al. Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr 2006; 84: 1033-1042
79. Johnson JB, Summer W, Cutler RG, et al. Alternate day calorie restriction improves clinical findings and reduces markers of oxidative stress and inflammation in overweight adults with moderate asthma. Free Radic Biol Med 2007; 42: 665-674
80. Couzin J. Cancer research. Can fasting blunt chemotherapy's debilitating side effects? Science 2008; 321: 1146-1147
81. Raffaghello L, Lee C, Safdie FM, et al. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. Proc Natl Acad Sci U S A 2008; 105: 8215-8220
82. Pamplona R, Barja G. Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. Biochim Biophys Acta 2006; 1757: 496-508
83. Sanz A, Gomez J, Caro P, et al. Carbohydrate restriction does not change mitochondrial free radical generation and oxidative DNA damage. J Bioenerg Biomembr 2006; 38: 327-333
84. Iwasaki K, Gleiser CA, Masoro EJ, et al. Influence of the restriction of individual dietary components on longevity and age-related disease of Fischer rats: the fat component and the mineral component. J Gerontol 1988; 43: B13-21
85. Sanz A, Caro P, Sanchez JG, et al. Effect of lipid restriction on mitochondrial free radical production and oxidative DNA damage. Ann N Y Acad Sci 2006; 1067: 200-209
86. Smith JJ, Kenney RD, Gagne DJ, et al. Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. BMC Syst Biol 2009; 3: 31
87. Barger JL, Kayo T, Pugh TD, et al. Short-term consumption of a resveratrol-containing nutraceutical mixture mimics gene expression of long-term caloric restriction in mouse heart. Exp Gerontol 2008; 43: 859-866
88. Baur JA, Pearson KJ, Price NL, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006; 444: 337-342
89. Hassan-Khabbar S, Cottart CH, Wendum D, et al. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. Liver Transpl 2008; 14: 451-459

Chapter 8

Preoperative fasting protects mice against hepatic ischemia/reperfusion injury: Mechanisms and effects on liver regeneration

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Abstract

We show that brief periods of fasting induce functional changes similar to those induced by long-term dietary restriction in mice, and these changes include protection from ischemia/reperfusion (I/R) injury. In this study, we investigated the mechanisms of protection induced by fasting, and we determined the effect on liver regeneration after partial hepatectomy. Partial hepatic ischemia (75 minutes) was induced in *ad libitum* fed mice and in 1 to 3-day fasted mice, and one-third or two-thirds hepatectomy was performed in *ad libitum* fed mice and 3-day fasted mice. Preoperative fasting for 2 or 3 days significantly decreased hepatocellular I/R injury. Hepatic gene expression of heme oxygenase-1 (HO-1), superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (Gpx1), and glutathione reductase (Gsr) was significantly up-regulated in 3 day-fasted mice at baseline and 6 hours after reperfusion. After reperfusion, P-selectin and interleukin-6 (IL-6) levels were significantly lower, and superoxide radical generation, lipid peroxidation, and neutrophil influx were significantly attenuated in 3-day fasted mice. Preoperative fasting did not affect liver regeneration after one-third hepatectomy. Hepatic gene expression of IL-6 and transforming growth factor-β1 was significantly higher in 3-day fasted mice before and after one-third hepatectomy. Tumor necrosis factor-α expression significantly increased after one-third hepatectomy in 3-day fasted mice. After a 3-day fast and two-thirds hepatectomy, liver regeneration and subsequent postoperative recovery were compromised. In conclusion, up-regulation of the stress response gene HO-1 and the antioxidant enzymes SOD2, Gpx1, and Gsr at baseline and a better response after reperfusion likely underlie the protection induced by fasting against hepatic I/R injury. Preoperative fasting may be a promising new strategy for protecting the liver against I/R injury during liver transplantation and minor liver resections, although its effect on extended hepatectomy warrants further exploration.

Introduction

The temporary occlusion of the hepatic inflow (Pringle maneuver) or the hepatic inflow and outflow (total hepatic vascular occlusion) are techniques routinely used during extended liver surgeries such as hepatic resection and liver transplantation.[1, 2] These prolonged interruptions of hepatic blood flow result in ischemia/reperfusion (I/R) injury.

Hepatic I/R injury is characterized by progressive hepatocellular injury and hepatocyte loss after reperfusion. It is considered to be a risk factor for potentially lethal primary or delayed non-function of the liver[3, 4] and for distant damage to organs such as the kidneys, heart, and lungs.[5, 6] Depending on the degree of hepatic injury, regenerative mechanisms are activated, and they restore liver function by recovering lost and damaged cells. To circumvent the consequences of hepatic I/R injury during liver surgery and liver transplantation, the development of a protective strategy against I/R is warranted.

Dietary restriction (DR), defined as reduced food intake without malnutrition, has been reported to extend the lifespan of several organisms, including nonhuman primates.[7] DR is associated not only with extended longevity but also with prolonged healthspan[8, 9] and improved resistance against multiple stressors.[10-12] We have shown recently that fasting can rapidly induce DR-like effects on the combined levels of gene expression, physiology, and stress resistance.[13] Fasting for 3 days is as effective as 1 month of DR in reducing I/R injury.

Although previous studies showed beneficial effects of fasting against hepatic I/R injury in liver transplant models,[14] the mechanism was not understood (with no connection whatsoever to DR), and the findings were contrary to current expectations. Studies with an isolated rat liver perfusion model later contested these findings,[15] and the issue has been mired in controversy to this day.

In the present study, we aimed to elucidate the mechanisms of protection induced by fasting against hepatic I/R injury. Because the postoperative outcome in the clinic is determined by the severity of the I/R injury and the regenerative capacity of the liver, we also investigated the effects of fasting on liver regeneration after partial hepatectomy (PH). Here we show that up-regulation of the antioxidant enzymes superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (Gpx1), and glutathione reductase (Gsr) and the stress response gene heme oxygenase-1 (HO-1) underlies the protection from fasting against hepatic I/R injury. In addition, we show that preoperative fasting does not affect liver regeneration after a minor liver resection, although it prevents adequate liver regeneration and subsequent postoperative recovery after extended hepatectomy.

Materials and Methods

Animals

Male C57BL/6 mice (~25 g) were obtained from Harlan (Horst, the Netherlands). All mice had

free access to water and chow (Special Diet Services, Witham, UK) unless stated otherwise. A 1- to 3-day water-only fasting regimen was applied to mice that were transferred to clean cages without food at 5:00 pm (n = 2-4 animals per cage). No deaths occurred during these fasting periods. The experimental protocol was approved by the animal experiments committee under the Dutch National Experiments on Animals Act, and it complied with Directive 86/609/EC (1986) of the Council of Europe.

Surgeries

All surgeries were performed between 9:00 am and 1:00 pm. Mice were anesthetized by isoflurane/N_2/O_2 inhalation and placed on a heating pad for maintenance of their body temperature. Partial (70%) hepatic I/R injury was induced by the occlusion of the blood flow to the left lateral and median liver lobes with a non-traumatic microvascular clamp for 75 minutes. After clamp removal, the restoration of blood flow in the ischemic liver lobes was monitored. Mortality associated with this amount of ischemic damage to the liver was not observed. PH was performed by resection of the left lateral liver lobe (one-third PH) or the left lateral and median liver lobes (two-thirds PH). All mice received 0.5 mL of phosphate-buffered saline subcutaneously. The mice were placed under a heating lamp until they recovered from anesthesia. Directly after surgery, all animals had free access to food and water.

