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## **Knee replacement surgery as a human clinical model of the effects of ischemia/reperfusion upon skeletal muscle**

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**Short title: Ischemia and reperfusion in knee surgery**

## ABSTRACT

### **Background:**

The temporal pattern of metabolic alterations in muscle tissue are not well characterised in man with respect to glutathione, amino acids and energy rich compounds during total ischemia and reperfusion. Here knee replacement surgery was used as a clinical model to elucidate this pattern.

### **Material and methods:**

Patients (n=15) undergoing elective knee replacement surgery employing tourniquet ischemia were studied. Muscle biopsies were taken from the quadriceps femoris muscle on the operated side preoperatively, at maximal ischemia and after 24 hours of reperfusion. The biopsies were analysed for glutathione, amino acids and energy rich compounds. In addition the patients were randomised to receive either glucose or a mannitol infusion during 24 hours following the tourniquet ischemia.

### **Results:**

During ischemia an increase in muscle lactate by 400 % ( $p < 0.05$ ) and a decrease in phosphocreatine by 70 % ( $p < 0.05$ ) were seen. During the subsequent 24 h reperfusion, muscle reduced glutathione and total glutathione decreased by 27 % and 22 % ( $p < 0.05$ ) respectively. The muscle amino acid pattern changed during ischemia with a rise in alanine by 65 % ( $p < 0.001$ ) and a decrease in glutamate by 29 % ( $p < 0.001$ ). During the reperfusion part of the study no differences attributable to the infusion of mannitol or glucose were seen.

### **Conclusions:**

During tourniquet ischemia and subsequent reperfusion, changes in glutathione metabolism indicating oxidative stress developed. Knee replacement surgery as a clinical model was useful during the ischemia period, while the reperfusion period was dominated by the general changes seen postoperatively.

## INTRODUCTION:

The metabolic effects of ischemia and reperfusion on skeletal muscle tissue have been extensively described in animal studies. The relevance of these findings for man is not obvious. Therefore we have investigated clinical situations involving ischemia followed by reperfusion. Changes in muscle glutathione, energy and amino acid metabolism have been described using major vascular surgery in order to characterise a human model [1, 2]. However the influence of abdominal aneurysm and aorto-bifemoral bypass surgery on lower limb muscle metabolism is attributable both to the surgical trauma and to the ischemia-reperfusion. The characteristic postoperative changes are dominating over the signs of major oxidative stress regarding muscle amino acid pattern as well as glutathione status. In addition, during the major vascular surgery procedures studied, no complete ischemia is at hand, related to the existence of collaterals and shunting. Therefore a clinical human ischemia-reperfusion model was explored using knee replacement surgery in tourniquet ischemia. This clinical model could possibly be more suitable for studies on ischemia and reperfusion in human skeletal muscle and the effects on glutathione metabolism, reflecting the scavenging status.

Application of a tourniquet and inflation up to double the systolic arterial blood pressure is described to induce ischemia with anaerobic metabolism and oxidative stress [3, 4]. Tourniquet ischemia is frequently used in orthopaedic surgery and metabolic tissue alterations are seen both during ischemia and during the following reperfusion such as decreased levels of phosphocreatine, increased lactate, increased glycogenolysis, and decreased pH [5-9]. After the release of tourniquet in knee replacement surgery an increased production of reactive oxygen species (ROS) is seen [10]. Although not always clinically significant, several studies show that an increased oxidative stress occurs during knee replacement surgery using tourniquet ischemia with activation of polymorphonuclear granulocytes and increased levels of H<sub>2</sub>O<sub>2</sub> in femoral vein blood from the reperfused leg [3, 11]. Moreover, increased expressions of ICAM-1 (intercellular cell-adhesion molecule-1) in muscle and of MnSOD (manganese superoxide dismutase), GST-mu (glutathione transferase-mu) in granulocytes and increases of IL6 and IL8 in plasma suggest an association between inflammation, redox markers and ischemia and reperfusion [12].

The aim of this study was to investigate the effects of total ischemia and reperfusion in a clinical model on muscle metabolism as reflected by glutathione status, amino acid pattern and

energy metabolism. A secondary aim was to study the effects of mannitol, a scavenging substance [13], on these parameters during the reperfusion period.

## **MATERIALS AND METHODS:**

### **Patients and protocol**

Patients (n=15) without any history of vascular disease, undergoing knee replacement surgery due to gonarthrosis were included in the study. The patients were non-smokers and not on corticosteroid therapy, and they were all metabolically healthy. The study has been carried out in accordance with the Declaration of Helsinki and the protocol has been approved by the Ethics Committee of the Karolinska Institute. Informed consent was obtained from the patients after informing them about the purpose, the nature and risk of the procedures in the study.

