STUDIES OF ISCHEMIA AND REPERFUSION IN MUSCLE AND LIVER ON GLUTATHIONE AND AMINO ACID METABOLISM IN MAN

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Abstract

Oxidative stress increases the formation of reactive oxygen species (ROS), which can induce damage and breakdown of enzymes and lipoproteins. This leads to impaired cellular function and ultimately to death of cells and eventually organ failure. ROS are thought to be responsible for the damage during reperfusion following ischemia. ROS are counteracted by scavengers (antioxidants) among which glutathione is one of the most important. Glutathione and oxidative stress during ischemia and reperfusion have been extensively investigated in animal models earlier. However, the relevance for human physiology especially during disease is not clear. The aim of the present studies was to characterise glutathione and amino acid metabolism in human skeletal muscle and liver during ischemia and reperfusion using surgical interventions as potential human models. In the first study abdominal aneurysm surgery with an occlusion time of about 65 minutes was used. Biopsies from the thigh muscle were obtained before ischemia, at maximal ischemia and after 10 min, 4, 24 and 48 hours of reperfusion. In a second study aorto bi-femoral surgery patients were studied because of the longer occlusion times up to 120 minutes with this procedure. Biopsies from both legs were obtained before ischemia, at maximal ischemia and at 10 min. and 24 h of reperfusion. In the third study knee replacement surgery was used as a model since the obtained ischemia is more complete than in the first 2 studies and because no severe metabolic stress due to abdominal surgery is present in this model. Muscle biopsies were taken before ischemia, at maximal ischemia and 24 h into reperfusion. In the last study the effect of ischemia and reperfusion on human liver was studied during liver resection surgery. In this study biopsies were obtained before ischemia, at maximal ischemia and at 5, 10, 15, 20, 25 and 30 minutes of reperfusion.

Results from the first two studies show that both reduced and total glutathione are decreased at 24 and 48 hours of reperfusion. No changes were observed at maximal ischemia or in oxidised glutathione at any point. The changes were however very similar to those previously obtained in muscle following abdominal surgery without ischemia to the muscle. Therefore the observed changes are mainly due to the surgical trauma. In the knee surgery model reduced and total glutathione were also only decreased after 24h of reperfusion in the patients receiving glucose during reperfusion. However in this model a complete ischemia is obtained without the metabolic stress of abdominal surgery. The decrease here is therefore more likely to be due to ischemia and reperfusion in combination with a minor surgical trauma. Ischemia of the human liver had no effect on glutathione levels at all. Changes in amino acid levels during muscle reperfusion were very similar as those seen in skeletal muscle following surgery without ischemia reperfusion. However the changes in amino acids seen at maximal ischemia are specific for the ischemia. Alanine is increased and glutamate decreased most likely to remove pyruvate to maintain the flux in the glycolysis. The branched chain amino acids behave differently in liver and muscle during maximal ischemia with and increase in liver and a slight decrease in muscle. Glutamate is decreased at ischemia in both muscle and liver, and decreases in reperfusion in muscle, while an increase is seen in liver at reperfusion. Glutamine is not affected by ischemia but decreases in muscle and increases in liver at reperfusion.

In conclusion, ischemia times of up to 120 minutes for skeletal muscle and about 30 minutes for liver do not affect glutathione levels in humans. During early reperfusion phase in the liver no changes are seen either. In skeletal muscle at 1 to 2 days after reperfusion decreases in glutathione can be observed which seem to a large extend to be caused by the surgical stress rather then the ischemic insult. However in a human model with minimal surgical stress also a decrease is observed suggesting a contribution of the IR. Amino acids are used during the ischemia to maintain the energy status of both skeletal muscle and liver in humans.

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I. **Effects on Skeletal Muscle Glutathione Status of Ischemia and Reperfusion Following Abdominal Aortic Surgery**
Bo Westman, Gunnar Johansson, Jia-Li Luo, Karin Söderlund, Jan Wernerman and Folke Hammarqvist

Ann Vasc Surg 2006; 20; 99-105

II. **Muscle glutathione metabolism during ischemia and reperfusion in patients undergoing aorto-bifemoral bypass surgery**
Bo Westman, Gunnar Johansson, Karin Söderlund, Jan Wernerman and Folke Hammarqvist

Acta Anaesthesiol Scand 2006; 50: 699-705

III. **Knee replacement surgery as a human clinical model of the effects of ischaemia/reperfusion upon skeletal muscle**
Bo Westman, Lars Weidenhielm, Olav Rooyackers, Jan Wernerman and Folke Hammarqvist

Clinical Science 2007, 113, 313-318

IV. **Glutathione and amino acid metabolism in human liver during warm ischemia and reperfusion**
Bo Westman, Anders Thörne, Olav Rooyackers, Katarina Fredriksson, Jan Wernerman and Folke Hammarqvist

Submitted to Annals of Surgery

ABBREVIATIONS

ROS	**=**	**Reactive Oxygen Species**
IR	**=**	**Ischemia/reperfusion**
H_2O_2	**=**	**Hydrogen peroxide**
$OH^{\cdot}$	**=**	**Hydroxyl radical**
$SO_2^{\cdot}$	**=**	**Superoxide**
PCr	**=**	**Phosphocreatine**
Cr	**=**	**Creatine**
ATP	**=**	**Adenosine triphosphate**
ADP	**=**	**Adenosine diphosphate**
GSH	**=**	**Reduced glutathione**
tGSH	**=**	**Total glutathione**
GSSG	**=**	**Oxidised glutathione**
Glu	**=**	**Glutamate**
Gln	**=**	**Glutamine**
Cys	**=**	**Cysteine**
Gly	**=**	**Glycine**
Ala	**=**	**Alanine**
BCAA	**=**	**Branched Chain Amino Acids**
TAA	**=**	**Total Amino Acids**
HPLC	**=**	**High-Pressure Liquid Chromatography**
SOD	**=**	**Super Oxide Dismutase**

INTRODUCTION

A historical perspective

In the light of developed surgical techniques and knowledge about pathophysiology it is interesting to briefly reflect the historical breakthroughs making it possible to perform advanced surgery. Development of anaesthesia and surgical technique in association with increased knowledge how to cope with pathophysiological changes has made it possible to perform advanced surgery despite increasing age of patients. The fascinating development started in modern time with the introduction of ether as an anaesthetic that made it possible to perform abdominal surgery on man under general anaesthesia, a door opener to the interior of the human body. Ether was used since the middle of the 19th century, first by doctor Crawford Williamson Long, who removed a tumour from the neck of Mr. James Venable under ether anaesthesia in the year 1842 [1]. Sadly enough Dr Williamson Long, did not write about this operation until 1848, at which time ether as an anaesthetic, already was made famous by a dentist in Boston USA, William Thomas Green Morton [2] who performed a painless tooth extraction on a patient in 1846, and rapidly reported on it. Interestingly enough, Thomas Morton, who claimed to be a graduate of the Baltimore College of dental surgery, was found out never to have attended dental or medical school. Furthermore, no records exist of him ever attending any university course – those were the times for pioneership.

Abdominal surgery made an important step forward in the 1870th with the development of several surgical techniques, such as partial gastrectomy, cholecystectomy and appendectomy. Among the pioneers in abdominal surgery, performing gastrectomies and cholecystectomies, was Theodor Billroth [3], a German surgeon educated in Berlin, who has his name written in surgical history by the two techniques describing partial resection of the lower gastric part, Billroth I and II.
With even better anaesthetic methods and surgical improvements, more and more advanced surgical procedures were introduced, including vascular surgery, both peripheral as well as cardiac surgery. Since the mid 20th century the technique for vascular repair advanced further and radiological interventions found a place in vascular repair initially with angioplasty of peripheral vessels and coronary vessels, but later also with endovascular repair of an abdominal aortic aneurysm. This un-necessitates the use of an abdominal approach when more suitable for endovascular repair, or less suitable for the abdominal approach.
The technique to replace the aneurysmatic part of the aorta with a graft via an abdominal incision is still common, and may so be for a long period of time because of the suitability of certain aneurysms to surgery rather than to radiological intervention.

Metabolic considerations

Life as we know it and the metabolic pathways in all forms of life have developed under many millions of years. The majority of the pathways we are sharing with all other living organisms. The metabolic pathways have adapted to the varying environments and life conditions that have existed on earth to meet the different needs for protection of essential systems and metabolic pathways. Understanding pathophysiological events in association with trauma, surgery and critical illness is important since this is a prerequisite for therapy aiming at modelling the response.

Nutrition, individual substrates, metabolic control and normoglycemia are important cornerstones for an optimal organ function. Also scavengers, with detoxifying properties

consumed during oxidative stress and inflammation are of importance [4,5]. Many compounds act as scavengers some of them supplied through food and some endogenously synthesised. Glutathione is an important endogenous scavenger, consisting of glutamate, cysteine and glycine, abundant in almost all tissues and involved in many important processes [6].

Ischemia/reperfusion, oxidative stress and ROS (Reactive Oxygen Species)

Life and cellular function are dependent on oxygen consuming (aerobic) metabolism, but during limited periods with shortage of oxygen, essential processes are maintained by energy rich compounds gained through anaerobic metabolism. Oxidative stress is defined as a situation when the redox-status of biological compounds is changed due to the combination of the action of ROS in relation to either a diminished or insufficient scavenging capacity, and occurs with excess oxygen supply, after chock, vascular occlusion (ischemia), radiation or toxin influence [7-9]. During reperfusion when oxygenised blood perfuse the tissue, the formation of ROS is enhanced [10], leading to an increased oxidative stress that damages cellular integrity, impairs energy metabolism and gives rise to organ failure [11]. The oxidative stress is counteracted by antioxidants, among which glutathione is the quantitatively most important.
ROS are formed via hypoxanthine to xanthine conversion catalysed by xanthine oxidase (fig 1). The ROS thus created are H_2O_2, $O2^{\cdot}$, and OH^{-} . ROS are highly reactive and short-lived substances that denaturate lipoproteins and proteins, inducing cellular breakdown (oxidative stress) [12-16]. Their instability is due to an unpaired electron in the outer orbital of the oxygen atom. ROS have important roles in normal metabolism in the defence against bacteria, where H_2O_2 is produced by neutrophil granulocytes to counteract bacterial infections. ROS have also important functions in the intracellular signalling system and many metabolic compounds can be defined as reactive oxygen species (NO, H, $OH^{\cdot}$, SO_2, H_2O_2) [15,17].
Ischemia with anaerobe metabolism decreases intracellular pH and if prolonged, the cells energy charge is depleted [18,19].
Moreover, IR initiates nucleotide breakdown which increases the intracellular concentration of hypoxanthine, a prerequisite for ROS formation (O_2-, H_2O_2- and OH-), the latter being the most reactive of the radicals. In the presence of iron or cupper as electron acceptors the ROS formation is further increased giving rise to $OH^{\cdot}$ (fig 2). The action of xanthine oxidase is prevented by allopurinol or oxypurinol [4,20]. The activity of xanthine oxidase varies between different tissues, and species. In man the highest activities are found in liver and intestine, whereas it is low in skeletal muscle [21,22]. The formation of oxygen free radicals in skeletal muscle can arise from the endothelium of the microvascular portion of the muscle, where the xanthine oxidase activity is higher [23], or from leukocytes in the small veins of the muscle [24].