Hepatocellular injury

Preoperatively fed mice (n = 4-5 per time point), 1-day fasted mice (n = 2-3 per time point), 2-day fasted mice (n = 6-7 per time point), and 3-day fasted mice (n = 7-8 per time point) were anesthetized, and blood was drawn by retro-orbital puncture before surgery (baseline) and 6 and 24 hours after reperfusion. Sera were analyzed for the serum alanine aminotransferase (ALAT) level at the Central Clinical Chemical Laboratory of the Erasmus University Medical Center. Hemorrhagic necrosis was assessed 24 hours after reperfusion in 3 μm thick Hematoxylin and Eosin stained liver sections from preoperatively fed mice (n = 8), 1-day fasted mice (n = 5), 2-day fasted mice (n = 3), and 3-day fasted mice (n = 11) at a magnification of 100x (with 5 random microscopic fields per section) by 2 independent observers blinded to the treatment. Hemorrhagic necrosis was characterized by the loss of the cellular architecture and the presence of erythrocytes in necrotic areas. The percentage of hemorrhagic necrosis per microscopic field was semiquantitatively determined with the following scoring system: 0% (absent), 0% to 25% (<25% necrosis per microscopic field), 25% to 50% (25% to <50% necrosis per microscopic field), 50% to 75% (50% to <75% necrosis per microscopic field), 75% to 100% (75% to <100% necrosis per microscopic field), or 100% (100% necrosis per microscopic field).

Immunohistochemistry

Frozen liver sections (5 μm) from preoperatively fed mice (n = 3-6 per time point) and 3-day fasted mice (n = 4-5 per time point) before (baseline) and 6 and 24 hours after reperfusion were stained with a monoclonal antibody against neutrophils and visualized with an alkaline

phosphatase secondary antibody. For proliferating cell nuclear antigen (PCNA), paraffin embedded (3 μm) liver sections from preoperatively fed mice (n = 4-6 per time point per treatment) and 3-day fasted mice (n = 4-6 per time point per treatment) before (baseline) and 24 and 48 hours after one-third or two-thirds hepatectomy were stained with a monoclonal antibody against neutrophils and visualized with a horseradish peroxidase-conjugated secondary antibody. In 5 microscopic fields per section, the number of neutrophils or the PCNA index (the percentage of PCNA-positive cells with respect to the total number of cells) was counted by independent observers blinded to the treatment at magnifications of 200 to 400x.

Superoxide radical production

Superoxide radical production in the livers of preoperatively fed mice (n = 4-8 per time point) and 3-day fasted mice (n = 4-5 per time point) before (baseline) and 6 and 24 hours after reperfusion was measured as described[16] with 10 μM dihydroethidium. At least 300 nuclei per section were counted in 2 to 3 consecutive sections by an independent observer blinded to the treatment.

Hepatic oxidative stress

Lipid peroxidation, an index for oxidative damage to lipids, was assessed by colorimetric determination of thiobarbituric acid reactive substances (TBARS) in livers from preoperatively fed mice (n = 4-6 per time point) and 3-day fasted mice (n = 4 per time point) before (baseline) and 6 and 24 hours after reperfusion with the QuantiChrom TBARS assay kit (DTBA-100, BioAssay Systems, Hayward, CA, USA) according to manufacturer's instructions. The TBARS concentration was expressed as the malondialdehyde (MDA) production (nM/mg of protein). Each sample was tested three times.

Liver weight/total body weight ratio

Wet liver weights and total body weights were determined at baseline and 5 days after one-third PH for preoperatively fed mice (n = 3-4 per time point) and 3-day fasted mice (n = 3-5 per time point), and for 3-day fasted mice refed for 5 days without PH (n = 4 per time point). The liver weight/total body weight ratio was calculated as the wet liver weight divided by the total body weight.

Quantitative RT-PCR

With the TRIzol reagent (Invitrogen, Breda, the Netherlands), total RNA was extracted from frozen liver tissues obtained from preoperatively fed mice (n = 3-4 per time point) and 3-day fasted mice (n = 3-5 per time point) before (baseline) and 6 and 24 hours after reperfusion, and from preoperatively fed mice (n = 4-6 per time point per treatment) and 3-day fasted mice (n = 3-6 per time point per treatment) before (baseline) and 24 and 48 hours after one-third or two-thirds hepatectomy. Total RNA was purified by a deoxyribonuclease treatment (RQ1 ribonuclease-free deoxyribonuclease, Promega Benelux B.V., Leiden, the Netherlands)

and reverse-transcribed to complementary DNA with random hexamer primers and Superscript II RT (both from Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. Quantitative RT-PCR was performed with a MyiQ single-color, real-time polymerase chain reaction detection system with SYBR Green incorporation (both from Bio-Rad Laboratories B.V., Veenendaal, the Netherlands; primer sequences are available upon request). The relative expression was calculated as $2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$ (ΔCt is the difference in the cycle threshold). Each sample was tested at least twice.

Statistical Analysis

The data are expressed as means ± SEM. Differences in groups were analyzed by Mann-Whitney U tests (SPSSv15). Differences were considered significant at $P \leq 0.05$.

Results

Preoperative fasting protected against hepatic I/R injury.

Six hours after reperfusion, serum ALAT levels were significantly lower in 2-day fasted mice (5603 ± 1446 U/L, $P = 0.004$) and 3-day fasted mice (6642 ± 1109 U/L, $P = 0.004$) versus *ad libitum* fed mice (16196 ± 456 U/L; Figure 1A). Twenty-four hours after reperfusion, serum ALAT levels remained significantly lower in 2-day fasted mice (1593 ± 598 U/L, $P = 0.03$) and 3-day fasted mice (1880 ± 332 U/L, $P = 0.02$) versus the control group (4356 ± 920 U/L). The histological examination of livers (Figure 1B) from 2-day fasted mice and 3-day fasted mice revealed significantly smaller areas of hemorrhagic necrosis in comparison with livers from fed mice (25% in 2-day fasted mice and 28% in 3-day fasted mice vs. 69% in fed mice, $P = 0.01$ vs. 2-day fasted mice and $P = 0.001$ vs. 3-day fasted mice).

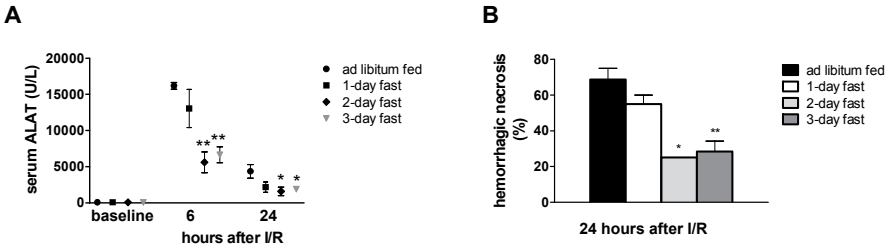

Figure 1. Hepatocellular injury. (A) After reperfusion, the serum ALAT concentration was significantly lower in mice preoperatively fasted for 2 or 3 days. (B) Twenty-four hours after reperfusion, livers from mice preoperatively fasted for 2 or 3 days had significantly less hemorrhagic necrosis. The data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. preoperatively fed mice.