All patients were given epidural anaesthesia with Xylocain<sup>R</sup> (Astra, Södertälje Sweden) during operation and during the following 24 hours. Ischemia was induced just prior to surgery by a tourniquet that was inflated to double the patients systolic arterial blood pressure. Muscle biopsies were obtained from the vastus lateralis portion approximately 15 cm above the knee on the leg to be operated on before ischemia, at maximal ischemia just before deflating the tourniquet and after 24 hours of reperfusion with a needle biopsy technique described by Bergström et al [14]. The biopsies after 24 hours of reperfusion were obtained under local anaesthesia down to the muscular fascia, but not into the muscular compartment.

The patients were randomised to receive either mannitol with an osmolality of 930 mosm/kg (Fresenius Kabi, Uppsala at a dose of 12.5 ml/kg/day; n=8) or glucose (Fresenius Kabi, Uppsala) at a concentration of 50 mg/ml, at a dose of 18.75 ml/kg/day; n=7). The infusions were given as a continuous infusion during 24 hours after the induction of tourniquet ischemia. The patients were allowed to drink non-caloric fluid during the postoperative period and were given Ringer's acetate to cover the basal need for fluid.

### **Muscle samples**

A percutaneous muscle biopsy of approximately 150 mg was obtained at each sampling occasion. The muscle biopsy was taken approximately 15-20 cm below the lower border of the tourniquet cuff. The muscle biopsy was divided into three smaller portions, weighed on an electro balance, then plunged into liquid nitrogen and stored at -80°C prior to analyse.

### **Biochemical analyses**

In a subgroup of patients (n=7) muscle samples were obtained preoperatively, at maximal ischemia and after 24 hours of reperfusion for analyses of lactate, ATP, creatine (Cr) and

phosphocreatine (PCr) as described in detail elsewhere [2, 15]. Total creatine (tCr) was calculated as the sum of Cr and PCr. In addition the mitochondria were assembled and the activities of citrate synthase and complexes I and IV were determined as described in detail elsewhere [16].

Glutathione and cysteine were analysed as described in detail previously [17]. The oxidised form of glutathione (GSSG) was calculated by the difference between total (tGSH) and reduced glutathione (GSH) divided by two. The redox-status of glutathione was expressed as the ratio between GSH and tGSH (GSH/tGSH). The concentrations of thiols are expressed as mmol/kg ww of muscle.

For amino acid analyses HPLC employing postcolumn derivatisation with ophthaldialdehyde was used [18]. The concentrations of amino acids were expressed as mmol/kg ww of muscle.

### **Statistical analyses**

The values are expressed as medians with lower and upper quartiles. All patients in the two groups were combined in this comparison. When comparing results during the reperfusion period with preoperative levels a nonparametric ANOVA (Freidman's ANOVA) comparing medians, was used followed by Wilcoxon test for post-hoc comparisons. Spearman rank test was used for correlations. A p-value < 0.05 was considered as statistically significant for comparisons.

## RESULTS

The patients included in the study were 71.0 (63.0 – 76.0) years old (mannitol 68.5 (63.5 – 74.3) and glucose group 73.0 (62.0 – 79.0) respectively). Ischemia time was 83.0 (56.0 – 92.0) minutes (mannitol 84.0 (75.0 – 93.0) and glucose group (78.0 (50.0 – 88.0)). The tissue was not exposed to glucose or mannitol until the reperfusion started after removal of the tourniquet. Therefore all samples obtained preoperatively and at maximal ischemia were combined in the calculations reflecting the preoperative and maximal ischemia condition. During the reperfusion period the values are presented for the combined group as well as for the two treatment groups.

### **Energy rich compounds and mitochondrial enzymes (Figure 1)**

Lactate increased during ischemia by 420 % as a sign of anaerobic metabolism ( $p < 0.05$ ). PCr decreased at maximal ischemia by 69 % ( $p < 0.05$ ) with a reciprocal increase in Cr by 91 % ( $p < 0.05$ ). At 24 h of reperfusion both PCr and Cr recovered back to the preoperative levels. tCr and ATP remained unchanged during the whole study period as compared to the preoperative values. Ischemia time and changes in lactate and PCr at maximal ischemia were not statistically correlated. The mitochondrial enzymes citrate synthase and Complexes I and IV were not affected by ischemia and reperfusion.