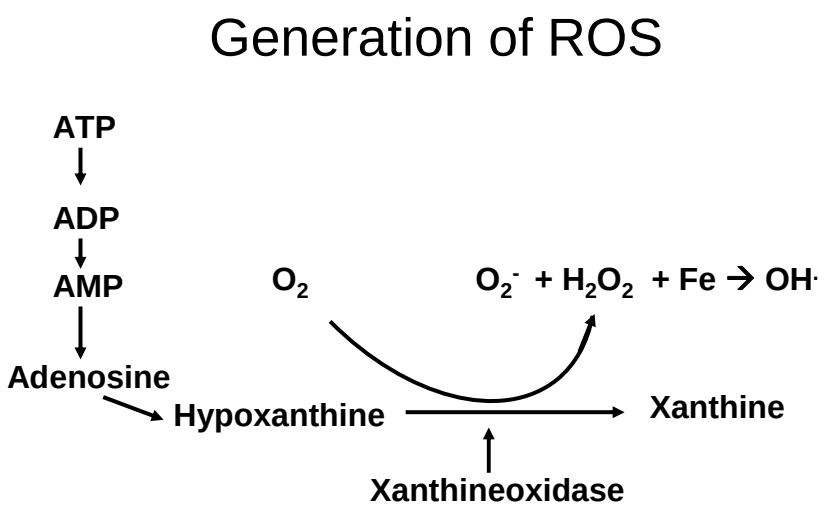


Fig 1. *Generation of ROS via the Hypoxanthine to Xanthine conversion, catalysed by Xanthine oxidase.*

The Fenton reaction; $H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^\cdot$

The Haber-Weiss reaction; $O2^{-\cdot} + H_2O_2 \rightarrow O_2 + OH^\cdot + OH^\cdot$

Fig 2. *The Fenton and Haber-Weiss reactions, generating highly reactive OH-.*

Scavengers

As ROS are highly unstable and react immediately with any hydrogen donor to be stabilised, they create new unstable reactive agents, which in turn can react with other hydrogen donors. In this way a chain reaction is started that could have fatal consequences to the cell. True scavengers are those compounds that react with ROS without generating further radicals and thus quenching the chain reaction mentioned above (fig 3). Normally ROS are counteracted by both endogenous and exogenous scavengers to create stability within the cell, acting through $2\ ROS^- + 2\ GSH \rightarrow 2\ ROSH + GSSG$. Example of endogenous scavengers are alfa-tocoferol (vitamin E), ascorbate (vitamin C), co-enzyme Q10 and folic acid, catalase, superoxide dismutase, glutathione, glutathione peroxidase, selenium and cysteine. Among the exogenous scavengers allopurinol, mannitol, vitamine E and glycine are available to be administered in conditions with reperfusion and increased production of ROS.

Oxidative stress and scavenging

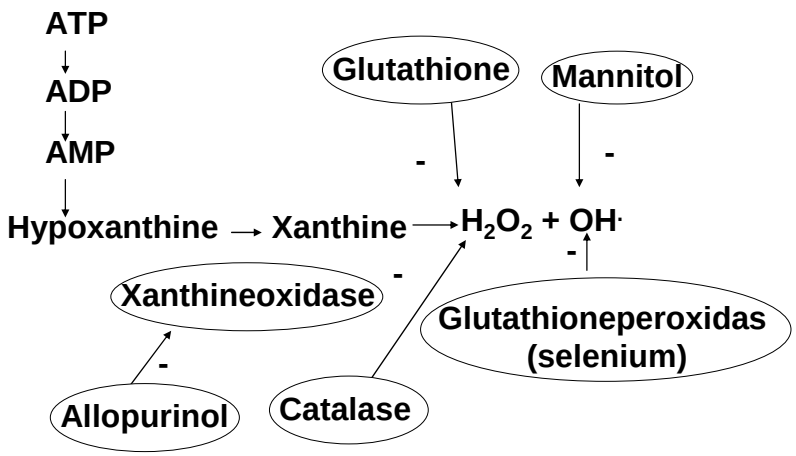


Fig 3. *Schematic overview of some ROS generation and anti-oxidant actions.*

There are several ways to study oxidative stress. ROS can be measured indirectly via analyses of products of ROS. Two such products are TBARS (Thio Barbiturate Reactive Substances) and MDA (Malone Di Aldehyde). Due to the high reactiveness and short life of ROS and their products, analyses of TBARS and MDA are said to be unreliable. Products of lipid oxidation and protein oxidation are also possible to measure, investigating oxidative stress.
Another way to study the effects of oxidative stress is to analyse one or more of the scavengers counteracting the oxidative stress. In this way it is possible to measure superoxide dismutase, catalase, and glutathione, as secondary markers of oxidative stress.
In this thesis work we have focused on measuring glutathione, the quantitatively most important endogenous scavenger, in human skeletal muscle and liver tissue.
What species and/or what tissue in a specific species to examine are matters to be decided on prior initiating a study. Skeletal muscle is a major source of protein to be utilised in other tissues in situations with increased physical stress as in burn injury, sepsis and surgical trauma [25-27]. Studies on ischemia and reperfusion in animal skeletal muscle have the advantages of being possible to standardise both in the way to achieve ischemia as well as the ischemia time. Theoretically one would believe no surgical stress to be present in such standardised animal models, but this is proved not to be the case as we know that ex-articulating the hip joint in a rabbit, cannot be achieved without applying a surgical trauma to that joint [19,28-30]. Effects of IR on glutathione metabolism have been studied extensively in animal models. Studies on ischemia and reperfusion in animal skeletal muscle have the advantages of being possible to standardise both in the way to achieve ischemia as well as the ischemia time. The results from animal studies concerning glutathione metabolism in skeletal muscle are not uniform, some showing no alterations in skeletal muscle GSH and GSSG contents [7], or increase in GSH and GSSG both at maximal ischemia as well as during reperfusion [31], or depleted GSH and

increased GSSG during reperfusion [32]. Studies on human are scarce. The overall aim of this thesis is to validate surgical interventions with IR as potential human models for studies of glutathione concentrations in skeletal muscle and liver.

Oxidative scavenging and surgery

To perform surgery, it is sometimes necessary to occlude the blood supply leading to ischemia of the organ or limb subjected to the surgical procedure, to avoid too much blood loss and to create a better vision of the surgical field. In abdominal aortic aneurysm surgery, the aorta is clamped above the neck of the aneurysm often just below the renal arteries to stop exsanguinations, and below the aneurysm of the aorta to minimise retrograde bleeding. In limb surgery as in knee replacement surgery, the blood is occluded by a tourniquet on the thigh, inflated up to double the arterial blood pressure. This stops all inflow of blood to the operation field, including collaterals and retrograde venous flow.
In liver resection surgery, the blood supply to the liver is occluded by the Pringle manoeuvre [33], clamping the hepatic artery and the portal vein. Furthermore the retrograde bleeding is minimised by occluding the inferior caval vein both above and below the liver. The ischemia thus achieved is followed by reperfusion, when ROS are potentially formed, necessitating an active ROS defence within the reperfused tissue. The defence consists of many scavenging subjects, amongst whom glutathione is the one of our interest, because of its abundance and importance in skeletal muscle and liver. Other endogenous scavengers against ROS are SOD (Super Oxide Dismutase) and catalase, counteracting $O_2^{\cdot}$ and H_2O_2 respectively. Counteracting the oxidative stress seen after ischemia with subsequent reperfusion, with anti- oxidants has been proven to decrease muscular oedema and restore the cells energy charge by preserving PCr and ATP in animals [28,29,34]. One substrate proven to be efficient against the effects of ROS in animal models is Mannitol, a large molecular sugar alcohol, clinically used as a diuretic to diminish intracranial pressure following head injury, acting both as a scavenger and as an osmotic agent [29,35,36]. The association between inflammation and oxidative stress has been addressed in previous studies [37-41].
In studies on skeletal muscle glutathione metabolism, lower levels of GSH has been described in laboratory models in animals with ischemia and reperfusion and after abdominal surgery and in critical illness in humans [40,42-44]. Furthermore, a changed redox-status has been described in IR and in critical illness [40,42].

Experimental IR in animals including exarticulation of the hip joint, leads to impaired metabolism and tissue oedema in skeletal muscle [45,46]. Furthermore, increased inflammatory response, with organ dysfunction may arise as in abdominal aortic aneurysm surgery on humans [9,47-49]. Other clinical conditions where ischemia (hypoperfusion) and reperfusion are at hand are cerebro-vascular lesions (stroke), cardiac infarction, during transplant surgery and after chock from various origins. Some studies have found beneficial effects by administering scavengers in ischemia and reperfusion [5,29,50], but most of the existing evidence of beneficial effects of scavengers are derived from animal studies. The importance of scavengers on humans under clinical conditions is less evident, and has not become a routine measure in everyday medicine up to this time.

Glutathione

Glutathione is a tripeptide consisting of L-glutamate, L-cysteine and glycine. Its actions and metabolism will be addressed in detail later. Shortly, glutathione is the quantitatively most important and abundant endogenous anti-oxidant in the human body. Glutathione deficiency leads to decreased action against oxidative stress with increased organ dysfunction [51]. Consequently, increased oxidative stress leads to decreased levels of reduced glutathione (GSH). Glutathione depletion is seen in inborn errors of metabolism like in glucose-6-phosphate dehydrogenase deficiency where NADPH is produced at lower levels decreasing the recycling of oxidised glutathione (GSSG) to reduced glutathione (GSH). These patients suffer from haemolytic anaemia.