Preoperative fasting reduced the inflammatory response after hepatic I/R

The development of hepatic I/R injury is a biphasic inflammatory process. In the first phase, reactive oxygen species are generated and induce oxidative damage to local macromolecules such as lipids and proteins,[17] whereas the second phase is characterized by the release of pro-inflammatory cytokines such as interleukin-6 (IL-6), the expression of adhesion molecules such as P-selectin, and the subsequent infiltration of neutrophils.[18] We investigated the effect of 3 days of preoperative fasting on the inflammatory response after hepatic I/R. Six hours after reperfusion, significantly fewer superoxide radicals were produced in livers from fasted mice (644 ± 41 vs. 1453 ± 235 in livers from fed mice, $P = 0.02$; Figure 2A).

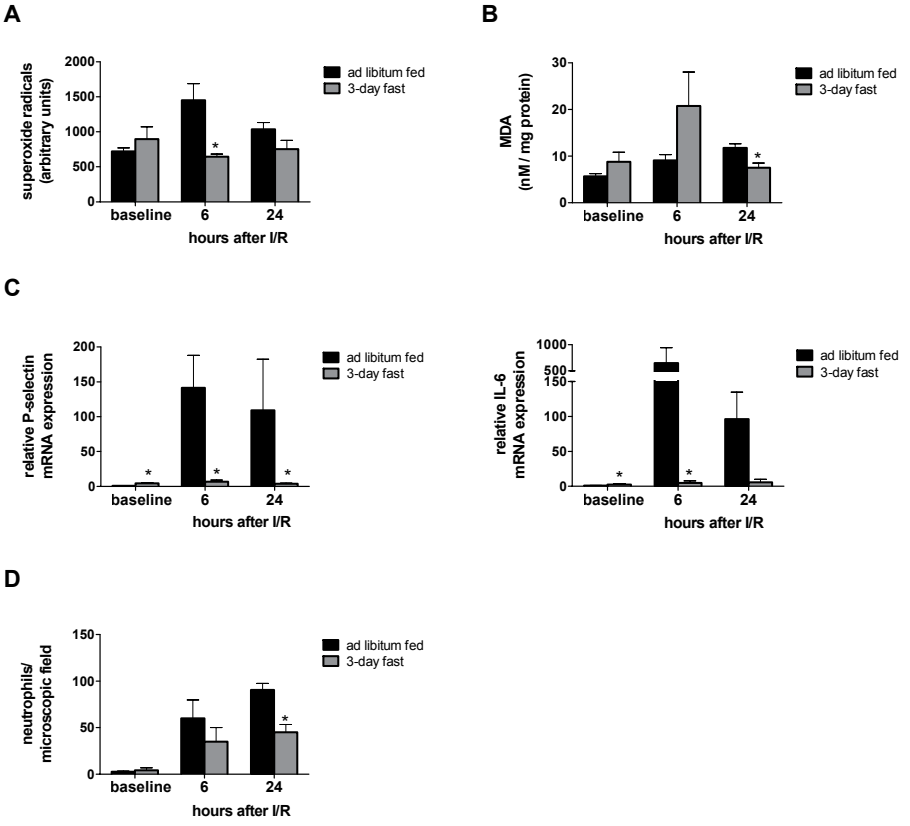

Figure 2. Inflammatory response. (A) Six hours after reperfusion, significantly fewer superoxide radicals were detected in livers from preoperatively fasted mice. (B) Less lipid peroxidation was found in livers from preoperatively fasted mice 24 hours after reperfusion. (C) Hepatic P-selectin (left graph) and IL-6 (right graph) mRNA expression levels were significantly lower in livers from preoperatively fasted mice. The data have been normalized for beta-2-microglobulin and are expressed relative to preoperatively fed mice at baseline. (D) Fewer neutrophils were detected in livers from preoperatively fasted mice. The data are expressed as means ± SEM. *$P < 0.05$ vs. preoperatively fed mice.

Significantly lower lipid peroxidation levels were found in livers from fasted mice 24 hours after reperfusion (7.5 ± 1.0 vs. 11.7 ± 0.9 nM MDA/mg of protein in livers from fed mice, $P = 0.02$; Figure 2B). Hepatic messenger RNA (mRNA) expression levels for P-selectin and IL-6 were significantly higher in fasted livers at baseline (P-selectin, 4.2 ± 1.4 vs. 0.7 ± 0.1 in livers from fed mice, $P = 0.03$; IL-6, 3.6 ± 1.2 vs. 1.1 ± 0.2 in livers from fed mice, $P = 0.05$; Figure 2C). Six hours after reperfusion, both inflammatory markers were significantly lower in livers from fasted mice (P-selectin, $P = 0.03$ vs. livers from fed mice; IL-6, $P = 0.02$ vs. livers from fed mice). Twenty-four hours after reperfusion, this difference remained significant for P-selectin ($P = 0.03$); for IL-6, P was not significant versus livers from fed mice (Figure 2C). Significantly fewer neutrophils were present in livers from fasted mice ($P = 0.02$) 24 hours after reperfusion (Figure 2D).

Fasting up-regulated antioxidant enzymes and the stress response gene HO-1

In response to hepatic I/R, heat shock proteins and antioxidants are produced that mitigate hepatocellular injury.[19, 20] We investigated the effect of 3 days of preoperative fasting on heat shock protein70 (HSP70) and HO-1 gene expression, and we determined its effect on the antioxidant enzyme activities of SOD2, Gpx1, Gsr, and SOD1 with quantitative RT-PCR. At baseline, HO-1 expression was up-regulated 8-fold in livers from fasted mice (8.8 ± 2.2 vs. 1.1 ± 0.2 in fed mice, $P = 0.01$; Figure 3A). Six hours after reperfusion, HO-1 expression significantly increased in livers from both groups versus baseline (fasted mice, 23.3-fold increase, $P = 0.01$; fed mice, 15.0-fold increase, $P = 0.02$), and there were significantly higher levels in livers from fasted mice versus those from fed mice (203.8 ± 51.9 vs. 16.5 ± 2.1, $P = 0.02$; Figure 3A). HO-1 expression did not significantly change in livers from fed mice 24 hours after reperfusion versus 6 hours after reperfusion, whereas in fasted mice, it returned to baseline. No significant difference in HSP70 expression was found at baseline (Figure 3B) or 6 hours after reperfusion, at which time HSP70 expression peaked in both groups before it returned to baseline values 24 hours after reperfusion. In livers from 3-day fasted mice, expression levels of SOD2, Gpx1, and Gsr were significantly up-regulated at baseline (SOD2, 2.7± 0.2 vs. 1.0 ± 0.1 in livers from fed mice, $P = 0.01$; Gpx1, 1.4 ± 0.1 vs. 1.0 ± 0.0 in livers from fed mice, $P = 0.03$; Gsr, 3.9 ± 0.8 vs. 1.0 ± 0.1 in livers from fed mice, $P = 0.01$; Figures 3C-E). In the fasted group, SOD2 expression increased 4.2 times (5.9 ± 1.9 vs. 1.4 ± 0.1 in livers from fed mice, $P = 0.03$), Gpx1 expression increased 2.5 times (1.5 ± 0.5 vs. 0.6 ± 0.0 in livers from fed mice, $P = 0.03$), Gsr expression increased 5.0 times (13.4 ± 4.8 vs. 2.7 ± 0.0 in livers from fed mice, $P = 0.03$), and SOD1 expression increased 3.6 times (4.0± 0.6 vs. 1.1 ± 0.2 in livers from fed mice, $P = 0.02$) 6 hours after reperfusion (Figure 3F). SOD2 (4.4 ± 1.5), Gpx1 (0.8 ± 0.1), Gsr (8.9 ± 3.1), and SOD1 expression levels (2.0 ± 0.2) decreased in livers from fasted mice 24 hours after reperfusion versus 6 hours after reperfusion. In livers from fed mice 24 hours after reperfusion, no significant changes in expression levels were observed in comparison with livers from fed mice 6 hours after reperfusion.