### **Thiols (Figure 2, and electronic supplement table 1)**

At maximal ischemia an increase was seen in GSH by 8% ( $p < 0.05$ ). At the same time the oxidised form of glutathione (GSSG) decreased by 21% ( $p < 0.01$ ), with a consequent increase in the redox-status of glutathione (GSH/tGSH) by 4% ( $p < 0.01$ ). At 24 h reperfusion decreases were seen in GSH and tGSH by 27% ( $p < 0.01$ ) and 22% ( $p < 0.01$ ) respectively, while an increase was seen in cystine (CySS), the oxidised form of cysteine by 23% ( $p < 0.01$ ).

At 24 h of reperfusion the effect of mannitol was studied. No changes in GSH and tGSH were seen in the patients randomised to mannitol, while decreases by 31% ( $p < 0.05$ ) and by 26% ( $p < 0.05$ ) respectively were seen in the control group as compared to the preoperative levels. There were no statistically significant changes between the two groups. (Data are given in an electronic supplement, Table 1).



**Amino acids (Figure 3 a and b, and electronic supplement table 2 and 3)**

Muscle glutamate decreased by 29 % ( $p < 0.001$ ) at maximal ischemia. At maximal ischemia increases in the muscle concentrations of threonine by 5 % ( $p < 0.05$ ), glutamine by 4 % ( $p < 0.05$ ), alanine by 65 % ( $p < 0.001$ ), lysine by 6 % ( $p < 0.05$ ), histidine by 10 % ( $p < 0.05$ ), and arginine by 6 % ( $p < 0.01$ ) were seen. Increases were also seen in the total sum of all amino acids (tAA) by 8 % ( $p < 0.05$ ), in the basic amino acids (lysine, histidine and arginine; BAA) by 8 % ( $p < 0.01$ ), in the aromatic amino acids (AAA) by 7 % ( $p < 0.05$ ) and in the essential amino acids (EAA) by 21 % ( $p < 0.01$ ).

After 24 hour of reperfusion the pattern was quite different with decreases seen in threonine by 23 % ( $p < 0.05$ ), serine by 18 % ( $p < 0.05$ ), glutamate 51 % ( $p < 0.001$ ), glutamine by 32 % ( $p < 0.01$ ), glycine by 15 % ( $p < 0.01$ ), ornithine by 50 % ( $p < 0.01$ ), lysine by 42 % ( $p < 0.01$ ), histidine by 27 % ( $p < 0.01$ ), arginine by 48 % ( $p < 0.01$ ), BAA by 41 % ( $p < 0.01$ ), EAA by 21 % ( $p < 0.01$ ), and in tAA by 28 % ( $p < 0.01$ ) in the combined group together with an increase in the sum of AAA by 15 % ( $p < 0.01$ ). (Data are given in an electronic supplement, Table 2). Between the two groups given mannitol or placebo no differences were seen in the muscle amino acid levels after the 24 h reperfusion period. (Data are given in an electronic supplement, Table 3).

No correlations were seen between changes in biochemical markers and length of ischemia.

## DISCUSSION

In our search for a human clinical model of ischemia and reperfusion, here knee replacement surgery was elucidated. The ischemia part of the model showed alterations in muscle amino acid and glutathione metabolism, which have not been described before. The reperfusion part of the model on the other hand showed changes in muscle amino acid and glutathione metabolism, which to a large extent represent what is generally seen postoperatively.

In our earlier efforts to use a human clinical model to study ischemia/reperfusion in skeletal muscle we have used major vascular surgery [1, 2]. In terms of amino acid and glutathione metabolism in muscle, these models have not been very different from what is seen following major surgical procedures in general. In abdominal aortic aneurysm repair the possibility of a too short ischemia time was discussed [2]. When similar results were obtained also when the ischemia time was doubled in aorto-bifemoral graft surgery, the fact that this type of vascular surgery does not represent total ischemia related to the existent of collaterals, was considered [1]. In the present model, knee replacement surgery, a total ischemia in terms of halted blood flow was obtained. Another advantage with the present protocol was that combined circulatory isolation was combined with the use of an epidural block for anaesthesia. Hence no interference from circulating hormones or neural impulses was at hand in the muscle during ischemia. At reperfusion the advantages during the ischemia time were not longer present. In the literature metabolic changes of ischemia followed by reperfusion during cross ligament repair in tourniquet ischemia has been reported [7]. Today this mode of surgical approach is obsolete, as this type of surgery is performed during arthroscopy not employing tourniquet ischemia.