The cysteine sulfhydryl group of glutathione is the site of antioxidant action, reducing oxygen derived free radicals by donating the hydrogen from the sulfhydryl group to the electrophilic site of the oxidant. Glutathione exerts most of its action on hydrogen peroxide, an oxygen derived radical formed from breakdown of purines and pyrimidines from breakdown of DNA, RNA and adenosine, giving excess nucleotides. Normally glutathione exists in more than 95 % in the reduced form (GSH) and less than 5 % in the oxidised form (GSSG). GSH is the monomeric and GSSG the dimeric form of glutathione. GSSG consists of 2 molecules of glutathione linked together by a disulfide bridge. The highest concentration of glutathione is found in the cytoplasm and in the mitochondria, where it exerts its actions against ROS. Glutathione tissue concentrations can increase by supplying precursors like acetyl-cysteine and glutamine. One in vitro study demonstrated that human intestinal epithelial cells pre-treated with glutamine and exerted to 2 hours of ischemia with subsequent reperfusion, had elevated levels of glutathione during the reperfusion as compared to cells not pre-treated with glutamine [52]. Another in vitro study described elevated glutathione concentrations in myelomonocytic cells, supplying them with glutamine, N-acetyl cysteine, glycine and alfa lipoic acid [53].

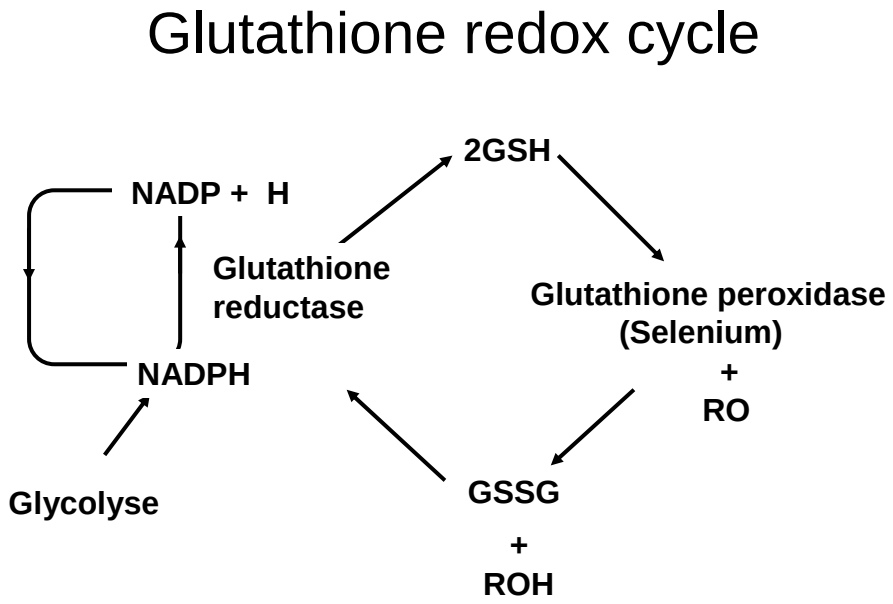


Fig 4. *Glutathione oxidation and reduction showing involved enzymes and the source of NADPH used as a reducing agent in reduction of GSSG to GSH. (RO = Oxidant, ROH = Reduced oxidant).*

Glutathione properties

Glutathione has many properties of which the most important is as an antioxidant, preferably acting against H_2O_2, protecting enzymes and lipoproteins intracellular and in cell membranes from oxidation and denaturation. Also the enzyme glutathione peroxidase, catalyzing the oxidation of GSH to GSSG, acts as an antioxidant reacting with a co-factor, selenium, to counteract the toxicity of OH- (fig 4). Glutathione also helps keeping other reducing agents such as Vitamin C in a reduced form [54], and can act as a transporter for cysteine. In clinical situations, glutathione has been proven to enhance sperm motility [55], and increase cellular glucose uptake in NIDDM (Non Insulin Dependent Diabetes Mellitus) patients [56]. Furthermore, glutathione has been shown to have immunomodulatory properties [57]. It is also involved in detoxification, as we know that acetylcysteine, a precursor to glutathione is useful against intoxication with paracetamol by enhancing glutathione production and by directly linking to the paracetamol molecule, making it detoxified and water soluble and thus possible to excrete via the urine. Moreover, protection against neurological conditions such as Morbus Parkinson, and Alzheimer's disease by glutathione has been showed [58].

Glutathione synthesis

Glutathione is almost not extracted from the ingested nutrients into the bloodstream, even though at least one study from 1991 on mice pre-treated with L-buthione-S, R-sulfoximine, a glutathione synthesis inhibitor, demonstrated elevated levels of glutathione in tissues after oral administration of glutathione [59]. Another study on mice showed increased glutathione levels in

plasma as well as in different tissues after orally administered glutahione [60]. The anti-oxidative active part of glutathione, cysteine, is not water-soluble. Therefore, it can not act as an antioxidant of its own. By binding to glutamate via the enzyme gamma-glutamylcysteine synthetase, the first and rate-limiting step in glutathione synthesis, it becomes water-soluble and non-toxic (fig 5). Formation of this bond requires activation of the gamma-carboxyl group, which is achieved by ATP. In the second step, catalysed by glutathione synthetase, ATP activates the carboxyl group of cysteine to enable it to condense with the amino group of glycine. Glutathione is mainly produced in the liver and exported for use to other tissues.

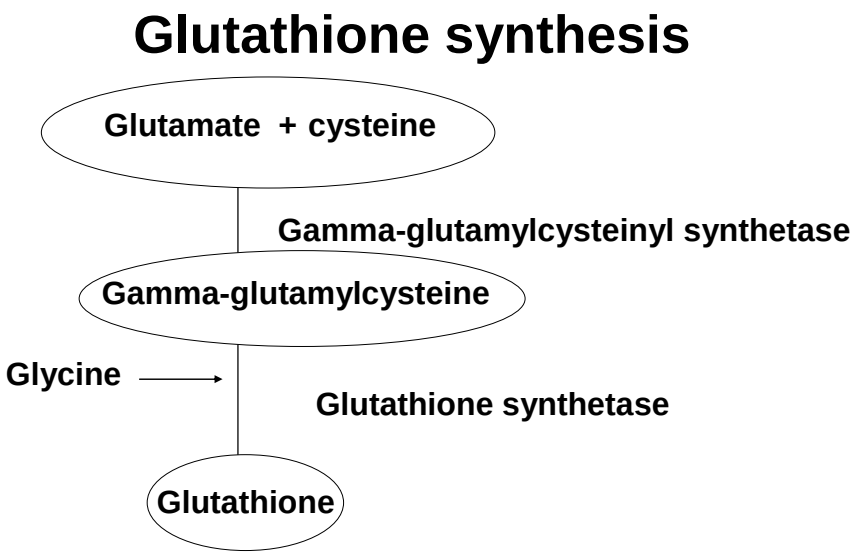


Fig 5. *Schematic presentation of the glutathione synthesis.*

Glutathione uptake

It is not quite clear how the active uptake of glutathione occurs, but inhibition of the membrane bound enzyme gamma-glutamyl transpeptidase, increases urinary excretion and plasma levels of glutathione, indicating that inhibition of this enzyme may lead to inhibited cellular uptake of glutathione [61,62]. Another study demonstrated active transport of glutathione against a concentration gradient in lung type II alveolar cells in neonatal rabbits, indicating an active transport mechanism for glutathione across cell membranes [63]. Passive transport of glutathione over cell membranes can also occur with esterified glutathione [64].

Glutathione degradation (Fig 6)

The degradation process of glutathione is not clear but most of the glutathione is exported out of the cell before degradation [6]. The degradation occurs then by activity of the gamma-glutamyl transpeptidase enzyme, cleaving the bond between glutamate and cysteine [65]. The remaining bond between cysteine and glycine can then be hydrolysed by other transpeptidases. GSSG can be excreted out from the cell without degradation [66,67], or in the presence of glutathione-reductase and NADPH, rapidly reduced to GSH within the cell to protect intracellular moieties from oxidation by GSSG, itself being an oxidant. Export of GSH out of the cell for utilization elsewhere is especially true, but not exclusive, for the liver [68].

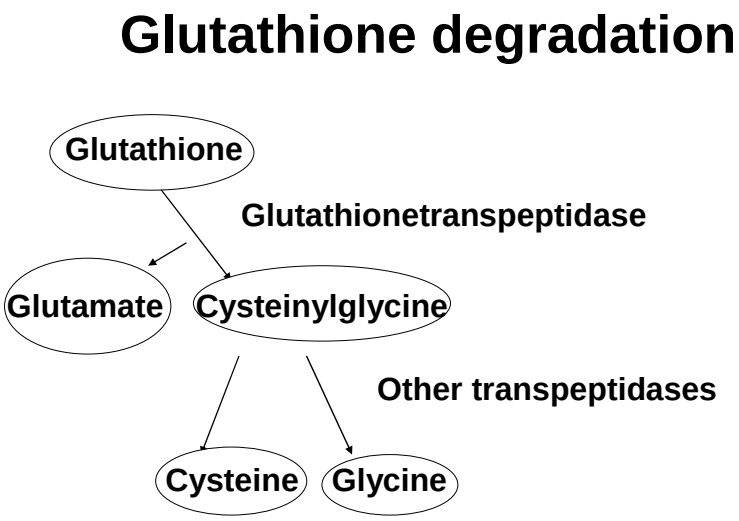


Fig 6. *Glutathione degradation schematically, showing enzymes and end products.*

Amino acids related to glutathione metabolism

Glutamine

Glutamine is a non-essential amino acid with essential properties. Glutamine is mainly produced in skeletal muscle by amination of glutamate. Glutamine accounts for more than 60 % of the free amino acid pool in the muscle. This means that the skeletal muscle in fact is a large glutamine pool, used by more central organs in case of surgical stress and trauma [69-71]. In case of physiological stress, glutamine is delivered from skeletal muscle to the intestine, blood cells, liver and kidneys [72]. This leads to lower muscle glutamine levels, a characteristic sign of muscle protein catabolism. Adding glutamine postoperatively reduces the nitrogen losses otherwise seen, and spares muscle glutamine with possible positive effects on muscle protein metabolism [73]. Glutamine is essential for cell proliferation, killer cell activation and production of interferon-gamma [74]. Glutamine is also utilised as a source for gluconeogenesis. Moreover, depletion in glutamine concentration leads to depletion in glutathione concentration. Adding

glutamine to postoperative TPN diminishes the decline in muscle glutathione levels postoperatively [75]**.** The functions of glutamine are summarised in a review article from 2002 [76]. Many of its important effects are explained by the fact that glutamine serves as an essential precursor for purine and pyrimidine synthesis and thus is important for the metabolism of all rapidly dividing cells, like immune competent cells, mucosal cells and cells involved in wound healing and proliferation. Furthermore it is said to inhibit apoptosis and stimulate intestinal immunity. Reports that glutamine reduces mortality in ICU patients through improved immunological competence has made it a standard nutrient in ICU patients nowadays [77]**.**

Glutamate

Glutamate is closely linked to glutamine. It is also linked to alfa-ketoglutarate, an intermediate in the citric acid cycle, and can thus be utilised for energy production in situations when glucose and glycogen are consumed. The reversible reaction Glutamate + pyruvat < -- > alfa-ketoglutarate + alanine, also creates alanine by amination of pyruvate. Glutamate is one of the three constituents of glutathione, and it is active as a neurotransmitter substance on it self [78] and also as a precursor to GABA (gamma-amino-butyric-acid), another potent neurotransmitter.