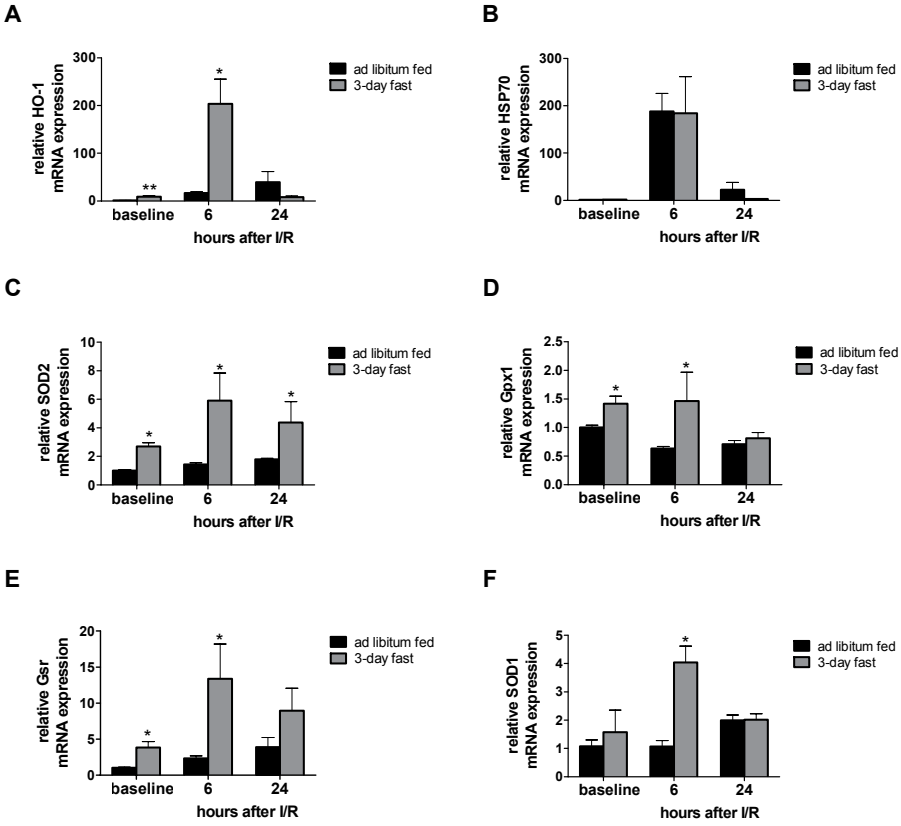

Figure 3. Hepatic gene expression of the stress response genes HO-1 and HSP70 and the antioxidant enzymes SOD2, Gpx1, Gsr, and SOD1. (A) In livers from preoperatively fasted mice, HO-1 levels were significantly up-regulated at baseline with maximum expression 6 hours after reperfusion. (B) HSP70 levels peaked 6 hours after reperfusion in both groups. (C) SOD2, (D) Gpx1, and (E) Gsr expression levels were significantly up-regulated in livers from preoperatively fasted mice at baseline and 6 hours after reperfusion. (F) Six hours after reperfusion, SOD1 peaked in livers from preoperatively fasted mice. The data have been normalized for beta-2-microglobulin and are expressed relative to preoperatively fed mice at baseline. The data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. preoperatively fed mice.

Fasting did not affect liver regeneration after PH

An important determinant of postoperative liver function is the capacity of the liver to regenerate. To investigate liver regeneration, we determined liver weight/total body weight ratios of preoperatively fed mice and 3-day fasted mice before and 5 days after one-third PH. As controls, the liver weight/total body weight ratios of 3-day fasted mice refed for 5 days without PH were determined. At baseline, significant decreases in the liver weight ($P = 0.05$) and the total body weight ($P = 0.05$) were observed in fasted mice versus fed mice (Figure 4A). Fasted mice had significantly lower liver weight/total body weight ratios (0.04 ± 0.00 vs.

0.05 ± 0.00 in fed mice, *P* = 0.05; Figure 4B). *Ad libitum* fed mice did not eat much during the first days after one-third PH, whereas fasted mice with or without one-third PH started eating rapidly when they regained access to food. On postresection day 5, remnant livers from fasted mice with or without one-third PH were significantly heavier than baseline values (fasting and one-third PH, *P* = 0.03; fasting without PH, *P* = 0.03), whereas the liver weight of fed mice was significantly lower after one-third PH (*P* = 0.03 vs. baseline). Remnant livers of fasted mice with or without one-third PH were significantly heavier versus fed mice after one-third PH on postresection day 5 (fasting and one-third PH, *P* = 0.02 vs. fed mice; fasting without PH, *P* = 0.01 vs. fed mice; Figure 4C). Fasted mice had significantly higher liver weight/total body weight ratios on postresection day 5 fasting and one-third PH, [0.06 ± 0.00, *P* = 0.01 vs. fed mice (0.04 ± 0.00); fasting without PH, 0.07 ± 0.00, *P* = 0.02 vs. fed mice; Figure 4D]. However, after a correction for the increase in the liver weight after fasting and 5 days of refeeding, no difference in the relative liver weight was observed between the fasted and fed groups on postoperative day 5 (Figure 4E). We next investigated hepatocyte proliferation with PCNA staining (Figure 4F). At baseline, the PCNA index for livers from fasted mice was 0.3%, and the index for livers from fed mice was 5.0% (*P* was not significant). After one-third PH, the PCNA index peaked at 48 hours without a significant difference between the 2 groups (22.5% vs. 34.2% in fed mice). Because one-third PH is a minor challenge that may not induce the full regenerative response,[21] we next investigated the effect of 3 days of fasting on regeneration after major liver resection. The removal of two-thirds of the liver resulted in similar PCNA indices for the remnant livers of the 2 groups 24 hours after resection (fed mice, 8.2%; fasted mice, 6.0%) vs. the remnant livers of these groups after one-third PH (fed mice, 9.5%; fasted mice, 12.5%; Figure 4F). However, at 48 hours, a lower PCNA index was found in livers from fasted mice (10.7%) versus livers from fed mice (22.6%) or mice after one-third PH.