The ischemia during 60-90 minutes was total as reflected by the increases of lactate and creatine levels with a reduction of phosphocreatine. Muscle ATP concentration was unaltered, reflecting that cellular energy status and indirectly the membrane potential were preserved. This fact is important for the interpretation of the rest of the results. A decrease in cellular ATP level is associated with necrosis and cellular disintegration [19], in which case the amino acid gradients across cell membranes may no longer be kept stable.

For glutathione a 10 % increase in reduced glutathione with an unaltered total glutathione pool in muscle was seen at maximal ischemia. This was also accompanied by a slight increase in the

redox ratio of glutathione. The physiological significance of this increase is not totally clear to us, but it indicates that the appearance of radical oxygen species was very low. A possible interpretation is that the redox ratio at maximal ischemia actually represents more or less a “maximal” value of the equilibrium between reduced glutathione and total glutathione in muscle tissue. This occurred in a situation when no oxygen was available. The redox status of cystine on the other hand was not different between basal situation and maximal ischemia.

The free amino acid pattern at maximal ischemia was different from what is seen at “maximal ischemia” during major vascular surgery. In this study all free amino acid concentrations except for glutamate were elevated. In contrast, during vascular surgery, immediately before reperfusion, most free amino acid concentrations decrease except for alanine [1, 2]. The ischemia pattern of muscle free amino acids seen here may be interpreted as a mobilisation of free amino acids from protein degradation in excess of ongoing synthesis. The most pronounced increases were seen in the alanine and glutamine concentrations. This may reflect transaminations to the carbon skeleton of pyruvate/lactate to form alanine and alpha-ketoglutarate/glutamate to form glutamine. The decrease in glutamate may consequently reflect transaminations into both glutamine and alanine production. As oxygen depending processes involving the Krebs cycle or an export via venous blood were not possible, the result was an increase in most amino acid concentrations. As the intracellular concentrations of the individual different amino acids are not proportional to their occurrence in muscle proteins, the percentage increases given must be considered in relation to the actual concentrations. This indicated for example *de novo* production of glutamine, which may explain the decrease in glutamate. The constant uptake of glutamate in muscle tissue described during all physiological situations so far investigated [20], which was now interrupted during ischemia, is also a factor to consider in this explanation.

When major vascular surgery was used as an ischemia model, the disadvantage of the possible incomplete ischemia and also the possibility of an incomplete epidural block must be considered. Although the aorta was clamped, it is well known that a collateral circulation exists. As for the completeness of the epidural block, during vascular surgery the patients were subjected to general anaesthesia in combination with epidural anaesthesia. Hence the efficacy of the epidural block may have been sufficient for postoperative pain relief without totally blocking the neurogenic part of the impact of surgery. The pattern of muscle free amino acid seen in major vascular surgery just before reperfusion is more in line with the general

postoperative pattern. This pattern develops characteristically over time [21, 22]. Initially there is an increase in alanine concentration together with a decrease in glutamate, while all other amino acids are unaltered. This pattern is also described during a combined stress hormone infusion [23]. At 6-12 hours postoperatively decreases in the free concentrations of a number of amino acids appear [21]. In particular the branched chain amino acid decrease at this time point. Later on, at 24 h after the traumatic insult, the characteristic postoperative pattern, with decreases in glutamate, glutamine and the basic amino acids together with increases of the aromatic and branched chain amino acids, is seen [21, 22]. With the result from the present study at hand, it is obvious that the amino acid pattern seen just before reperfusion during major vascular surgery was merely dominated by the changes in the amino acid patterns in response to trauma rather than to ischemia.

A secondary aim of this study was to use a scavenging substance, mannitol, to investigate whether the postoperative glutathione pattern may be modified. Administration of mannitol, a free radical scavenger diminishes oedema in the muscular compartment and prevents compartment syndrome, mostly by its hyperosmolar properties, and has been shown to reduce muscular necrosis and increase restitution of energy production by free radical scavenging. This has been shown in an animal model in which preparation of the hind-limb followed by clamping of surgically isolated vessels outruled the influence of circulation through collaterals [13]. In that model positive effects were seen both of mannitol and allopurinol leading to reduced signs of reperfusion injury. Moreover, mannitol has been shown to reduce the negative effects of reperfusion elicited by reactive oxygen species in acute vascular surgery [24, 25] and in models of ischemia reperfusion [26-28].

As stated above the reperfusion part of the knee replacement model was less convincing, as the traumatic insult elicited by the surgical procedure probably was the dominating influence upon the muscle glutathione and amino acid metabolism during this time period. Nevertheless, a slight different pattern was seen in the two groups. At 24 h of reperfusion the decrease in total and reduced glutathione concentrations in muscle attained statistical significance in the control group, but not in the mannitol group. Still the difference between the two groups was numerically minimal and it was not statistically significant. Considering the small numerical difference and the imperfection of the reperfusion part of the model, the significance of this finding is not clear. Evaluation of mannitol treatment as an adjunct during reperfusion in man

must be done employing a more appropriate model to be conclusive. For the muscle free amino acid patterns no differences between the two groups were seen.