Cysteine

Cysteine, one of the constitutives of glutathione, is a thiol, a sulphur containing amino acid. The sulphur part of the cysteine can protect other sulphur groups in lipoproteins and enzymes from oxidation. As mentioned earlier, cysteine is not water soluble and highly toxic, so to make the scavenging entities of cysteine possible, it has to be linked to other amino acids to be able to exert its actions. Except for glutathione, cysteine is also acting as a reducing agent in the combination with an acetyl group in N-acetylcystein, which is commercially available and used in paracetamol intoxications to detoxify paracetamol by making it water soluble and excreted via the urine. It is also used as a mucus dissolving agent in pulmonary diseases.

Glycine

Lately increasing interest has been directed against glycine as an amino acid with immuno-modulatory capacities, by reducing TNF-alfa (tumor necrosis factor-alfa) production, and enhance IL-10 (interleukine-10) production [79]. The action being blocking of Ca^{2+} channels in the cellmembrane and thus inhibiting the cell's action. For example, one study showed anti-angiogenic properties by blocking of Ca^{2+} channels in the cellmembrane [80]. It has also been proved to be beneficial in treatment of various alcohol induced liver diseases by inactivating the Kuppfer cells by Ca^{2+} channel blocking [81]. Furthermore, glycine is also of major importance in the synthesis of proteins, peptides, purines, ATP, nucleic acids, porphyrins, hemoglobin, glutathione, creatine, bile salts, one-carbon fragments, glucose, glycogen, and L-serine and other amino acids. Glycine also acts as a neurotransmitter in the central nervous system (CNS). Moreover, it has been proven to increase survival rate and decrease TNF-alfa in rat livers subjected to 120 minutes of warm ischemia [82].

IR and energy metabolism in skeletal muscle

The mitochondria are known as the power plants of the cells. They act as a cell within the cell with their own DNA, which codes for 13 different proteins. The main function of the mitochondria is to produce energy. The energy production arises from the citric acid, or Kreb's cycle and the electron (respiratory) chain in the membrane of the mitochondrion. In the presence of oxygen, glucose and fatty acids are utilised to produce energy. In the absence of oxygen (anaerobe energy metabolism), glucose is metabolised to pyruvate to create ATP. Lactate is then formed from pyruvate, catalysed by lactate dehydrogenase. The anaerobe glycolysis pathway is less sufficient and gains approximately 2/3 as much ATP as the aerobe glycolysis.

In critically ill patients, mitochondrial content decreases twofold, a decrease that is accompanied by a decrease in energy rich phosphates and increased anaerobic energy production in leg skeletal muscle but not in intercostals skeletal muscle [83]. The ATP level in skeletal muscle is said to be relative constant even during ischemia and reperfusion, provided that the ischemia time is not prolonged. Ischemia of more than 6 hours in rabbits hindlimbs depletes the cells energy charge, with a decrease in ATP, leading to an irreversible loss of the cell's energy charge [19].

A comparison between energy metabolism in healthy humans and patients with peripheral vascular disease (intermittent claudication) demonstrated lower ATP and PCr in claudicants at rest as compared to the healthy population [84].

AIMS OF THE STUDIES

Overall aim of the thesis

IR has potential damaging effects if the oxidative stress during reperfusion exceeds the scavenging capacity. This has however mainly been shown in animal models allowing prolonged ischemia times. The relevance of these findings for human physiology, especially in the clinical setting is not well enough studied. The overall aim of this thesis was to elucidate the effects of IR on GSH and its related amino acids in human tissues. To enable this, we validated several surgical procedures with IR as potential models for such studies.

Specific aims

- To study vascular occlusion of about 60 minutes and the effects on glutathione and amino acids in skeletal muscle in patients subjected to abdominal aortic surgery to elucidate if this was a suitable model to study IR in man.

- To study the effect of longer vascular occlusion of about 120 minutes on glutathione and amino acids in aorto-bifemoral by-pass surgery.

- To study if a more complete ischemia in conjunction with a less pronounced surgical trauma affects glutathione and amino acids status during ischemia and reperfusion, and in addition to study the effect of Mannitol, a scavenger of ROS, on glutathione metabolism.

- To describe tissue-specific metabolism of glutathione and amino acid concentrations in liver during ischemia and reperfusion. Liver resection surgery was used as a human model for liver IR.

MATERIALS AND METHODS

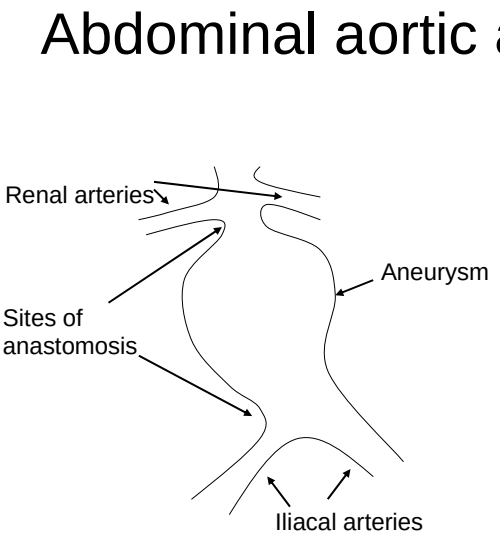


Fig 7. *An illustration of an abdominal aortic aneurysm with an aneurysmatic sac and the sites of anastomosis.*

Surgical techniques

Abdominal aneurysm surgery **(fig 7)**

In abdominal aortic aneurysm surgery the abdomen is opened with a midline incision from the xiphoide process down to the symphysis of the pubic bone, exposing the abdominal cavity. The intestines are than held to the right and the retroperitoneum is incised just to the left of the aorta to expose the aneurysm. The aorta is thereafter clamped at the neck of the aneurysm just below the exit of the renal arteries and on both iliacal arteries to minimise bleeding during the surgical procedure, and thereafter opened up. After division of the aorta both proximal and distal, a graft, made of PTFE (polytetraflourethylene) or Gortex ® is inserted with suturing to the proximal neck of the aorta and the distal “non-aneurysmatic” aorta or the iliacal arteries if the aorta is aneurysmatic down to the bifurcation of the iliacal arteries. When the graft is safely in place, the blood flow is allowed back by taking of the clamps, beginning with the distal ones.

Aorto-bifemoral surgery

In the aorto-bifemoral surgery group, abdominal incision is performed in a similar way as in the abdominal aortic aneurysm patients. The aorto-bifemoral patients do not have any aneurysm though. Also in this group, access to the retroperitoneum is necessary to be able to occlude the aorta and perform an anastomosis between the abdominal aorta and the femoral arteries on both sides. This surgical procedure includes therefore also bilateral inguinal incisions to dissect the femoral arteries. This makes the clamping times (ischemia times) of the aorta longer compared to those in abdominal aortic aneurysm surgery.

Knee replacement surgery

Knee replacement surgery is usually performed in patients with severe arthrosis of the knee joint. Arthrosis means that the cartilage in the joint is destroyed, which could arise from different reasons and give the patient pain not only at strain, but also at rest. The knee replacement surgery in this study is performed with epidural anaesthesia (EDA) and a blood less operation field. The bloodless operation field is achieved by first elevating the leg to be operated on to try to empty it from as much blood as possible. An inflatable tourniquet is then applied around the thigh, and inflated to double the arterial blood pressure measured in one of the arms. Here after the knee joint is opened up and the destroyed cartilage is removed before a new joint, made of titanium and plastic is placed to substitute the original joint. After the skin has been closed, and a draping of the wound applied, the inflatable tourniquet is released and the blood again allowed to flow down the extremity.

Liver resection surgery

In liver resection surgery, the abdomen is opened via a subcostal incision below the right costal margin, often crossing also the midline on to the left subcostal margin. This gives optimal access to the hepatic area. The inferior caval vein both above and below the liver is dissected free and ligated with rubber ligatures to avoid retrograde bleeding from the resection margin. Furthermore, the portal vein and the hepatic artery are both occluded with a surgical atraumatic clamp to further diminish perioperative blood loss. In fact, this is a surgical method that has been recommended in cases with large central hepatic tumours to minimise the hepatic blood loss [85]. The resection of the diseased liver lobe is than performed with help of an ultrasound knife during total liver ischemia.

Summary of study protocols

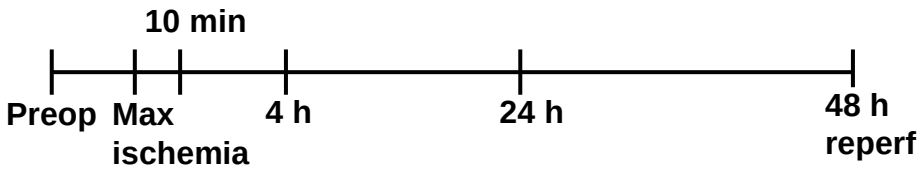
The patients included in this thesis work were informed about the studies and approved participation. All studies and protocols were approved by the ethics committee of the Karolinska Institutet, Stockholm, Sweden.