Fasting differentially affected cytokine and growth factor expression after PH

Because liver regeneration is initiated by the expression of genes involved in hepatic growth and proliferation, such as tumor necrosis factor-alpha (TNF-α)[22] and IL-6,[23] we investigated the effects of preoperative fasting on these markers with qRT-PCR. At baseline, TNF-α expression was significantly lower in livers from fasted mice (0.4 ± 0.1 vs. 1.1 ± 0.2 in fed mice, *P* = 0.03). Twenty-four hours after one-third PH, TNF-α expression significantly increased in livers from fasted mice (8.6 ± 2.1 vs. 1.6 ± 0.6 in fed mice, *P* =0.009), before it returned toward baseline values 48 hours after resection (Figure 5A). Removal of two-thirds of the liver slightly increased TNF-α expression in the livers of fasted mice at 24 hours (11.1 ± 5.6), and the expression remained elevated 48 hours after resection. Although TNF-α expression remained unaffected in livers from fed mice after one-third PH, it increased to an extent similar to that in livers from fasted mice after two-thirds PH (Figure 5A). Before PH, IL-6 expression was significantly up-regulated in livers from fasted mice (3.6 ± 1.2 vs. 1.1 ± 0.2 in fed mice, *P* = 0.05), and it remained elevated until 24 hours after one-third PH (6.8 ± 1.4 vs. 1.6 ± 0.1 in fed mice, *P* = 0.01); after that, the levels dropped by 48 hours; Figure 5B). After one-third PH, IL-6 expression remained

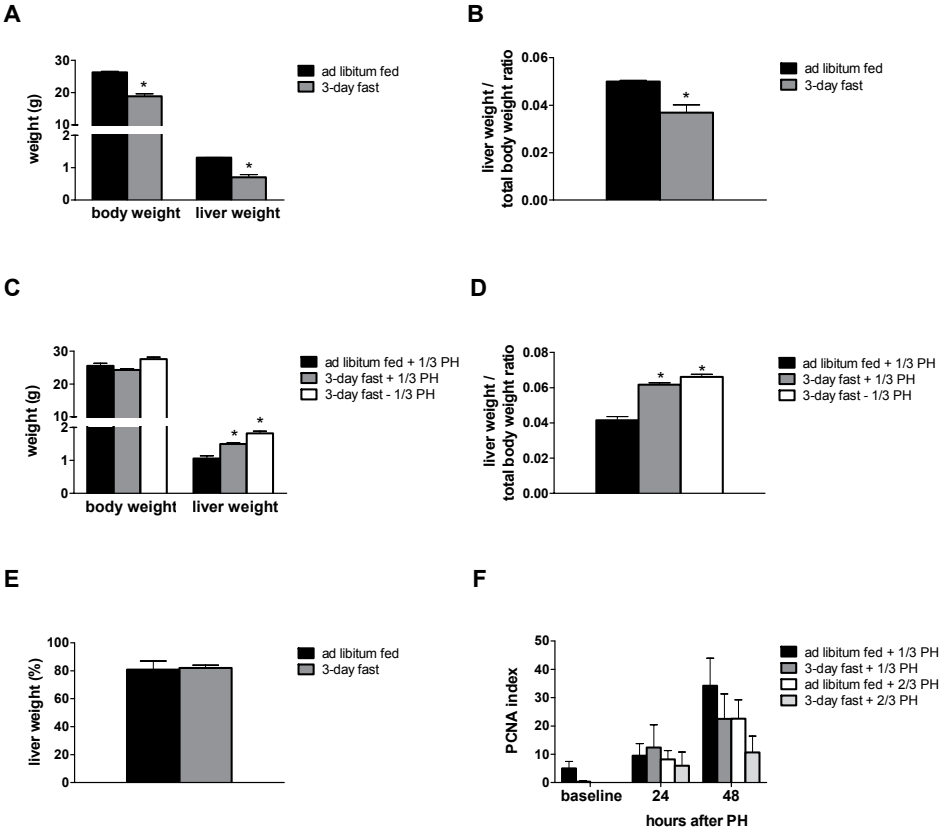

Figure 4. Liver regeneration. (A) The liver and total body weights and (B) the liver weight/total body weight ratios were significantly decreased in 3-day fasted mice at baseline. (C) Livers from preoperatively fasted mice with or without one-third PH were significantly heavier on postoperative day 5. (D) On post-resection day 5, the liver weight/total body weight ratio was significantly increased in preoperatively fasted mice with or without one-third PH. (E) The relative liver weight on postoperative day 5 is expressed as a percentage of the weight at baseline (the *ad libitum* fed group) or as a percentage of the liver weight after 3 days of fasting and 5 days of refeeding without one-third PH (the fasted group) to compensate for the increase in the liver weight by fasting and refeeding alone. No significant change in the relative liver weight was observed between the 2 groups. (F) Hepatocyte proliferation was assessed with the PCNA index. Fasting did not affect the regenerative response after one-third hepatectomy. Forty-eight hours after resection, the PCNA index was 47% lower in the fasted group after two-thirds hepatectomy versus one-third hepatectomy. The data are expressed as means ± SEM. *$P < 0.05$ vs. preoperatively fed mice.

unaffected in livers from fed mice, whereas after two-thirds PH, its expression increased to an extent similar to that in fasted mice after two-thirds PH (Figure 5B). Transforming growth factor-beta 1 (TGF-β1) is one of the factors involved in the termination response of liver regeneration.[24] Before PH, expression levels were significantly up-regulated in fasted livers (1.8 ± 0.4 vs. 1.0 ± 0.1 in livers from fed mice, $P = 0.02$; Figure 5C). After one-third PH,

significantly higher expression levels were found in livers from fasted mice (24 hours, 3.0 times higher vs. fed mice, $P = 0.009$; 48 hours, 2.8 times higher vs. fed mice, $P = 0.03$), whereas after two-thirds PH, no significant difference was found between the 2 groups (Figure 5C).

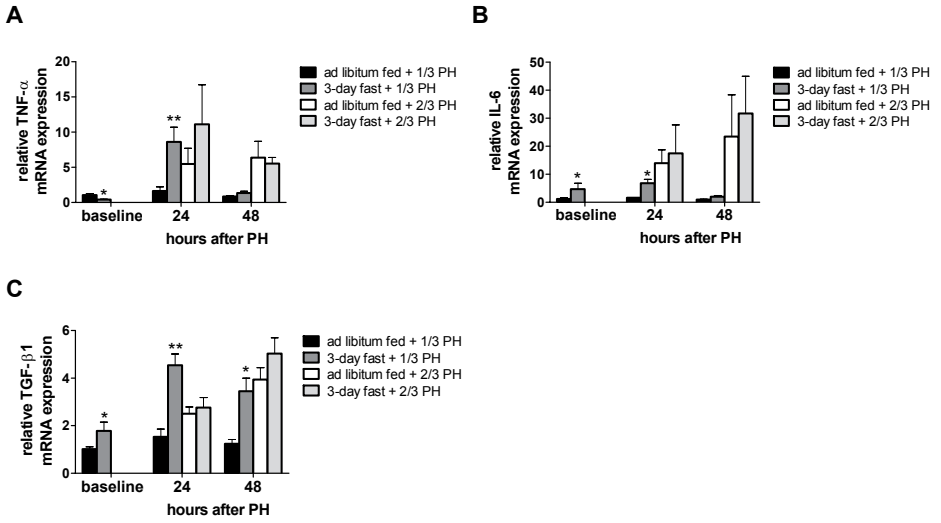

Figure 5. Growth factor kinetics after PH. (A) Hepatic mRNA expression levels of TNF-α peaked 24 hours after one-third or two-thirds PH in livers from preoperatively fasted mice. (B) IL-6 and (C) TGF-β1 expression levels were already significantly up-regulated at baseline and remained so until 24 hours after one-third PH, whereas these levels increased until 48 hours after two-thirds PH. The data have been normalized for beta-2-microglobulin and are expressed relative to preoperatively fed mice at baseline. The data are expressed as means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. preoperatively fed mice.