In summary knee replacement surgery during tourniquet ischemia was used as a human clinical model of ischemia and reperfusion. The ischemia part of the model worked fine and original findings concerning the increase in glutathione redox status and the general increase in free amino acid concentrations after 60-90 minutes of ischemia were described. The reperfusion part of the knee replacement model worked less good, and here the findings in muscle glutathione and in free amino acid status were mostly attributable to the surgical trauma.

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## Figure legends

### Figure 1

Concentrations of energy rich compounds and activities of mitochondrial enzymes in skeletal muscle preoperatively (pre), at maximal ischemia (max), and after 24 hour of reperfusion (reperf) in patients (n=15) undergoing knee replacement surgery employing tourniquet ischemia. The symbols are explained on the figure, left hand y-axis (atp = adenosinetriphosphate, pcr = phosphocreatine, ct = creatine, tcr = total creatine, lac = lactate, cs = citrate synthase), right hand y-axis (compl I = complex I, compl IV = complex IV). \* denotes different from preoperative values,  $p < 0.05$ .

### Figure 2

Concentrations of thiols (mmol/kg ww) and the redox ratio (%) of glutathione in skeletal muscle preoperatively (pre), at maximal ischemia (max), and after 24 hour of reperfusion (reperf) in patients (n=15) undergoing knee replacement surgery employing tourniquet ischemia. The symbols are explained on the figure, left hand y-axis (cysh = cysteine, tcys = total cysteine, cyss = cystine, gsh redox = the ratio gsh/tgsh), right hand y-axis (gsh = reduced glutathione, tgsh = total glutathione, gssg = reduced glutathione). \*, \*\* denotes different from preoperative values,  $p < 0.05$  and  $p < 0.01$  respectively.

### Figure 3

Concentrations of selected amino acids in skeletal muscle preoperatively (pre), at maximal ischemia (max), and after 24 hour of reperfusion (reperf) in patients (n=15) undergoing knee replacement surgery employing tourniquet ischemia. The symbols are explained on the figure, panel a (glu = glutamate, gln = glutamine, gly = glycine, ala = alanine, lys = lysine), panel b (thr = threonine, ser = serine, orn = ornithine, his = histidine, arg = arginine). \*, \*\*, \*\*\* denotes different from preoperative values,  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  respectively.

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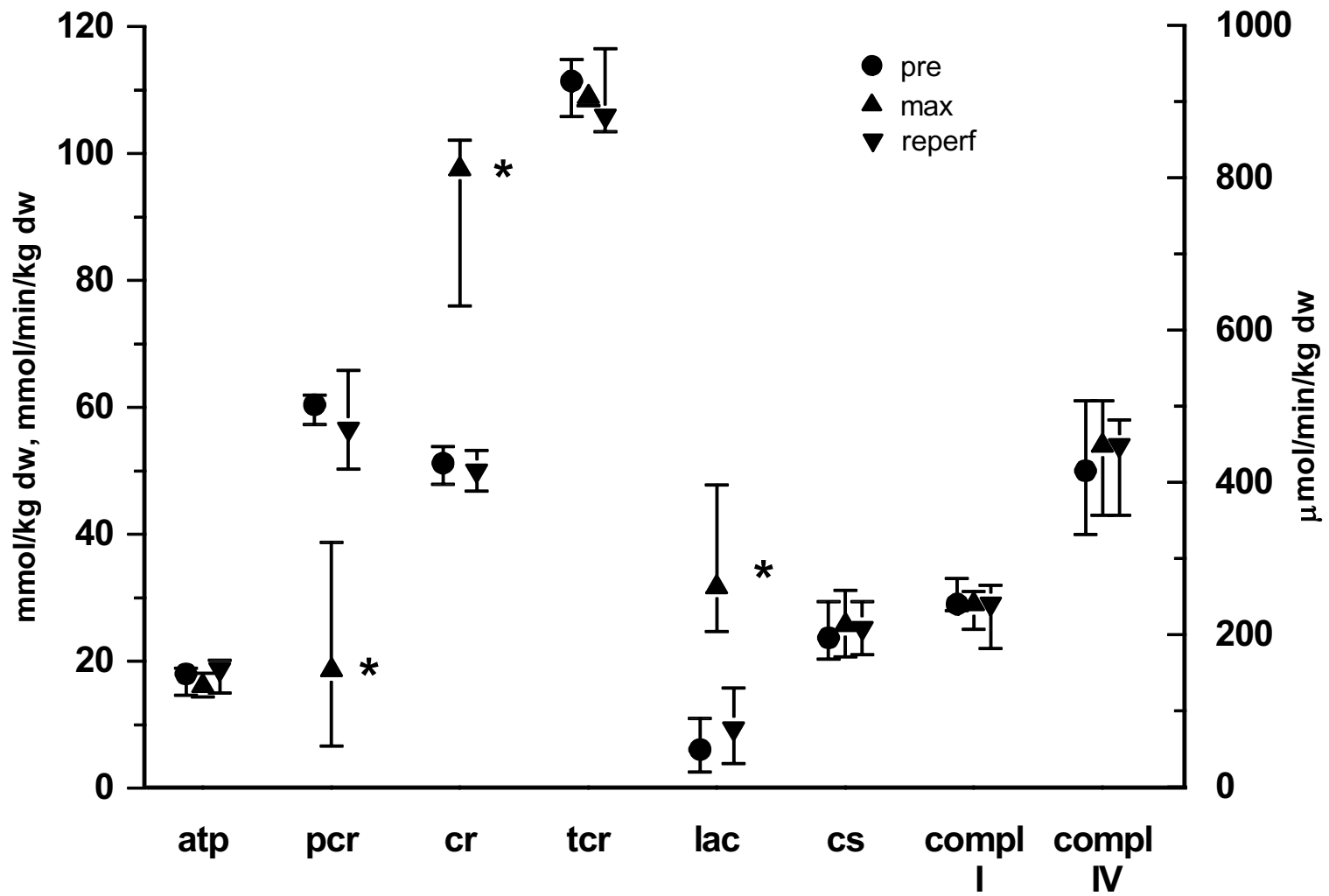