Study protocols

Study I

Study protocol abdominal in aortic aneurysm surgery

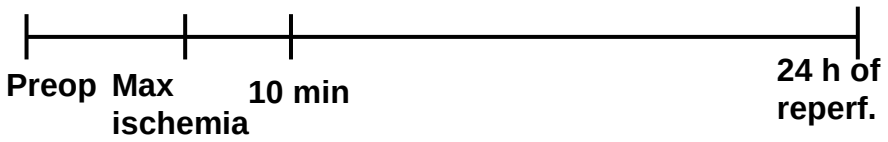
Muscle biopsies taken at each occasion for analyses of glutathione, amino acids and energy rich content



Study II

Study protocol in aorto-bifemoral surgery study

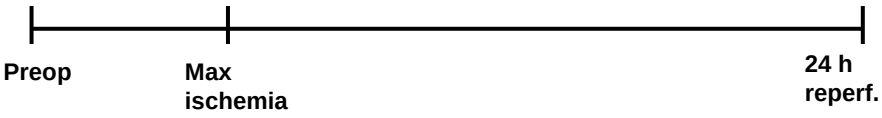
Muscle biopsies for analyses of glutathione, energy rich Content and amino acids were obtained at each sampling occasion from both legs. At 10 minutes only energy rich contents were analysed



Study III

Study protocol in knee replacement study

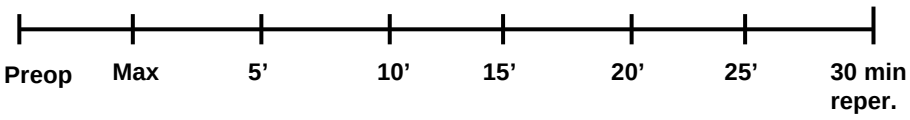
Sampling occasions in the knee replacement study Glutathione, amino acids and energy rich content were analysed at every sampling occasion



Study IV

Study protocol in liver resection surgery

Biopsy occasions. The biopsies were analysed for glutathione, amino acids and lactate



Patient characteristics (Table I)

Study I

Ten patients scheduled for elective abdominal aortic aneurysm surgery were included. Muscle biopsies were obtained from the thigh muscle, about 15 cm above the knee, according to the study protocol above. Analyses for glutathione, amino acids and energy rich compounds were made from the specimens.

Study II

Twelve patients with iliac occlusion and subjective signs of peripheral vascular disease (intermittent claudication), were included in the study. Muscle biopsies were obtained from the lateral part of the thigh muscle about 15 cm above the knee on both legs according to the study protocol above. The biopsies were analysed for glutathione, amino acids and energy rich compounds.

Study III

Patients (n=15) with gonarthrosis were included in the study. They were randomised to receive either glucose 50 mg/ml (Fresenius Kabi, Uppsala, Sweden) 18.75 ml/kg/day or Mannitol (Fresenius Kabi) with osmolality 930 mosmol/kg, 12.5 ml/kg/day.
Ischemia was induced using an inflatable tourniquet around the thigh on the leg that was subjected to surgery. The tourniquet was inflated to double the blood pressure measured in the patient's right arm. Muscle biopsies were obtained from the lateral portion of the quadriceps femoris muscle on the operated side, according to the study protocol. The muscle biopsies were analysed for glutathione, amino acids and energy rich compounds.

Study IV

Six patients (4 females and 2 males), between 41 and 72 years old with colorectal cancer metastasis of the liver, suitable for hemi-hepatectomy and otherwise considered healthy, were included in the study. Liver biopsies were obtained from both liver lobes according to the study protocol. The biopsies were later analysed for glutathione, amino acids and lactate.

Table I. *Study overview with patient characteristics.*

	AAA	ABF	Knee	Liver
Male / Female	9/1	8/4	6/9	2/4
Age (years)	74 (72-83)	68 (64-73)	71 (63-76)	55 (52-57)
Ischemia times (min)	65 (60-77)	113 (99-120)	83 (56-92)	28 (15-36)
Amino acids				
Thiols				
Energy rich compounds				
Lactate				
Mitochondrial enzymes				

AAA = Abdominal aortic aneurysm study
ABF = Aorto-bifemoral study
Knee = Knee replacement study and
Liver = Liver resection study. Grey field indicates which analyses were performed

Values are given as median and range.

Sample preparation

In study I, II and III, muscle biopsies were obtained from the lateral part of the quadriceps femoris muscle at each sampling occasion. One was immediately plunged into liquid nitrogen for later analyse of energy rich compounds. The other was weighed on an electrobalance, than frozen in liquid nitrogen and stored at – 80° C prior analyse of glutathione and amino acids. In study IV, liver tissue biopsies were collected, immediately frozen in liquid nitrogen and stored at – 80° C until analysed.

Analyses of thiols (study I, II, III and IV)

The thiols were analysed according to a method described earlier [86]. The protein content was denaturated by addition of 10 % sulphosalicylic acid (SSA). Neutralisation was achieved by adding excess $NaHCO_3$. Part of the sample was mixed with mBBr (monobromo-bimane), incubated in dark for 10 minutes, and the reaction was thereafter stopped by addition of 100 % SSA, to analyse reduced glutathione. In another part of the sample oxidised thiols were reduced with dithiothreitol before the mBBr to measure total glutathione. Hereafter both samples were analysed by HPLC (High Pressure Liquid Chromatography). The oxidised

glutathione was calculated as the difference between total and reduced glutathione, divided by 2. The concentrations of thiols are expressed as mmol/kg wet weight muscle.

Analyses of Amino Acids (study I, II)

The muscle portion was homogenised in 4 % SSA containing nor-leucine as an internal standard. Thereafter the specimen was centrifuged (12.000*g* for 15 minutes at 4° C). The supernatant from the centrifugate was then stored at – 80° C before analysed. Amino acid separation was done on an Ultropae 8 lithium form ion exchange resin (Biochrom, Cambridge, UK) with the use of lithium citrate buffers and using an automatic amino acid analyser (Alpha Plus, LKB Bromma, Sweden). After postcolumn derivatisation with ophtaldialdehyde, amino acids were detected by fluorescence at excitation 350 nm and emission 420 nm [86]. Amino acid concentrations are expressed as mmol/kg wet weight muscle.

Analyses of Amino Acids (study III and IV)

The frozen liver biopsies were homogenised in 4 % SSA containing norvaline as an internal standard. Samples were analysed on a HPLC after precolumn derivatisation with ophtaldialdehyde as described previously [87]. The concentrations of amino acids are expressed as mmol/kg wet weight of liver.

Analyses of energy-rich compounds and lactate Study I, II, II and (IV)

The freeze dried muscle specimens were dissected free of all visible connective tissue and fat. Hereafter the sample was pulverised and the powder was extracted with 0.5 M $HCLO_4$ containing 1.0 mM ethylenediaminetetraacetic acid. A neutralization was then performed with 2.1 M $KHCO_3$. Analyses for ATP, PCr, Cr and lactate were performed according to a method described by Harris et al [88]. The concentrations of energy-rich compounds were expressed as mmol/kg dry weight muscle.

Analyses of mitochondrial enzymes (study III)

Mitochondrial enzymes, citrate synthase, complex I and IV were analysed according to a method described in detail by Fredriksson and collaborators [83]. Briefly the specimen were homogenised in a KCL buffer and used for analyses of mitochondrial enzymes citrate synthase, complex I and IV by use of a spectrophotometric assay on a Konelab 20 analyser (Thermo Clinical Labsystems, Vantaa, Finland). Mitochondrial enzymes values are expressed as mmol/kg dry weight muscle.

Statistical analyses

The material in these studies was considered not normally distributed according to the Kolmogorov-Smirnov and Lilliefors test. Friedman's analysis of variance for non-parametric statistics followed by Scheffe's post hoc test (study I) and Wilcoxons test (study II, III and IV) were used for statistical analyses. All values in the studies are expressed as medians and percentiles. A p-value < 0.05 was considered statistically significant.

RESULTS

Summary of results

Energy rich compounds and lactate (table II)

An increase was present in lactate at maximal ischemia in all studies. The increase seen in the patients in study II with peripheral vascular disease was less profound, indicating a well developed blood shunting via collaterals. PCr decreased and Creatine increased at maximal ischemia in skeletal muscle, whereas total Creatine was left un-affected. No alterations were seen in ATP during ischemia and subsequent reperfusion. Comparing the results from all skeletal muscle samples, revealed correlations between the increase in lactate and alanine at maximal ischemia (fig 8), ($p<0.001$), and between the change in lactate and BCAA at maximal ischemia (fig 9), ($p<0.01$). No correlation was found between the increase in lactate and ischemia time looking at all legs examined (fig 10). When looking at the aortic aneurysm patients (study I), a correlation between ischemia time and lactate increase at maximal ischemia was evident (see paper I).

Glutathione (table III)

tGSH and GSH (fig 11) decreased during reperfusion, while GSSG (fig 12) and the redox ratio of glutathione were unchanged in skeletal muscle. In the knee replacement study (III), the patients receiving glucose during reperfusion showed declines in both GSH and tGSH, while those who received Mannitol, a free radical scavenger, showed unaffected levels of GSH and tGSH. However, the difference between these two groups did not reach any statistical significance. In liver tissue no changes in GSH, GSSG or the redox ratio of glutathione were seen.

Amino acids (table IV)

At maximal ischemia there was an increase in alanine, and a decrease in BCAA in study I and II and glutamate in skeletal muscle, while in liver increases in both alanine and BCAA were seen, accompanied by a decrease in glutamate. At reperfusion alanine and BCAA were normalised in both skeletal muscle and liver, while glutamate decreased continuously during reperfusion in skeletal muscle, in contrast to liver tissue were a continues incline was seen during reperfusion.
Glutamine was not affected by ischemia in neither skeletal muscle nor liver, but decreased in skeletal muscle and increased in liver tissue during reperfusion.