Discussion

Long-term DR is associated with extended longevity and improved stress resistance in multiple experimental models. We have shown recently that the beneficial effects of DR are induced rapidly. Short-term DR and brief periods of fasting induce many of the transcriptional changes observed after long-term DR, and both protect against I/R injury.[13] The objective of the present study was to elucidate the mechanisms of protection induced by preoperative fasting. In addition, we investigated the effect of preoperative fasting on liver regeneration after PH. Fasting (for both 2 and 3 days) offered significant protection against hepatic I/R induced hepatocellular injury. Moreover, we show that the beneficial effects of 3 days of fasting are likely achieved by the overexpression of genes encoding for the antioxidant defense enzymes SOD2, Gpx1, and Gsr and the stress response gene HO-1 at baseline and by a more expeditious and pronounced response after reperfusion. Finally, we show that preoperative fasting does

not affect liver regeneration after one-third hepatectomy, but compromises liver regeneration and subsequent postoperative recovery after two-thirds hepatectomy.

In agreement with previous studies,[25-28] we found that hepatic I/R resulted in increased superoxide radical formation, increased lipid peroxidation, up-regulation of P-selectin and IL-6 mRNA expression levels, and increased neutrophil infiltration. Although at baseline these markers were significantly elevated in livers from fasted mice, 3 days of fasting significantly reduced each of these inflammatory markers after hepatic I/R. HO-1 overexpression at baseline and after reperfusion is a critical factor in protection against hepatic I/R injury. Up-regulation of HO-1 expression after hepatic I/R has been shown to reduce graft injury in liver transplant patients.[29] In addition, pharmacologically induced baseline HO-1 overexpression decreases I/R-mediated hepatocellular injury in several animal models. For example, pretreatment with the HO-1 inducer cobalt protoporphyrin reduces hepatocellular injury after reperfusion in rat and mouse models.[30-32] In contrast, inhibition of HO-1 expression in the liver at baseline and after reperfusion results in higher serum ALAT levels, more hepatocellular necrosis and apoptosis, higher neutrophil numbers, and increased pro-inflammatory cytokine synthesis.[31] Our finding that 3 days of fasting induced HO-1 and strongly increased its expression after reperfusion indicates that HO-1 plays an important role in the beneficial effects of preoperative fasting.

Mitochondria are considered a major intracellular source of reactive oxygen species generation during hepatic I/R injury.[17] To minimize oxidative stress, these organelles contain a variety of antioxidant enzymes, and some can translocate to the cytoplasm. Overexpression of these enzymes protects I/R injury-prone organs such as the liver and heart.[33, 34] We have demonstrated that fasting for 3 days significantly increases the expression of the antioxidants SOD2, Gpx1, and Gsr at baseline and after reperfusion. These data suggest that protection against hepatic I/R injury by preoperative fasting is in part mediated by increased resistance against oxidative stress. In addition, the slight increase in IL-6 and P-selectin expression at baseline after 3 days of fasting points to the induction of a low-grade inflammatory response by fasting. It is possible that this low-grade inflammatory response contributes to the induction of cytoprotective and antioxidant genes and preconditions the liver to a stronger response after injury.

Because I/R injury in the clinic occurs when the liver needs to regenerate to maintain function, we investigated the effects of preoperative fasting on liver regeneration. Because hepatic ischemia impairs liver regeneration after PH[35] we chose to study PH *per se* without concomitant hepatic ischemia.

In accordance with previous observations,[36, 37] we found a decrease in the liver weight after 3 days of fasting. Five days after one-third PH, the livers of preoperatively fasted mice gained more weight than the livers of fed mice. This increase could not be explained by an increase in hepatocyte proliferation because the PCNA index did not differ significantly in the remnant livers of the 2 groups after resection. It has been reported that fasting followed by refeeding increases liver weight.[38-40] In our hands, mice fasted for 3 days and refed for 5 days had a liver weight/total body weight ratio comparable to that of 3-day fasted mice 5 days after

one-third PH. After a correction was made for the increase in the liver weight after 3 days of fasting and 5 days of refeeding, the liver weights were similar in preoperatively fed mice and fasted mice 5 days after one-third PH. These data show that preoperative fasting does not affect liver regeneration after one-third hepatectomy. After one-third hepatectomy, TNF-α, IL-6, and TGF-β1 showed a stronger and more expeditious response in the livers of preoperatively fasted mice. This suggests that, to compensate for the hepatic injury caused by fasting at baseline, higher TNF-α and IL-6 levels facilitate an enhanced regenerative response. This is followed by an increase in TGF-β1 expression to terminate this enhanced regeneration response. The net result is a proliferative response that is similar to the response in livers of fed mice.

Because one-third hepatectomy is a minor challenge[21] and can easily be covered by the regenerative capacity of the liver, we increased the resection volume to two-thirds in order to test the regenerative capacity of the fasted liver under more stressful conditions. We found that 48 hours after resection, the PCNA index was 47% lower in the preoperatively fasted group after two-thirds hepatectomy versus one-third hepatectomy. In addition, 2 days after two-thirds hepatectomy, these mice showed signs of decreased well-being, such as hunched posture and ruffled fur. This indicates that although preoperative fasting protects against hepatic I/R injury and has no effect on liver regeneration after one-third hepatectomy, liver regeneration and subsequent postoperative recovery and well-being after extended hepatectomy are compromised.

Two-thirds hepatectomy induced a stronger response of TNF-α, IL-6, and TGF-β1 than one-third hepatectomy. It has been shown that the preservation of glycogen metabolism after hepatectomy is an important determinant for animal survival.[41] Depletion of the glycogen stores by fasting and PH up-regulates the expression of IL-6, which stimulates glycogenesis by the liver.[42] Because two-thirds PH depletes liver glycogen stores more severely than one-third PH, it may be that the balance between the functions of IL-6 in proliferation and inflammation shifts toward an inflammatory-mediated response in fasted mice after an extended liver resection, but not after a minor resection. Thus, the decrease in well-being, the decrease in proliferation, and the high IL-6 levels 48 hours after two-thirds hepatectomy collectively suggest that preoperative fasting prevents adequate liver regeneration after major liver resection.

In conclusion, this study shows that preoperative fasting ameliorates hepatic I/R injury via up-regulation of baseline levels of the antioxidant enzymes SOD2, Gpx1, and Gsr and the stress response gene HO-1 and via a more expeditious and pronounced response of these genes after I/R injury. Baseline up-regulation of IL-6 and TGF-β1 suggests that preoperative fasting acts as a low-level stressor and preconditions the liver for other types of stress such as hepatic I/R injury. Preoperative fasting is a new and promising noninvasive strategy for protecting the liver against the detrimental effects of hepatic I/R injury during liver transplantation and minor liver resections, although its effect on extended hepatectomy warrants further exploration.