Fig 1

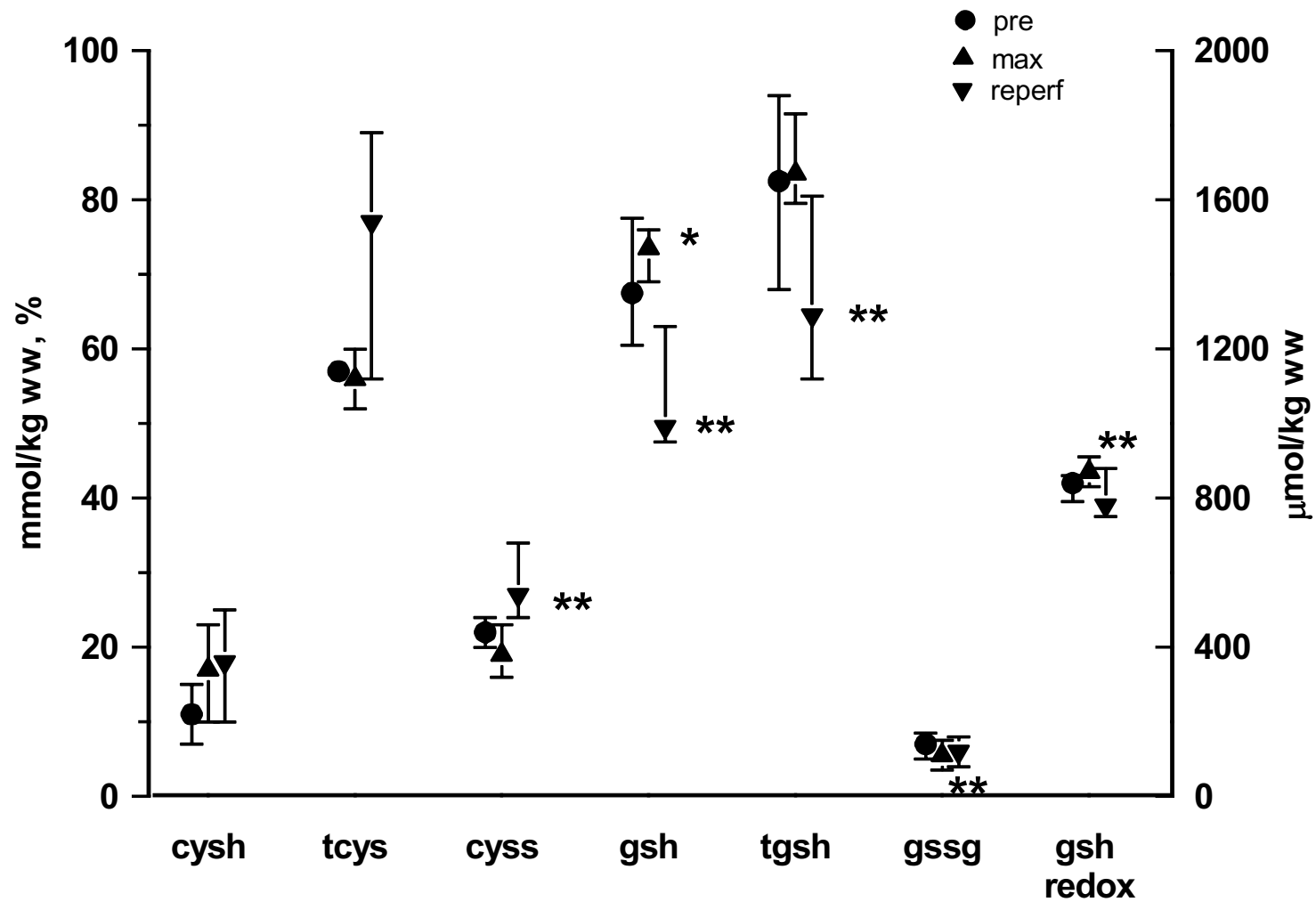