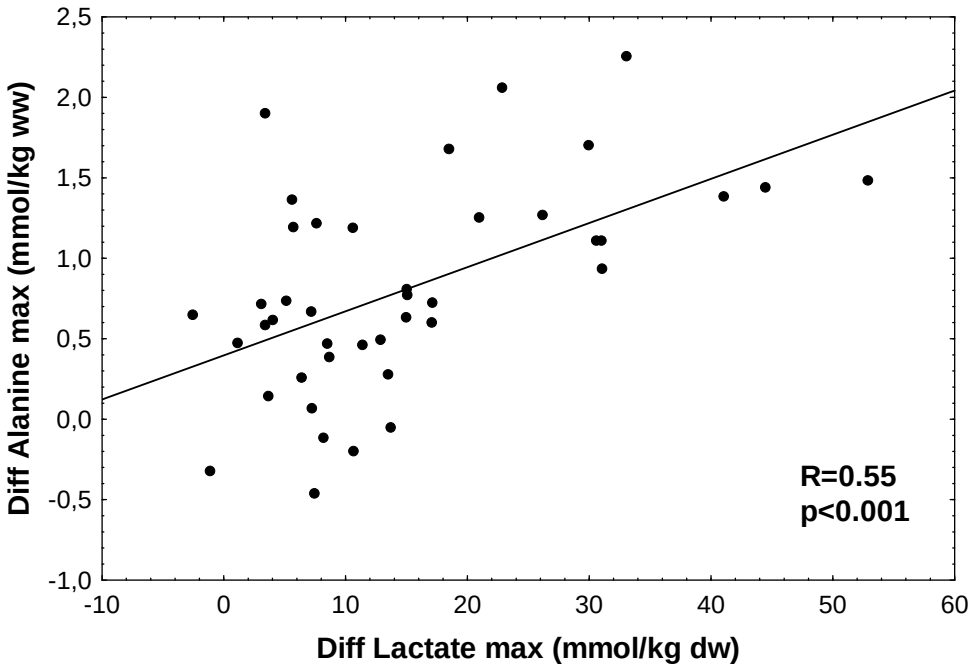


Fig 8. *Correlation between the increases in lactate and alanine at maximal ischemia R=0.55, p<0.001.*

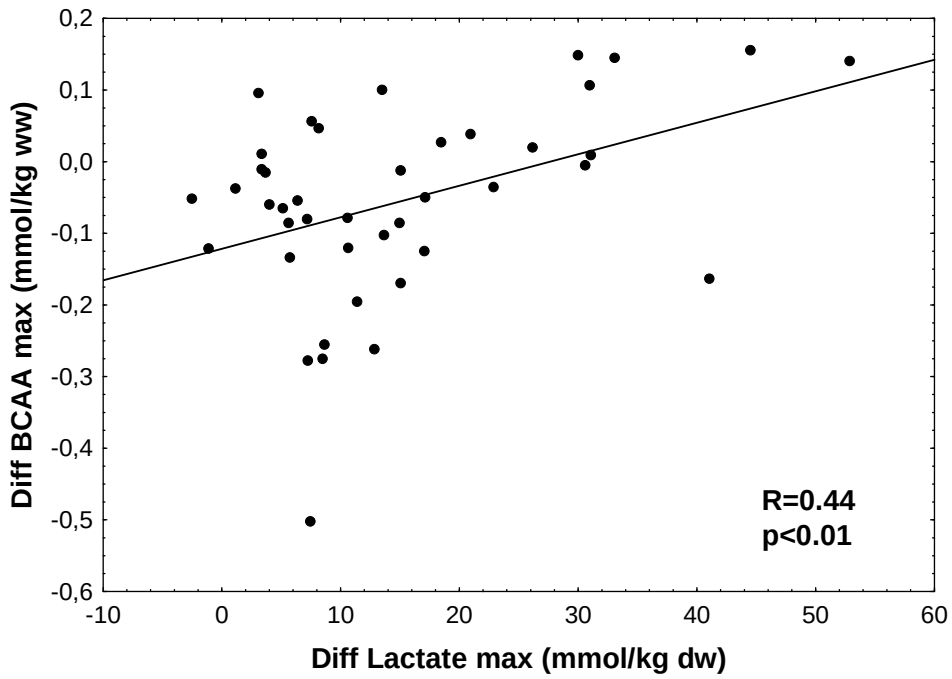


Fig 9. *Correlation between the changes in lactate and BCAA at maximal ischemia R=0.42, p<0.01.*

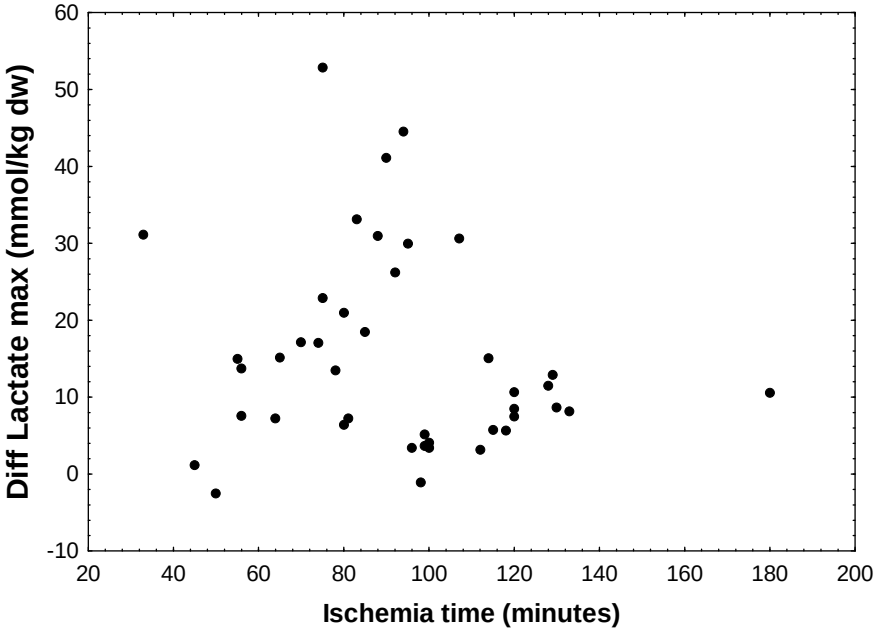


Fig 10. *The increase in lactate at maximal ischemia compared to ischemia time in muscle samples from study I, II, and III. No correlation was seen in the total material but when doing subgroup analyses a correlation was seen in the AAA-group (R=0.77, p<0.01) while the Knee group had a correlation close to statistically significant (R=0.44) and the ABF group had not (R=0.01).*

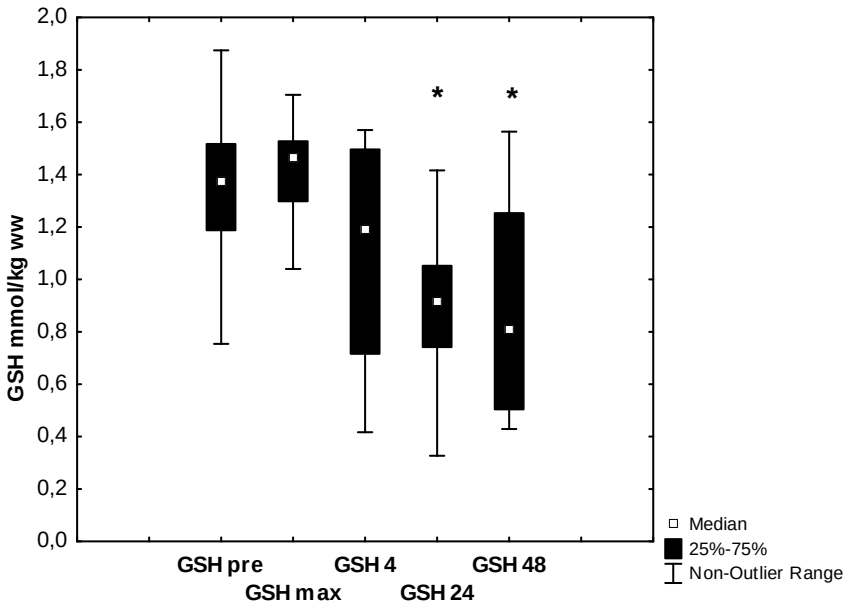


Fig 11. *A summary of reduced glutathione alterations in skeletal muscle in all studies during ischemia and reperfusion. * denotes statistical diffenrences compared to baseline.*

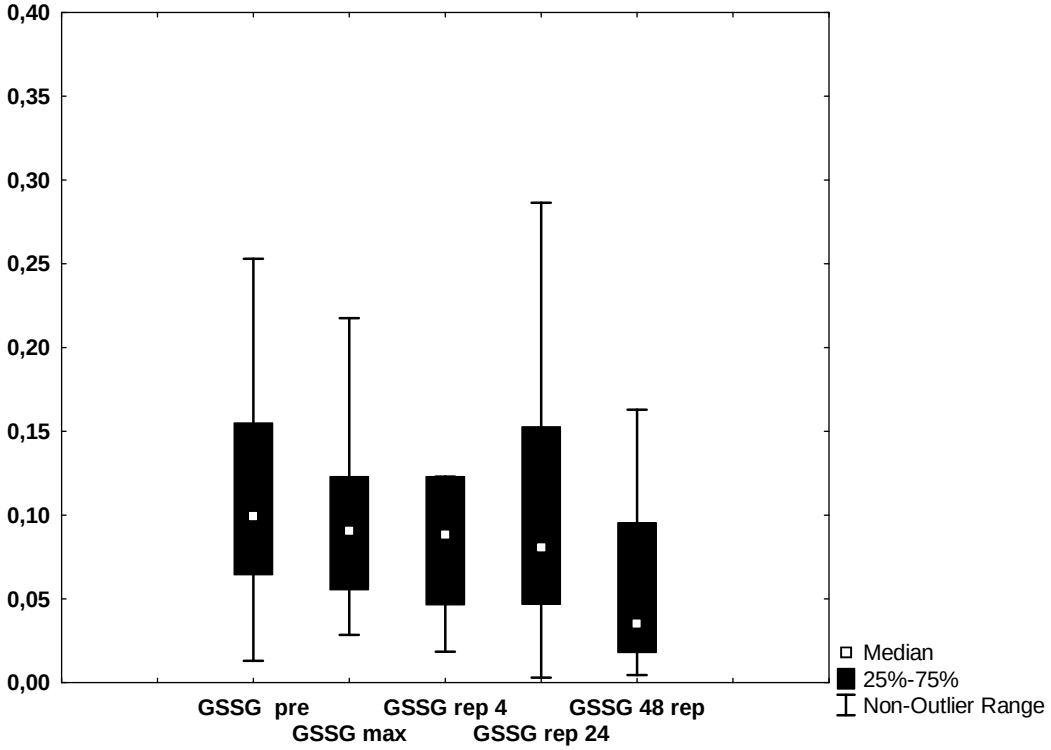


***Fig 12**. Summary of GSSG pattern over time in muscle during reperfusion.*

Table II. *A summary of percentage changes in lactate and energy rich compounds at maximal ischemia in the four studies, NM = Not Measured, NS = Not Significant*