Acknowledgements

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References

1. Laca L, Olejnik J, Vician M, et al. The effects of occlusive techniques on the short-term prognosis after liver resections. Hepatogastroenterology 2006; 53: 576-579
2. van der Bilt JD, Livestro DP, Borren A, et al. European survey on the application of vascular clamping in liver surgery. Dig Surg 2007; 24: 423-435
3. Briceno J, Marchal T, Padillo J, et al. Influence of marginal donors on liver preservation injury. Transplantation 2002; 74: 522-526
4. Uemura T, Randall HB, Sanchez EQ, et al. Liver retransplantation for primary nonfunction: analysis of a 20-year single-center experience. Liver Transpl 2007; 13: 227-233
5. Weinbroum AA, Hochhauser E, Rudick V, et al. Direct induction of acute lung and myocardial dysfunction by liver ischemia and reperfusion. J Trauma 1997; 43: 627-633; discussion 633-625
6. Lee HT, Park SW, Kim M, et al. Acute kidney injury after hepatic ischemia and reperfusion injury in mice. Lab Invest 2009; 89: 196-208
7. Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science 2009; 325: 201-204
8. Lakowski B, Hekimi S. The genetics of caloric restriction in Caenorhabditis elegans. Proc Natl Acad Sci U S A 1998; 95: 13091-13096
9. Lin SJ, Kaeberlein M, Andalis AA, et al. Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature 2002; 418: 344-348
10. Raffaghello L, Lee C, Safdie FM, et al. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. Proc Natl Acad Sci U S A 2008; 105: 8215-8220
11. Vigne P, Tauc M, Frelin C. Strong dietary restrictions protect Drosophila against anoxia/reoxygenation injuries. PLoS One 2009; 4: e5422
12. Hall DM, Oberley TD, Moseley PM, et al. Caloric restriction improves thermotolerance and reduces hyperthermia-induced cellular damage in old rats. FASEB J 2000; 14: 78-86
13. Mitchell JR, Verweij M, Brand K, et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell 2010; 9: 40-53
14. van Ginhoven TM, Mitchell JR, Verweij M, et al. The use of preoperative nutritional interventions to protect against hepatic ischemia-reperfusion injury. Liver Transpl 2009; 15: 1183-1191
15. Stadler M, Nuyens V, Seidel L, et al. Effect of nutritional status on oxidative stress in an ex vivo perfused rat liver. Anesthesiology 2005; 103: 978-986
16. van Eijsden RG, Eijssen LM, Lindsey PJ, et al. Termination of damaged protein repair defines the occurrence of symptoms in carriers of the m.3243A > G tRNA(Leu) mutation. J Med Genet 2008; 45: 525-534
17. Jassem W, Heaton ND. The role of mitochondria in ischemia/reperfusion injury in organ transplantation. Kidney Int 2004; 66: 514-517
18. Monson KM, Dowlatshahi S, Crockett ET. CXC-chemokine regulation and neutrophil trafficking in hepatic ischemia-reperfusion injury in P-selectin/ICAM-1 deficient mice. J Inflamm (Lond) 2007; 4: 11
19. Terajima H, Thiaener A, Hammer C, et al. Attenuation of hepatic microcirculatory failure during in situ xenogeneic rat liver perfusion by heat shock preconditioning. Transplant Proc 2000; 32: 1111
20. Gonzalez-Flecha B, Cutrin JC, Boveris A. Time course and mechanism of oxidative stress and tissue damage in rat liver subjected to in vivo ischemia-reperfusion. J Clin Invest 1993; 91: 456-464
21. Mitchell C, Nivison M, Jackson LF, et al. Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration. J Biol Chem 2005; 280: 2562-2568
22. Webber EM, Bruix J, Pierce RH, et al. Tumor necrosis factor primes hepatocytes for DNA replication in the rat. Hepatology 1998; 28: 1226-1234

23. Cressman DE, Greenbaum LE, DeAngelis RA, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science 1996; 274: 1379-1383
24. Zimmermann A. Regulation of liver regeneration. Nephrol Dial Transplant 2004; 19 Suppl 4: iv6-10
25. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. Faseb J 1990; 4: 3355-3359
26. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, et al. Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. Transplantation 1993; 55: 1265-1272
27. Llacuna L, Mari M, Lluis JM, et al. Reactive oxygen species mediate liver injury through parenchymal nuclear factor-kappaB inactivation in prolonged ischemia/reperfusion. Am J Pathol 2009; 174: 1776-1785
28. Lee SM, Park MJ, Cho TS, et al. Hepatic injury and lipid peroxidation during ischemia and reperfusion. Shock 2000; 13: 279-284
29. Geuken E, Buis CI, Visser DS, et al. Expression of heme oxygenase-1 in human livers before transplantation correlates with graft injury and function after transplantation. Am J Transplant 2005; 5: 1875-1885
30. Amersi F, Buelow R, Kato H, et al. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 1999; 104: 1631-1639
31. Tsuchihashi S, Livhits M, Zhai Y, et al. Basal rather than induced heme oxygenase-1 levels are crucial in the antioxidant cytoprotection. J Immunol 2006; 177: 4749-4757
32. Kato H, Amersi F, Buelow R, et al. Heme oxygenase-1 overexpression protects rat livers from ischemia/reperfusion injury with extended cold preservation. Am J Transplant 2001; 1: 121-128
33. Zwacka RM, Zhou W, Zhang Y, et al. Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-kappaB activation. Nat Med 1998; 4: 698-704
34. Yoshida T, Watanabe M, Engelman DT, et al. Transgenic mice overexpressing glutathione peroxidase are resistant to myocardial ischemia reperfusion injury. J Mol Cell Cardiol 1996; 28: 1759-1767
35. Selzner M, Camargo CA, Clavien PA. Ischemia impairs liver regeneration after major tissue loss in rodents: protective effects of interleukin-6. Hepatology 1999; 30: 469-475
36. Jenniskens FA, Jopperi-Davis KS, Walters LC, et al. Effects of fasting on tissue contents of coenzyme A and related intermediates in rats. Pediatr Res 2002; 52: 437-442
37. Sumimoto R, Fukuda Y, Nishihara M, et al. Liver glycogen in fasted rat livers does not improve outcome of liver transplantation. Transpl Int 1996; 9: 541-545
38. Wiegand RD, Rao GA, Reiser R. Dietary regulation of fatty acid synthetase and microsomal glycerophosphate acyltransferase activities in rat liver. J Nutr 1973; 103: 1414-1424
39. Meikle AW, Klain GJ. Effect of fasting and fasting-refeeding on conversion of leucine into CO 2 and lipids in rats. Am J Physiol 1972; 222: 1246-1250
40. Liang G, Yang J, Horton JD, et al. Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. J Biol Chem 2002; 277: 9520-9528
41. Hubner RH, Leopold PL, Kiuru M, et al. Dysfunctional glycogen storage in a mouse model of alpha1-antitrypsin deficiency. Am J Respir Cell Mol Biol 2009; 40: 239-247
42. Gewiese-Rabsch J, Drucker C, Malchow S, et al. Role of IL-6 trans-signaling in CCl induced liver damage. Biochim Biophys Acta 2010; 1802: 1054-1061