Fig 2

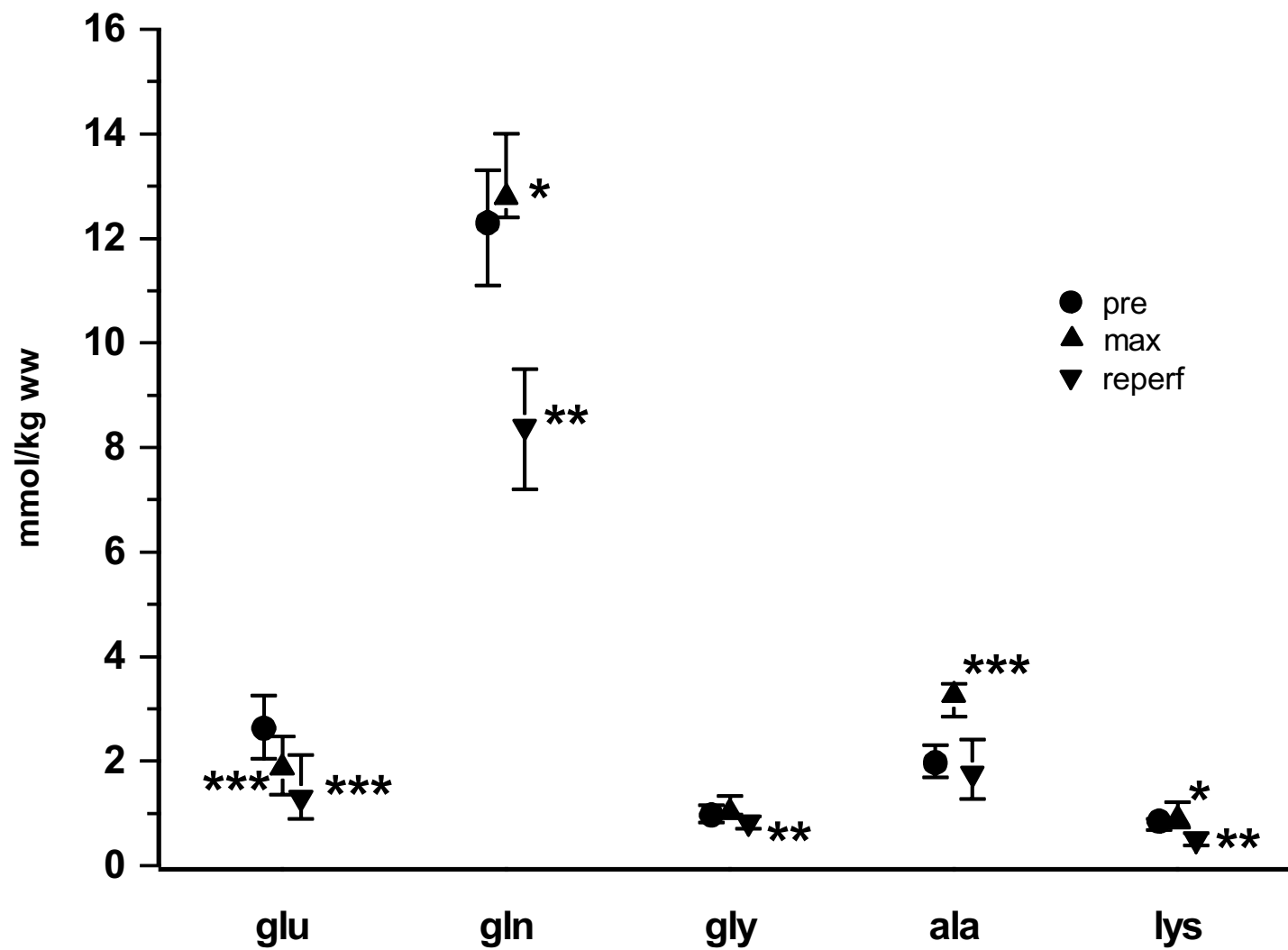


Fig 3a