	Lactate	**ATP**	**PCr**	**Cr**	**tCr**
Study I	+274	NS	-37	+57	NS
Study II	+101	NS	-21	+17	NS
Study III	+420	NS	-69	+91	NS
Study IV	+267	NM	NM	NM	NM

Table III. *A summary of percentage changes in reduced glutathione during reperfusion in the four studies. NS = Not Significant.*

	tGSH	**GSH**	**GSSG**	**Redox**
Study I 24 hours	-46	-48	NS	NS
Study I 48 hours	-43	-44	NS	NS
Study II 24 hours	-32	-41	NS	NS
Study III 24 hours	-22	-27	NS	NS
Study IV 30 minutes	NS	NS	NS	NS

Table IV. *Percentage changes of some amino acids in the studies during ischemia and reperfusion as compared to preischemic values. NS = Not Significant.*

		Gln	**Glu**	**Ala**	**BCAA**	**TAA**
Study I	Max	NS	-33	+21	-25	NS
	24 hours	-23	-54	NS	NS	-34
	48 hours	-54	-41	NS	NS	-37
Study II	Max	NS	-31	+31	-33	NS
	24 hours	-25	-41	NS	NS	-21
Study III	Max	NS	-18	+48	NS	NS
	24 hours	-32	-50	-17	NS	-29
Study IV	Max	NS	-22	+104	+67	+25
	30 minutes	+25	+72	NS	NS	+23

GENERAL DISCUSSION

Energy rich compounds and lactate

The results from our studies show that routine vascular surgery and liver surgery do elicit various degrees of ischemia as measured by increase of lactate at maximal ischemia. Lactate increased at maximal ischemia in skeletal muscle and liver, the highest concentrations seen in tourniquet ischemia and the lowest in aorto-bifemoral surgery. The increase in lactate is a proof of anaerobic metabolism. In aorto-bifemoral surgery with vascular occlusion of 113 minutes lactate increased by 100 % at maximal ischemia, due to an incomplete ischemia because of well developed collateral blood vessels shunting blood to the extremities even when the aorta is clamped. Lactate even continued to increase after 10 minutes of reperfusion and remained elevated after 24 hours of reperfusion compared to basal values indicating an abnormal tissue oxygenation in the immediate postoperative period despite improved ankle blood pressures. The other patients demonstrated higher increases in lactate at maximal ischemia, increases that were normalised early during reperfusion. A situation with impaired blood supply to the extremities as in the aorto-bifemoral study patients due to various degrees of vascular occlusions exposes the tissue to ischemic preconditioning, which could improve the tissue's resistance to oxidative stress [89-92]. During ischemia in skeletal muscle, decreases in PCr are seen, accompanied by concomitant increases in Creatine. These changes are reversed after 24 hours of reperfusion. tCr is thus logically unaffected at maximal ischemia. The most important energy source of the cell, ATP, remains unaffected during both ischemia and reperfusion in skeletal muscle in these clinical studies, being of high priority to the cell. Aerobe glycolyse produce more ATP than anaerobe glycolyse. So in situations with oxygen deficit, ATP has to be maintained by PCr donating phosphate to ADP, keeping ATP normalised.
It has been demonstrated in animal studies that prolonged skeletal muscle ischemia can deplete the cell of ATP and cause an irreversible loss of the cells energy charge [19].

Glutathione

Abdominal surgery of moderate size has been demonstrated to decrease glutathione concentration in skeletal muscle without any measurable changes in GSSG or the redox ratio of glutathione (GSH/tGSH ratio) [43].
In the present studies clamping of the aorta for 60 minutes with subsequent reperfusion in combination with surgical stress due to the abdominal aortic aneurysm surgery, did not further influence the changes seen after abdominal surgery [93]. The possibility that the ischemia time was too short to detect any changes in GSH was considered. However, a prolongation of the clamping time to 120 minutes during aorto-bi femoral surgery had no further impact on the glutathione concentrations in skeletal muscle, as compared to abdominal surgery and abdominal aortic surgery [43,94]. In both these studies a complete ischemia is not guaranteed which might explain the lack of any effect of ischemia. In addition, the dramatic effect of the abdominal surgery on the decreased GSH post-surgery, might be disguising any effect of IR itself. The third study was therefore designed to obtain complete ischemia of the leg muscle without any major stress from abdominal surgery. However, also in this study glutathione concentrations changed no more than has been demonstrated in abdominal vascular surgery [93-95]. On the other hand, the lack of any abdominal surgery in this study indicates that IR itself might be responsible for at least part of the decrease in GSH seen post-surgically.
The changes seen in muscle glutathione status in the present studies may be multifactorial. The influence of fasting preoperatively can not be an explanation to the changes seen since fasting for a longer period of time, up to three days, did not affect glutathione status in muscle

in healthy volunteers [96]. Moreover, it has been proven that neither general anaesthesia nor epidural anaesthesia affect human skeletal muscle amino acid metabolism, excluding that as a cause for the alterations seen [97-99].
Surgical stress without IR has been shown to decrease muscle glutathione levels, a change that may be explained by the link between glutathione and amino acid metabolism and the consistent decrease in muscle glutamine and glutamate following surgery [43,100,101]. In severe oxidative stress the ability to reduce GSSG is impaired due to increased anaerobe glycolysis with lower NADPH production. An accumulation of tissue GSSG is seen and this leads to increased efflux of this oxidised compound out of the cell [66].
It is interesting to note that in the knee replacement study, the patients receiving Mannitol, a scavenger of ROS [5,29,36], did not show any decrease in GSH or tGSH, while the patients that received glucose, did (study III). This difference did not reach statistical significance comparing the two groups, but nevertheless it is an interesting observation indicating that exogenous scavengers may protect the levels of tissue glutathione.

In our present studies no changes were seen regarding the redox-status of glutathione. On the other hand, prolonged total ischemia (2 and 4 hours respectively) followed by reperfusion in an animal model demonstrated alterations in GSH concentration and the redox (GSSG/GSH) status of muscle glutathione after 4 hours of ischemia. These changes were not seen in the group subjected to 2 hours of ischemia [32], proposing that prolonged ischemia with subsequent reperfusion induces a more profound oxidative stress in muscle tissue.

In study IV we wanted to investigate whether the effect of IR could be different in another human tissue, e.g. the liver. In our study on warm liver ischemia and reperfusion in man, glutathione was un-affected during ischemia up to 36 minutes with subsequent reperfusion during 30 minutes. In animal studies on warm liver IR and glutathione concentrations different results have been demonstrated [14,67,102], showing oxidative stress to occur by elevated lipid peroxidation and increased GSSG and GSH concentrations in hepatic vein plasma. In other studies no evidence of oxidative stress was at hand, neither any alteration in glutathione concentration.
One study investigated the difference between inflow obstruction and inflow-outflow obstruction to liver and the difference in metabolic response depending on the site of liver biopsy in rats. Results showed different distribution of oxidative damage depending on the type of occlusion made after 30 minutes of either inflow or inflow-outflow occlusion, and a difference also between central and peripheral biopsies, showing a decrease in GSH concentration after, an inflow-outflow obstruction in central biopsies but not after inflow obstruction alone, and not peripheral in liver tissue [103]. This implicates that both the choice of tissue (plasma or liver tissue) examined and the location of the biopsy from the liver are of importance when investigating oxidative stress in the liver.
In a rat study, GSH decreased in liver tissue subjected to 30 minutes of warm ischemia. Furthermore it was noted that this decrease could be counteracted by the administration of alanyl-glutamine infusion 3 days prior to the ischemic insult [104].
To summarise, our study, as well as several other animal studies show that liver glutathione metabolism is of high priority, showing liver tissue GSH to be un-affected by ischemia and reperfusion.
Examining plasma from hepatic veins during liver ischemia and reperfusion and liver central tissue after inflow/outflow obstruction on the other hand demonstrated altered glutathione concentrations in liver and hepatic vein plasma.

Amino acids

The amino acid pattern during reperfusion largely mimics that seen postoperatively in non-vascular abdominal surgery [43,100,101], whereas the changes at maximal ischemia seem to be specific for ischemia. These changes are not described earlier to our knowledge.
Furthermore, a decreased protein synthesis is at hand in the skeletal muscle postoperative.
The stable concentrations of cysteine and glycine during ischemia and reperfusion could be explained by their toxicity and by their importance in other metabolical pathways such as cell signalling, transport amino acids, immuno modulation and prevention of apoptosis. In the tourniquet ischemia patients however, the oxidised form of cysteine, cystine, increased during reperfusion as an indicia of oxidative stress.
Moreover, increase of alanine was present at maximal ischemia, probably because of its relationship to pyruvate, an intermediate in the Kreb's cycle, increasing in concentration during anaerobe energy production. Simultaneously glutamate decreased in both skeletal muscle and liver. The increase in alanine and decrease in glutamate at maximal ischemia in the liver and skeletal muscle is an interesting finding. Another interesting finding comparing the total results from the studies, is the strong correlation (R=0.55) between the increase in lactate and alanine at maximal ischemia. The hypothesis being that pyruvate is produced during anaerobe glycolysis, and excess pyruvate inhibits further glycolysis. There are two ways to handle the excess of pyruvate, one is to convert pyruvate to lactate catalysed by lactate dehydrogenase, and secondly, by amination of pyruvate to form alanine. The aminogroup is most probable donated by glutamate, thus creating alfa-ketoglutarate and alanine from glutamate and pyruvate. It was also clear that the basic amino acids as well as the total sum of amino acids decreased during the reperfusion in skeletal muscle.

In liver, glutamate decreased at maximal ischemia and increased thereafter continuously during reperfusion, while glutamine was unaffected at maximal ischemia and increased during reperfusion. This could be explained by a net uptake of glutamine in the liver during reperfusion and deamination of glutamine giving rise to glutamate. In addition elective surgery has been shown to decrease protein synthesis, further increasing amino acids concentrations [105].
Alanine and the branched chain amino acids valine and isoleucine, increased at maximal ischemia and then normalised during reperfusion. The total sum of amino acids increased during reperfusion in the liver.
The reason for the alterations in alanine and glutamate seen at maximal ischemia is addressed above. The increase in BCAA at maximal ischemia could be due to increased proteolysis giving rise to increased BCAA that are not deaminated in liver tissue [106]. It has to be remembered though that the reperfusion studied in liver is considerable shorter compared to skeletal muscle. This makes any comparison between liver and skeletal muscle during reperfusion hazardous.