Chapter 9

Summary, discussion and future directions

References

1. Linden PK. History of solid organ transplantation and organ donation. Crit Care Clin 2009; 25: 165-184, ix
2. Ojo AO, Wolfe RA, Held PJ, et al. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation 1997; 63: 968-974
3. McLaren AJ, Jassem W, Gray DW, et al. Delayed graft function: risk factors and the relative effects of early function and acute rejection on long-term survival in cadaveric renal transplantation. Clin Transplant 1999; 13: 266-272
4. Joo JD, Kim M, D'Agati VD, et al. Ischemic preconditioning provides both acute and delayed protection against renal ischemia and reperfusion injury in mice. J Am Soc Nephrol 2006; 17: 3115-3123
5. Iliodromitis EK, Kremastinos DT, Katritsis DG, et al. Multiple cycles of preconditioning cause loss of protection in open-chest rabbits. J Mol Cell Cardiol 1997; 29: 915-920
6. Rahman IA, Mascaro JG, Steeds RP, et al. Remote ischemic preconditioning in human coronary artery bypass surgery: from promise to disappointment? Circulation 2010; 122: S53-59
7. Dirksen MT, Laarman GJ, Simoons ML, et al. Reperfusion injury in humans: a review of clinical trials on reperfusion injury inhibitory strategies. Cardiovasc Res 2007; 74: 343-355
8. Hausel J, Nygren J, Lagerkranser M, et al. A carbohydrate-rich drink reduces preoperative discomfort in elective surgery patients. Anesth Analg 2001; 93: 1344-1350
9. Svanfeldt M, Thorell A, Hausel J, et al. Effect of "preoperative" oral carbohydrate treatment on insulin action--a randomised cross-over unblinded study in healthy subjects. Clin Nutr 2005; 24: 815-821
10. Nygren J, Soop M, Thorell A, et al. Preoperative oral carbohydrate administration reduces postoperative insulin resistance. Clin Nutr 1998; 17: 65-71
11. Hausel J, Nygren J, Thorell A, et al. Randomized clinical trial of the effects of oral preoperative carbohydrates on postoperative nausea and vomiting after laparoscopic cholecystectomy. Br J Surg 2005; 92: 415-421
12. Noblett SE, Watson DS, Huong H, et al. Pre-operative oral carbohydrate loading in colorectal surgery: a randomized controlled trial. Colorectal Dis 2006; 8: 563-569
13. Diks J, van Hoorn DE, Nijveldt RJ, et al. Preoperative fasting: an outdated concept? JPEN J Parenter Enteral Nutr 2005; 29: 298-304
14. Selzner M, Camargo CA, Clavien PA. Ischemia impairs liver regeneration after major tissue loss in rodents: protective effects of interleukin-6. Hepatology 1999; 30: 469-475
15. Streetz KL, Luedde T, Manns MP, et al. Interleukin 6 and liver regeneration. Gut 2000; 47: 309-312
16. Sanchez-Capelo A. Dual role for TGF-beta1 in apoptosis. Cytokine Growth Factor Rev 2005; 16: 15-34
17. Mitchell C, Nivison M, Jackson LF, et al. Heparin-binding epidermal growth factor-like growth factor links hepatocyte priming with cell cycle progression during liver regeneration. J Biol Chem 2005; 280: 2562-2568
18. Park Y, Hirose R, Coatney JL, et al. Ischemia-reperfusion injury is more severe in older versus young rat livers. J Surg Res 2007; 137: 96-102
19. Selzner M, Selzner N, Jochum W, et al. Increased ischemic injury in old mouse liver: an ATP-dependent mechanism. Liver Transpl 2007; 13: 382-390
20. Shinmura K, Tamaki K, Bolli R. Short-term caloric restriction improves ischemic tolerance independent of opening of ATP-sensitive K+ channels in both young and aged hearts. J Mol Cell Cardiol 2005; 39: 285-296
21. Azhar G, Gao W, Liu L, et al. Ischemia-reperfusion in the adult mouse heart influence of age. Exp Gerontol 1999; 34: 699-714
22. Chen G, Bridenbaugh EA, Akintola AD, et al. Increased susceptibility of aging kidney to ischemic injury: identification of candidate genes changed during aging, but corrected by caloric restriction. Am J Physiol Renal Physiol 2007; 293: F1272-1281

23. Park KM, Kim JI, Ahn Y, et al. Testosterone is responsible for enhanced susceptibility of males to ischemic renal injury. J Biol Chem 2004; 279: 52282-52292
24. Burne MJ, Haq M, Matsuse H, et al. Genetic susceptibility to renal ischemia reperfusion injury revealed in a murine model. Transplantation 2000; 69: 1023-1025
25. Terpstra AH. Differences between humans and mice in efficacy of the body fat lowering effect of conjugated linoleic acid: role of metabolic rate. J Nutr 2001; 131: 2067-2068
26. van Ginhoven TM, de Bruin RW, Timmermans M, et al. Pre-operative dietary restriction is feasible in live-kidney donors. Clin Transplant 2011; 25(3):486-94
27. Ikizler TA, Hakim RM. Nutrition in end-stage renal disease. Kidney Int 1996; 50: 343-357
28. Wojtovich AP, Brookes PS. The endogenous mitochondrial complex II inhibitor malonate regulates mitochondrial ATP-sensitive potassium channels: implications for ischemic preconditioning. Biochim Biophys Acta 2008; 1777: 882-889
29. Hubner RH, Leopold PL, Kiuru M, et al. Dysfunctional glycogen storage in a mouse model of alpha1-anti-trypsin deficiency. Am J Respir Cell Mol Biol 2009; 40: 239-247
30. Guo F, Cavener DR. The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. Cell Metab 2007; 5: 103-114
31. Yang R, Wek SA, Wek RC. Glucose limitation induces GCN4 translation by activation of Gcn2 protein kinase. Mol Cell Biol 2000; 20: 2706-2717
32. Montie HL, Kayali F, Haezebrouck AJ, et al. Renal ischemia and reperfusion activates the eIF 2 alpha kinase PERK. Biochim Biophys Acta 2005; 1741: 314-324
33. Muaddi H, Majumder M, Peidis P, et al. Phosphorylation of eIF2alpha at serine 51 is an important determinant of cell survival and adaptation to glucose deficiency. Mol Biol Cell 2010; 21: 3220-3231
34. Liu Y, Laszlo C, Liu Y, et al. Regulation of G(1) arrest and apoptosis in hypoxia by PERK and GCN2-mediated eIF2alpha phosphorylation. Neoplasia 2010; 12: 61-68
35. van Huizen R, Martindale JL, Gorospe M, et al. P58IPK, a novel endoplasmic reticulum stress-inducible protein and potential negative regulator of eIF2alpha signaling. J Biol Chem 2003; 278: 15558-15564
36. Robert F, Williams C, Yan Y, et al. Blocking UV-induced eIF2alpha phosphorylation with small molecule inhibitors of GCN2. Chem Biol Drug Des 2009; 74: 57-67
37. Brunet A, Sweeney LB, Sturgill JF, et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004; 303: 2011-2015
38. Cohen HY, Miller C, Bitterman KJ, et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science 2004; 305: 390-392
39. Wang Y, Ji HX, Xing SH, et al. SP600125, a selective JNK inhibitor, protects ischemic renal injury via suppressing the extrinsic pathways of apoptosis. Life Sci 2007; 80: 2067-2075
40. Sunayama J, Tsuruta F, Masuyama N, et al. JNK antagonizes Akt-mediated survival signals by phosphorylating 14-3-3. J Cell Biol 2005; 170: 295-304

Chapter 10

Nederlandse samenvatting

Chapter 11

Dankwoord

List of abbreviations

List of publications

Curriculum Vitae

PhD portfolio