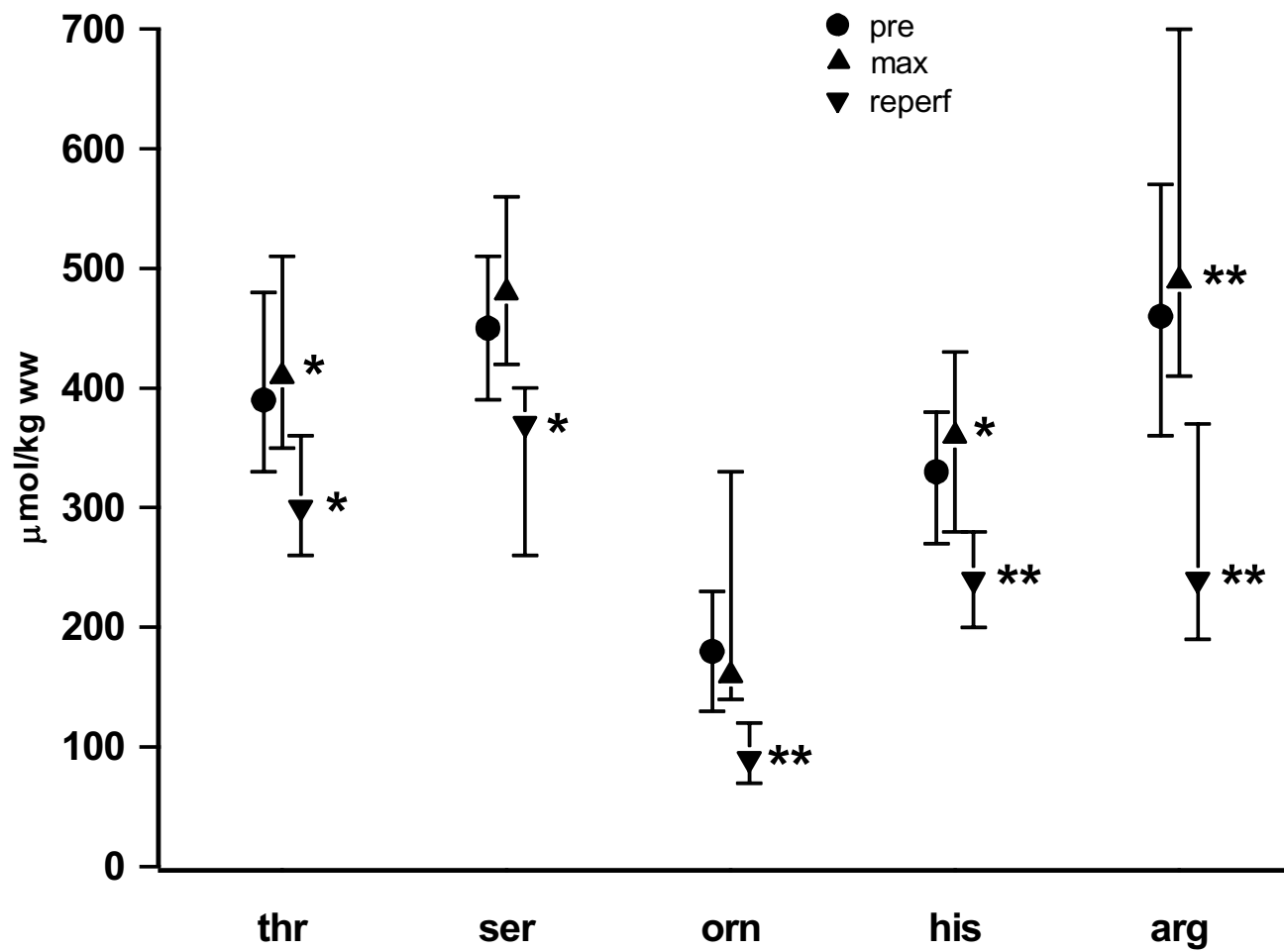


Fig 3b