In summary, at maximal ischemia, glutamate decreases to keep anaerobe glycolysis going, by amination of pyruvate to alanine. Thus one building stone of glutathione is diminished. In muscle, the fall in glutamate continuous at reperfusion, due to the flux of glutamine to splanchnicus, possibly explaining the decrease in GSH during muscle reperfusion. In liver on the other hand, the fall in glutamate at maximal ischemia, is followed by a rise at reperfusion, possibly due to a net uptake of glutamine to the liver for deamination to glutamate and urea production. In this way, liver GSH will not suffer from any lack of this important building stone, which might explain the unaffected glutathione concentration in liver during reperfusion. We must bear in mind though that the reperfusion time examined in the liver did not exceed 40 minutes.

CONCLUSIONS

We conclude that abdominal surgery on the aorta with ischemia and subsequent reperfusion, affects skeletal muscle glutathione, amino acids and energy rich compounds, but that this effect is similar during reperfusion to that seen after non-vascular abdominal surgery, without IR. Furthermore we conclude that knee replacement surgery with a more complete ischemia and subsequent reperfusion, affects glutathione and amino acids in skeletal muscle in the same way. However, since this procedure does not have the severe metabolic stress from abdominal surgery, part of the decreases in GSH seen might be IR related. It is also concluded that liver ischemia/reperfusion, affects liver amino acid metabolism, but not glutathione metabolism.

- We conclude that vascular occlusion of about 60 minutes with subsequent reperfusion in abdominal aortic aneurysm surgery, affect glutathione metabolism with decreased GSH and tGSH levels but not GSSG and glutathione redox status in skeletal muscle at reperfusion. This is not different from what previously has been seen postoperative in abdominal non-vascular surgery without IR. Amino acids are influenced by the ischemia and consequent anaerobe metabolism. We cannot exclude oxidative stress as a contributing factor for the glutathione changes seen.

- Increasing vascular occlusion to almost 120 minutes did not have any further effect on glutathione or amino acids concentrations in skeletal muscle. The changes seen most likely are attributable to the surgical trauma and possibly ischemia and reperfusion.

- Making the ischemia more complete with a tourniquet during 85 minutes, and omitting abdominal surgery, did not change anything concerning glutathione and amino acid concentrations in skeletal muscle. The changes seen in the knee replacement patients are not as pronounced as in the aortic patients, and thus possibly more the results of IR. Supplying Mannitol, a scavenger of ROS to 8 of the 15 patients suppressed the anticipated GSH decrease, but the difference between the two groups was not significant.

- Focusing on another target organ of ischemia and reperfusion, the liver, did not alter the metabolic response. Here, the liver did not show any alterations in glutathione concentrations during 30 minutes of reperfusion. Regarding amino acids, the pattern differed in the liver compared to skeletal muscle with an increase of BCAA at maximal ischemia and increases in both glutamate and glutamine during reperfusion, showing the organs different tasks concerning amino acids and protein metabolism.

General conclusion

- Alterations were seen in skeletal muscle glutathione concentrations, attributable to both the surgical trauma and possibly IR. In the liver glutathione did not change, indicating a high priority in liver to glutathione homeostasis, and possibly a too short reperfusion time.
- Amino acid alterations due to anaerobe metabolism were seen at maximal ischemia, while a typical postoperative pattern was at hand during reperfusion in muscle. In the liver probable organ specific alterations were seen both during ischemia and reperfusion. This has not been shown earlier in man to our knowledge.

Future perspectives

To be able to address the question whether or not oxidative stress occurs in human skeletal muscle and liver during elective routine surgery, more studies with measures of glutathione, superoxide dismutase, catalase and MDA are warranted. Furthermore, situations with even longer ischemia times would be helpful, as would emergency patients with circulatory chock, acute pancreatitis and peritonitis, with an inflammatory onset and hypothetically impaired peripheral circulation. These conditions are relevant situations in which oxidative stress has an important role for the pathophysiological alterations and in extension also for the clinical outcome.

SAMMANFATTNING PÅ SVENSKA

Bakgrund

Högreaktiva fria syreradikaler bildas i vävnad under såväl fysiologiska som patologiska förhållanden. Den negativa effekten av dessa motverkas av scavengers (antioxidanter).
I situationer med ökad bildning av syreradikaler som vid chock av olika ursprung, sepsis, trauma och ischemia/reperfusion (cirkulationsstopp/cirkulationspåsläpp) utsätts vävnaden för oxidativ stress. Detta kan leda till att vävnadens förråd av både kroppsegna (endogena) och exogena antioxidanter inte räcker till, vilket kan leda till att DNA, RNA, protein och cellmembran skadas med celldöd och ytterst organsvikt/multiorgansvikt som följd.
Att förstå hur det förhåller sig hos människa under kliniskt vanliga tillstånd innefattande ischemi och reperfusion kan öka kunskaperna om mekanismerna bakom oxidativ stress. Vidare kan vi få en bild av om och hur planerad rutinkirurgi med avstängning och påsläpp av cirkulationen, påverkar det kroppsegna kvantitativt viktigaste försvaret mot oxidativ stress, glutathion. Glutathion är en tripeptid, bestående av 3 aminosyror, glutamat, cystein och glycin. Glutathion förekommer i alla vävnader i kroppen, men i högsta koncentration i ögats lins, lever och tolvfingertarm. I skelettmuskulatur är nivåerna lägre.

Syfte

Att utvärdera om glutathion och aminosyra koncentrationerna i skelettmuskulatur och lever påverkas av ischemi och den efterföljande reperfusionen som ett mått på oxidativ stress vid elektiv kirurgi, samt att utvärdera dessa kirurgiska ingrepp som modeller att studera IR hos människa under kliniska betingelser.

Metoder

I studie I studerades patienter (n=10) uppsatta för elektiv operation av bukaorta aneurysm. Muskelprover togs före operation, vid maximal ischemi, efter 10 minuter, 4, 24 och 48 timmars reperfusion och analyserades avseende glutathion, aminosyror och energi rika föreningar, inklusive laktat.
I studie II undersöktes patienter (n=12) planerade för aorto-bifemoral by pass kirurgi på grund av varierande grad av förträngningar i bäcken artärerna. Muskel prover togs från båda benen, före operation, vid maximal ischemi, efter 10 minuter och 24 timmars reperfusion. Glutathion, aminosyror och energi rika föreningar analyserades.
I studie III studerades patienter (n=15) som var planerade att genomgå operation av knäled med insättning av konstgjord led till följd av avancerad förslitning i densamma. 8 av patienterna gavs Mannitol, en antioxidant under reperfusionen, medan 7 av patienterna fick glukos. Muskelprover togs före operation, vid maximal ischemi och efter 24 timmars reperfusion för analys av glutathion, aminosyror och energirika föreningar.
I studie IV undersöktes patienter (n=6) som var planerade att genomgå operation med borttagande av ena halvan av levern till följd av cancer. Leverbiopsi togs före ischemi, vid maximal ischemi och efter 5, 10, 15, 20, 25 och 30 minuters reperfusion. Analyser avseende glutathion, aminosyror och laktat gjordes.

Resultat

I skelettmuskulatur sågs en minskning av det reducerade glutathionet (GSH) och det totala glutathionet (tGSH) under reperfusionen, medan det oxiderade glutathionet (GSSG) och glutathionets redox kvot (GSH/tGSH), inte påverkades. I studie III sågs ingen sänkning av GSH under reperfusionen hos de patienter som gavs Mannitol, medan de som fick glukos uppvisade en sänkning. Denna skillnad var dock inte statistiskt signifikant, men likväl noterbar. I lever sågs inga förändringar i GSH, GSSG eller glutathionets redox kvot.
Aminosyrapåverkan i skelettmuskulatur under reperfusionen liknade det man ser efter operation utan ischemi. Vi maximal ischemi sågs en ökning i alanin, och en reduktion av glutamat och grenkedjade aminosyror (valin, leucin och isoleucin) i muskulatur, som tecken på anaerob glykolys. Under reperfusionen postoperativt sågs typiska minskningar i glutamat och glutamin i muskel. I levervävnad sågs ökningar av både alanin och grenkedjade aminosyror vid maximal ischemi, medan glutamat sjönk. Under reperfusionen normaliserades alanin och grenkedjade, samtidigt som glutamat och glutamin ökade.
Laktat ökade i studie I och III med 274 respektive 420 % vid maximal ischemi. I studie II var ökningen vid maximal ischemi 101 %, en ökning som tilltog ytterligare efter 10 minuters reperfusion till 166 % och fortfarande efter 24 timmars reperfusion var 50 % förhöjt jämfört med det preoperativa utgångsvärdet. PCr minskade och Cr ökade vid maximal ischemi medan ATP inte påverkades i skelettmuskulatur. I lever ökade laktat med 264 % vid maximal ischemi och normaliserades sedan vid reperfusionen.

Slutsats

Elektiv kirurgi av bukaorta och knäled med ischemi och reperfusion påverkar glutathion koncentrationen på ett sätt som är förenligt med oxidativ stress, men liknande mönster ses också efter bukkirurgi utan ischemi och reperfusion. Aminosyraförändringarna som sågs under reperfusionen, skilde sig inte från vad som tidigare har setts efter bukirurgi utan ischemi och reperfusion. Cellens energi laddning (ATP) påverkades inte. Aminosyra förändringarna som sågs vid maximal ischemia uppstår till följd av anaerob glykolys i både muskel och lever. Skillnaden i grenkedjade aminosyror beror troligen på en skillnad i koncentrationen av transaminaser för dessa mellan lever och muskel.
Elektiv leverkirurgi med ischemi och reperfusion påverkade inte lever glutathion koncentrationen. Aminosyra koncentrationerna förändrades under både ischemi och reperfusion. Detta finns inte beskrivet under dessa betingelser hos människa tidigare.
De prövade skelettmuskel modellerna uppvisar således förändringar som kan vara betingade av oxidativ stress, men också kan vara en produkt av det kirurgiska traumat.

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